Pulmonary exposure to nanoparticles during pregnancy : Effects in prenatally exposed offspring

PhD thesis

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TABLE OF CONTENTS

TABLE OF FIGURES AND TABLES

PREFACE

This PhD project was performed at the National Research Centre for the Working Environment. The four supervisors were Karin Sørig Hougaard, Ulla Vogel and Håkan Wallin from the National Research Centre for the Working Environment, and Ole Andersen from Department of Science, Systems and Models, Roskilde University.

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The global gene expression by using DNA microarrays was sponsored by and analyzed at Mutagenesis Section, Health Canada, under the supervision of Sabina Halappanavar.

During a week-long stay at the Department of Chemical Toxicology, Division of Environmental Medicine at the Norwegian Institute of Public Health, I learned the Comet analysis with the high throughput protocol, developed by Gunnar Brunborg and Kristine Bjerve Gutzkow, with the support of OMOCS LSHB-CT-2006-037575.

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- The Mutagenesis Section, Health Canada;
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ABBREVIATIONS AND ACRONYMS

GD	Gestation day
PND	Post natal day
In vivo	Experimentation using a living organism (Latin for "within the living")
In vitro	Experimentation in a test tube or Petri dish (Latin for "within the glass")
Ex vivo	Experimentation in tissue in an artificial environment outside the organism (Latin for "out of the living")
In silico	Performed on computer or via computer simulation
NRCWE	National Research Centre for the Working Environment
EU	European Union
IARC	Agency for Research on Cancer
OECD	Organisation for Economic Co-operation and Development
USEPA	U.S. Environmental Protection Agency
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (EU law)
TiO2	Titanium dioxide
BAL	Bronchoalveolar lavage
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
cRNA	Complementary RNA
miRNA	micro RNA
RT-PCR	Real-time polymerase chain reaction
ROS	Reactive oxygen species
AGD	Anogenital distance
TEM	Transmission electron microscopy
BET	Gas Absorption Surface Area Analysis
BeWo	Human choriocarcinoma cell line (placenta model)
RA	Retinoic acid
LPS	Lipopolysaccharide
Il-1β	Interleukin-1 beta cytokine
NF-κB	Nuclear factor kappa-B
TLR4	Toll-like receptor 4
Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1

SUMMARY

Uncontrolled dispersion of engineered nanoparticles may affect human health. Human exposure to nanoparticles in the ambient air is associated with increased risk of pulmonary and cardiovascular diseases, as well as lung cancer and allergy. Particulates in the ambient air are also associated with adverse pregnancy outcomes, systemic inflammation and increased DNA damage in exposed children. Several animal studies with diesel exhaust and diesel exhaust particles report effects such as altered growth, immune function, neurofunction and sexual differentiation. Similar effects can be anticipated from exposure to engineered nanoparticles.

Nanoparticles are more reactive compared to their larger counterparts with similar particle mass. When nanoparticles are inhaled, they deposit deep into the lung, and because of inefficient clearance, they tend to persist for a long time. A portion of nanoparticles may translocate into the blood stream and deposit in other organs in the exposed body. Some airborne nanoparticles induce inflammation, oxidative stress and damage to DNA. Only few animal studies with prenatal exposure address potential effects to nanoparticles.

The purpose of this PhD work was to assess the developmental toxicity of two model nanoparticles: titanium dioxide (UV-Titan, designed for use in the paint and lacquer industry) and carbon black (Printex 90, printing ink used as a model for carbon-based nanoparticles). Pregnant mice were exposed by inhalation or intratracheal instillation. The effects on mothers and offspring were assessed.

The endpoints include: classical gestational and developmental parameters; evaluation of maternal lung inflammation in bronchoalveolar lavage; analysis of gene expression in maternal lung, liver and in offspring liver; analysis of DNA strand breaks by comet assay in maternal lung, liver and in offspring liver; and developmental neurotoxicity in the offspring.

Exposures were designed to be comparable to each other. The total inhaled masses were similar for UV-Titan and Printex 90, and the highest intratracheally instilled Printex 90 dose was similar to the estimated deposited pulmonary (alveolar) dose in Printex 90 exposure. However, aerosolized particles during inhalation exposures were different; UV-Titan was more agglomerated compared to Printex 90. Pulmonary exposure to the tested nanoparticles induced persistent pulmonary inflammation and acute phase response in the exposed mothers and inflammation related reaction in the offspring. Inhalation exposure to Printex 90 induced DNA strand breaks in the liver of exposed mothers and offspring, even weeks after the end of exposure. No DNA damage was observed after UV-Titan inhalation exposure or intratracheal instillation of Printex 90. Prenatal exposure to nanoparticles induced minor neurobehavioral changes in the exposed offspring after reaching adulthood. Despite the observed effects, the exposure did not affect gestational and developmental parameters.

RESUMÉ (SUMMARY IN DANISH)

Ukontrolleret spredning af nanopartikler kan muligvis have en sundhedsskadelig effekt. Menneskelig eksponering for nanopartikler i luften er associeret med en forhøjet risiko for lunge- og hjertekarsygdomme, lungekræft og allergi. Partikler i luften er også associeret med for tidlig fødsel, reduceret fødselsvægt, lille størrelse for gestationsalder alder, systemisk inflammation, og forhøjet DNA skade i eksponerede børn. Mange dyrestudier med dieseludstødning og dieseludstødningspartikler viser effekter som ændret vækst, immunofunktion, neurofunktion samt seksuel differentiering. Lignende effekter kan forventes efter eksponering for nanopartikler.

På grund af deres større relative overfladeareal er nanopartikler mere reaktive i forhold til større partikler med lignende partikelmasse. Nanopartikler, der indåndes, deponeres dybt i lungen og bliver der i lang tid på grund af ineffektiv clearance. En andel af nanopartiklerne translokerer muligvis til blodbanen og til andre organer i kroppen. Nogle typer nanopartikler inducerer inflammation, oxidativt stress og DNA skade. Formålet med dette PhD arbejde var at vurdere de to følgende modelpartiklers udviklingsmæssige toksicitet: Titaniumdioxid (UV-Titan, udviklet til brug for farve- og lakindustrien) samt carbon black (Printex 90, tryksværte, brugt som model for carbonbaserede nanopartikler).

Drægtige mus blev eksponeret ved inhalation eller intratracheal instillation. Effekter på mødre og afkom blev vurderet. Endpoints omfattede de klassiske drægtigheds- og udviklingsparametre; vurdering af maternel lungeinflammation i bronchoalveolar lavage; analyse af gene ekspression i maternel lunge, lever og lever fra afkom; analyse af DNA strengbrud udført vha. comet assay i maternel lunge, lever, og lever fra afkom; samt udviklingsneurotoksicitet i afkom.

Eksponeringerne var designet til at være sammenlignelige med hinanden. Den samlede inhalerede masse var sammenlignelig både for UV-Titan og Printex 90. Den højeste dosis af Printex 90 i instillationsforsøget var designet til at være så tæt på den inhalerede masse som muligt. Der var dog forskel på aerosoliseringen af de to partikeltyper i inhalationseksponeringen idet UV-Titan blev mere agglomereret i forhold til Printex 90. Pulmonal eksponering for begge de testede nanopartikler inducerede vedholdende lungeinflammation, akut fase response i de eksponerede mødre samt en inflammationsrelateret reaktion i deres afkom. I leveren hos eksponerede mødre og deres afkom gav inhalationseksponering for Printex 90 DNA strengbrud, som var tilstede flere uger efter endt eksponering. Der blev ikke fundet DNA skade efter UV-Titan inhalationseksponering eller intratracheal instillation af Printex 90. Forskelle i genotoksicitet blev sandsynligvis forårsaget af forskellige partikel deposition, udbredelse og reaktivitet. Prænatal eksponering for nanopartikler forårsagede adfærdsændringer i eksponeret voksent afkom. Trods de observerede effekter, påvirkede eksponeringen ikke drægtigheds- og udviklingsparametre.

LIST OF PAPERS INCLUDED

Paper I

Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice.
Hougaard KS, Jackson P, Jensen KA, Sloth JJ, Loschner K, Larsen EH, Birkedal RK, Vibenholt A, Boisen AM, Wallin H & Vogel U.
Particle and Fibre Toxicology, 2010, 7:16.

Paper II

Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: A toxicogenomic study.
Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard KS, Vogel U, Yauk CL & Wallin H.
Environmental and Molecular Mutagenesis, 2011, *52(6):425-39*.

Paper III

Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan): Effects in prenatally exposed offspring on hepatic DNA damage and gene expression.
Jackson P, Halappanavar S, Hougaard KS, Yauk C, Williams A, Lamson JS, Andersen O, Wallin H and Vogel U.
Nanotoxicology, 2011, DOI:10.3109/17435390.2011.633715.

Paper IV

An Experimental Protocol for Maternal Pulmonary Exposure in Developmental Toxicology.
Jackson P, Lund SP, Kristiansen G, Andersen O, Vogel U, Wallin H & Hougaard KS.
Basic & Clinical Pharmacology & Toxicology, 2011, 108: 202-207.

Paper V

Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: Effects on liver DNA strand breaks in dams and offspring.
Jackson P, Hougaard KS, Boisen AMZ, Jacobsen NR, Jensen KA, Møller P, Brunborg G, Gutzkow KB, Andersen O, Loft S, Vogel U and Wallin H.
Nanotoxicology, 2011, DOI: 10.3109/17435390.2011.587902.

Paper VI

Prenatal exposure to carbon black (Printex 90): Effects on sexual development and neurofunction.
Jackson P, Vogel U, Wallin H and Hougaard KS.
Basic & Clinical Pharmacology & Toxicology, 2011, DOI: 10.1111/j.1742-7843.2011.00745.x.

1. BACKGROUND

Man has always been exposed to ambient airborne nanoparticles, e.g. from wildfires or volcanic eruptions. With the events of industrialization and urbanization, ambient air pollution has grown from being a localized and temporal problem to a more regional and recurring problem. The emergence of nanotechnology provides a new source of exposure to airborne nanoparticles (Oberdöster et al. 2005).

1.1 Ambient air pollution

Uncontrolled dispersion of engineered nanoparticles may affect human health, similarly to what has been found for exposure to particles in ambient air. Particulates (in contrast to gases) are suspected to be the major factor driving the adverse effects. Human exposure to ultrafine particles in the ambient air has been associated with increased risk of lung cancer, allergy, pulmonary and cardiovascular disease (Brunekreef et al. 2009; Delfino et al. 2005; Krewski et al. 2009; Pope, III and Dockery 2006). Occupational exposure to particulate air pollution, especially diesel exhaust, increases risk for ischaemic heart disease (Toren et al. 2007).

Less is known of the effects of nanoparticle exposure of women during pregnancy, and the effects in the developing organism before and after birth. Epidemiological studies indicate that exposure to environmental air pollutants (especially particulates) is associated with adverse pregnancy outcomes, such as premature birth, reduced birth weight, small size for gestational age, recently reviewed in (Shah and Balkhair 2010), stillbirth (Pope et al. 2010), and postnatal respiratory deaths (Šrám et al. 2005). Several mechanisms, including particle induced oxidative stress and pulmonary and placental inflammation, have been suggested (Kannan et al. 2007). DNA damage is reported to increase after maternal exposure to ambient air pollution during pregnancy, reviewed in (Kannan et al. 2007; Neri et al. 2006). Effects include total DNA adducts in placenta tissue (Šrám et al. 1999), bulky DNA adducts and micronuclei in umbilical blood of newborns (Pedersen et al. 2009) and stable chromosomal aberration frequencies in cord blood (Bocskay et al. 2005). Increased systemic inflammation and levels of urinary 8-oxodeoxyguanosine (a marker of oxidative damage to DNA) have been reported in children 'born and raised' in areas with high air pollution (Calderon-Garciduenas et al. 2008; Švecová et al. 2009). Genomic analysis of children and their parents exposed to air pollution suggest that children respond differently than adults, with differences at the transcriptome level. DNA integrity and suppression of immune function are the possibly affected presences (van Leeuwen et al. 2008).

Exposure to environmental air pollutants is suspected to cause effects on the nervous and reproductive system. Maternal exposure during pregnancy to traffic-related or industrial air pollution has been associated with adverse effects to the children, related to cognitive and perceptual performance, motor function and language skills (Freire et al. 2010; Tang et al. 2008). It is difficult casually to link exposure to air pollution to negative reproductive outcomes in man. Animal studies are important for testing this hypothesis.

Developmental toxicity of diesel exhaust has been assessed in animal studies. Prenatal exposure to diesel exhaust is not necessarily fetotoxic; it may inflict health effects. Gestational exposure to diesel exhaust changed immunological action in mouse placentas (Fujimoto et al. 2005).

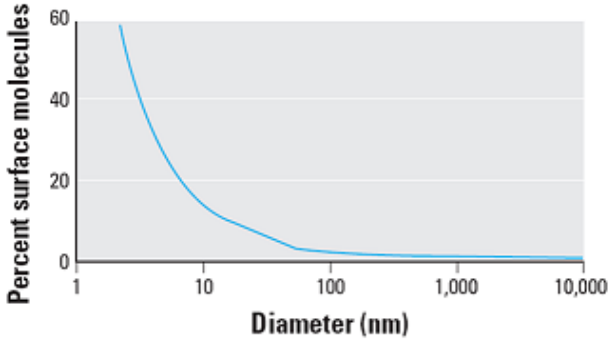
Prenatal exposure to diesel exhaust altered offspring body weight, immunological functions and sexual differentiation, reviewed in (Hougaard et al. 2008). Maternal oral exposure to diesel exhaust particles increased the frequency of DNA deletions in retinal pigment epithelium in the prenatally exposed offspring eyes (Reliene et al. 2005). More recent animal data indicate that diesel exhaust may affect male reproductive function after birth (Hemmingsen et al. 2009; Li et al. 2008; Ono et al. 2007). Several animal studies report changes in the brain and neurofunction due to diesel exhaust exposure (Masao Sugamata 2006b; Suzuki et al. 2010; Takeda et al. 2004; Yokota et al. 2009). Most studies used whole diesel exhaust, such as exposure to exhaust gases in combination with diesel exhaust particles. However, a numerically large fraction of the particulates in diesel exhaust is ultrafine in size, and recent studies indicate that the particulate fraction may be important for toxicity (Hougaard et al. 2008). Adverse effects of prenatal exposure to engineered nanoparticles *in vitro* and *in vivo* are summarized by (Ema et al. 2010; Hougaard et al. 2011). The effects in animals are altered immune response, neurodevelopment and reproductive function (Fedulov et al. 2008; Lamoureux et al. 2010; Shimizu et al. 2009; Takahashi et al. 2010; Takeda et al. 2009; Yoshida et al. 2009; Yoshida et al. 2010). The results are presented in Table 1.

Particle	Vehicle	Physical and chemical characteristics	Animal	Exposure			Results		Ref
				Route	Time	Dose	Maternal	Offspring	
DEP TiO_2 CB	PBS	-	BALB/c mice	IN	GD 14	50 50 250 (µg/animal/day)	↑BAL PMN ↑IL-1b, TNF-α, IL-6 Pathway: Infl. Response Immune & DNA regulation Pregnant>Normal	>12-wk ♀offspring (OVA challenged) ↑AHR ↑AI ↑EOS =↑allergic susceptibility	1
TiO_2	Saline with 0.05%Tween 80 Sonicated>30min	Sigma-Aldrich Japan Inc. Particle size 25-70 nm Surface area 20-25 m^2/g Anatase	Slc:ICR mice	SC	GD 6, 9, 12, 15	100 (µg/animal/day)	-	GD 16, PND 2, 7, 14, 21 1♂offspring ↑↓ gene expression in brain Genes related to development& function of CNS	2
TiO_2	Saline with 0.05%Tween 80 Sonicated>30min	Sigma-Aldrich Japan Inc. Particle size 25-70 nm Surface area 20-25 m^2/g Anatase FE-SEM ∅ 20-100 nm DLS (27 nm & 2429 nm)	Slc:ICR mice	SC	GD 6, 9, 12, 15	100 (µg/animal/day)	-	6-wk-old ♂offspring ↑DA and metabolites in brain	3
TiO_2	Saline with 0.05%Tween 80	Sigma-Aldrich Japan Inc. Particle size 25-70 nm Surface area 20-25 m^2/g Anatase FE-SEM ∅ 45 nm	Slc:ICR mice	SC	GD 3, 7, 10, 14	100 (µg/animal/day)	-	4&6-wk-old ♂offspring NP in brain & testes ↓Body weight Abnormal testicular morphology ↓DSP Apoptosis in olfactory bulb	4
TiO_2	Distilled water Ultrasonic 20min	Sigma-Aldrich USA Inc. Particle size <25 nm Surface area 200 m^2/g Anatase 97.7% pure	SD rats	OG	GD 2-21 PND 2-21	100 (mg/kg BW/day)	-	↓PPR ↓Synaptic plasticity in hippocampus	5
CB	Saline with 0.05%Tween 80 Sonicated 5min	14 nm	Slc:ICR mice	IT	GD 7 & 14	200 (µg/animal/day)	-	No effects on gestation 5, 10&15-wk-old ♂offspring Abnormal testicular morphology ↓DSP	6

Table 1. Effects of prenatal exposure to nanoparticles tested *in vivo*.
Nanoparticles (NP), Diesel exhaust particles (DEP), Titanium dioxide (TiO2), Carbon black (CB), Ovalbumin (OVA, allergen model), Phosphate buffered saline (PBS), Field emission-type scanning electron microscope (FE-SEM), Dynamic light scattering (DLS),Gestation day (GD), Postnatal day (PND, day after birth), Airway Hyperresponsiveness (AHR), Allergic airway inflammation (AI), Week (Wk), Intranasal insufflations (IN), Intraperitoneal injection (IP), Inhalation exposure (IH), Intratracheal instillation (IT), Subcutaneous injection (SC), Oral gavage (OG), Bronchoalveolar lavage (BAL), Polymorphonuclear neutrophils (PMN), Eosinophils (EOS), Proinflammatory cytokines (IL-1b, TNF-α, IL-6), Central nervous system (CNS), Dopamine (DA), Daily sperm production (DSP), Paired pulse reaction (PPR)
1) Fedulov et al. 2008; 2) Shimizu et al. 2009; 3) Takahashi et al. 2010; 4) Takeda et al. 2009; 5) Gao et al. 2011; 6) Yoshida et al. 2010.

1.2 Nanotoxicology and nanoparticles

Innovations in the field of 'Nanotechnology' have allowed creation of new materials with new, exciting properties for a vast range of applications (energy production, electronics, biomaterials, medicine, cosmetics and others). 'Nanomaterial' is defined as insoluble or bio-persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm. (Article 2.1.k of Regulation EC/1223/2009 on Cosmetic Products). Solid matter in the atomic and molecular size range has a large, specific surface reactivity and surface area to volume ratio (Figure 1). Therefore, nanomaterials have unique properties compared with their larger counterparts of similar particle mass (Duffin et al. 2007). Changes in material properties affect the biological interactions, and therefore 'Nanotoxicology' has emerged a new discipline in toxicology, being the study of toxicity of nanomaterials.



Particle diameter (µm)	Particle number (cm^{-3})	Particle surface area ($µm^2/cm^3$)
5	153,000,000.00	12,000
20	2,400,000.00	3,016
250	1,200.00	240
5,000	0.15	12

Figure 1. Particle number and particle surface area per 10 µg/m^3 airborne particles.
The larger surface area gives the particles a larger reaction area compared to bigger particles, which may increase the toxicological effects, (modified from Oberdöster et al. 2005).

Based on the size, particles deposit in different regions of the pulmonary tract (Figure 2). Nanoparticles deposit deeper in the respiratory system, and because of inefficient clearance by alveolar macrophages and the mucociliary escalator, they interact with epithelial and interstitial sites, leading to greater biological reactivity (Oberdöster et al. 2005). Translocation of nanoparticles from the lung into circulation is considered to be slow, and it has been reported that only a fraction of a percent gets beyond the lung cavity and regional lymph nodes, depending on the particle or agglomerate size (Kreyling et al. 2010; Kreyling et al. 2002; Kreyling et al. 2009; Sadauskas et al. 2009; van Ravenzwaay et al. 2008). A portion of inhaled particles are swallowed and deposited in the gastric system (Jacobsen et al. 2009), however nanoparticles do not seem to pass readily over the gastrointestinal mucosa in rodents (Carr et al. 1996; Kreyling et al. 2002). Some airborne nanoparticles are known to elicit oxidative stress and inflammatory reactions (Borm et al. 2006), which can further induce oxidative damage to DNA (Møller et al. 2010a). Therefore, bio-durable nanoparticles consisting of matter without known significant specific toxicity have been suggested to fulfil the European Union (EU) criteria for category 2 of carcinogenic substances (Roller 2009). On the basis of scientific evidence, the International Agency for Research on Cancer (IARC) has classified some materials that are commonly marketed as nanoparticles as possibly carcinogenic to humans (Baan et al. 2006).

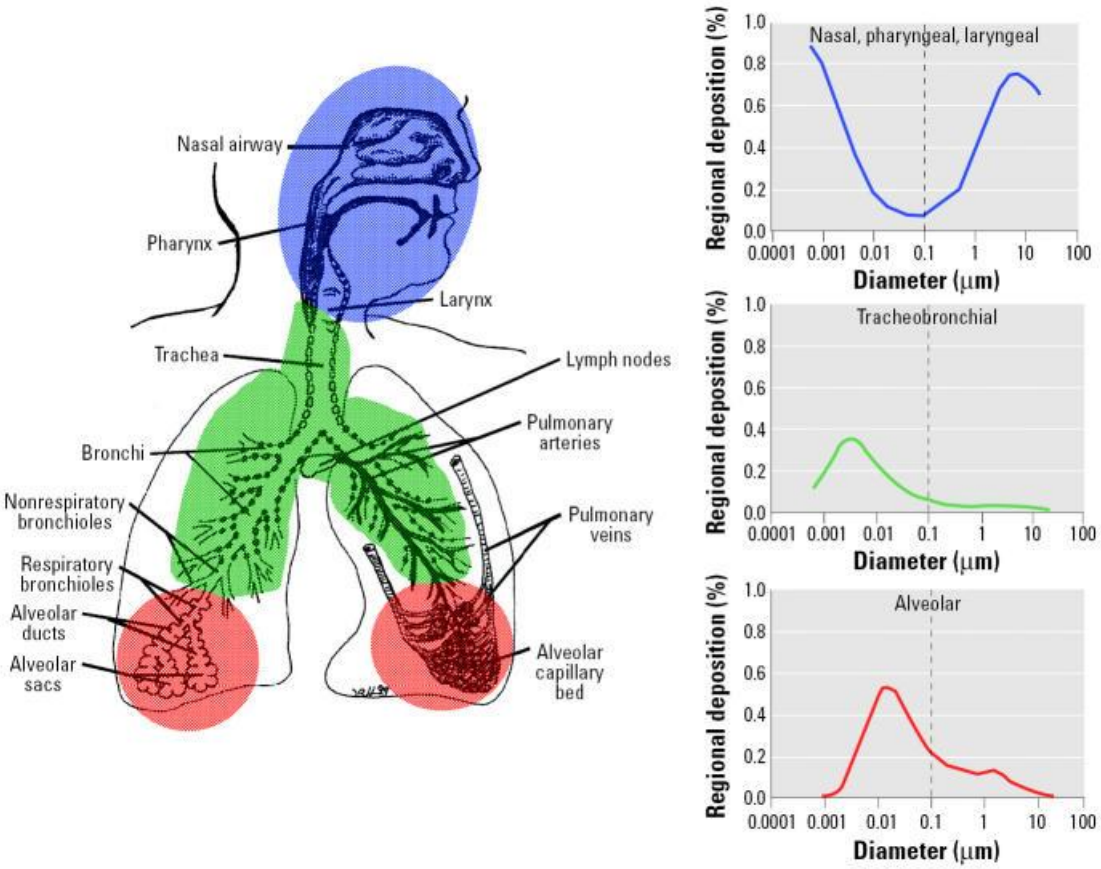


Figure 2. Lung deposition based on particle size.
Large particles around 10 µm deposit almost entirely in the upper airways (nasal, pharyngeal and laryngeal region). Smaller particles penetrate deeper into the lung, based on particle size (2-10 µm tracheobronchiolar region and <2 µm alveolar region), (modified from Oberdöster et al. 2005).

1.3 Questions addressing maternal pulmonary exposure to nanoparticles

Based on findings in epidemiological and animal studies, the questions addressing maternal pulmonary exposure to nanoparticles were:

- Does maternal exposure to nanoparticles affect gestation, development during lactation and sexual maturation?
- Does prenatal exposure to nanoparticles alter global gene expression in the offspring in liver (first exposed organ), evaluated by Microarray analysis?
- Does prenatal exposure to nanoparticles induce genotoxicity in the exposed offspring measured as levels of DNA strand breaks in liver?
- Does exposure to nanoparticles induce developmental neurotoxicity?

2. PARTICLE EXPOSURE

In this project, we investigated two types of nanoparticles and two means of exposure.

2.1 Particles

We investigated the effect of nanoparticles by using two commonly used model particles: titanium dioxide and carbon black. We have addressed the effects of maternal pulmonary exposure during pregnancy on offspring exposed prenatally. Pulmonary exposure was the only primary exposure route considered in this work, because of the relevance to exposure to ambient air and occupational exposure.

2.1.1 Titanium dioxide (UV-Titan)

Titanium dioxide (TiO2) is an industrial product, which is produced in large quantities world-wide. It is used as a white pigment in cosmetics, food, plastics, paints and other products, and used as a UV-filter in cosmetics. The titanium dioxide used in the study was UV-Titan L181 (Kemira, Pori, Finland), supplied by The Danish Association of the Paint and Lacquer industry. This material was chosen as a model nanoparticle to be applied by the paint industry in new paints and lacquers. TiO2 particles are generally considered to be inert. However, when the particle size is reduced to the nanoscale, it becomes reactive (Sager et al. 2008). The activity of different crystalline forms of TiO2 (rutile, anatase, brookite) varies. The anatase form appears to be more reactive compared to the rutile form, and therefore may be potentially more toxic (Jiang et al. 2008; Warheit et al. 2007). Physico-chemical characteristics such as particle aggregation, crystal phase, and surface modifications contribute to particle induced toxicity (Johnston et al. 2009); thus, it is difficult to generalise the TiO2 toxicity.

Pulmonary exposure to TiO2 nanoparticles induces lung inflammation in mice and rats (Bermudez et al. 2004; Grassian et al. 2007; Ma-Hock et al. 2009; van Ravenzwaay et al. 2008; Hougaard et al. 2010). Of notable importance, nanosized TiO2 particles have been detected in lung tissue up to four weeks after inhalation exposure (Hougaard et al. 2010; Kreyling et al. 2010). Thus, acute as well as chronic effects of exposure must be considered. TiO2 nanoparticles are suspected to be both genotoxic (Johnston et al. 2009) and carcinogenic (Mohr et al. 2006). TiO2 dust, when inhaled, has recently been classified by the International Agency for Research on Cancer (IARC) as being possibly carcinogenic to humans (Group 2B). However, currently, there is no evidence of TiO2 related cancer in the occupational setting (Boffetta et al. 2004; Ellis et al. 2010; Fryzek et al. 2003).

2.1.2 Carbon black (Printex 90)

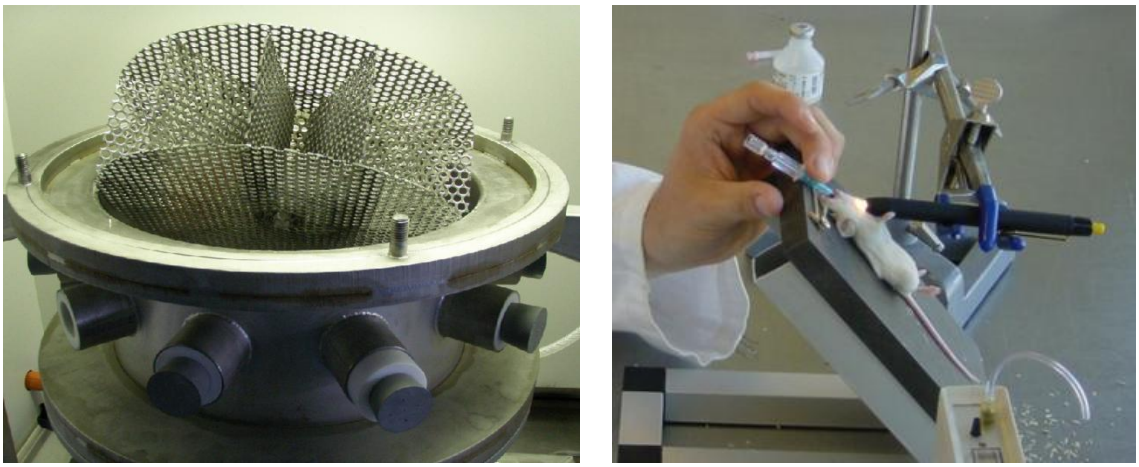
Carbon black is a well-characterized, carbonaceous core-particle that has been used extensively as a model for diesel emission particles without adhered chemicals and metals. The carbon black used in this study was Printex 90 (Degüssa, Frankfurt Germany). This material is marketed as printing ink pigment. Printex 90 was used as a positive control in many nanotoxicology studies. It consists of carbon with less than 1% organic and inorganic impurities (Borm et al. 2005; Brown et al. 2000; Jacobsen et al. 2007; Wilson et al. 2002).

Health effects reported after exposure to carbon black are assumed to be caused by the insoluble particle core rather than by associated compounds. It is well known that pulmonary exposure to carbon black by instillation or inhalation induces inflammatory response *in vivo* in

rats (Brown et al. 2000; Driscoll et al. 1997; Elder et al. 2005; Gallagher et al. 2003; Renwick et al. 2004; Sager and Castranova 2009; Wilson et al. 2002) as well as mice (Jacobsen et al. 2009; Saber et al. 2005; Totsuka et al. 2009). Carbon black nanoparticles possess an intrinsic potential to generate reactive oxygen species (Folkmann et al. 2009; Jacobsen et al. 2007; Jacobsen et al. 2010; Wilson et al. 2002; Yang et al. 2009), induce DNA strand breaks and oxidatively generated DNA damage (Jacobsen et al. 2008b; Jacobsen et al. 2009), are reported to be mutagenic (Driscoll et al. 1996; Driscoll et al. 1997; Jacobsen et al. 2007; Jacobsen et al. 2010; Totsuka et al. 2009), and induce lung tumors in rats (Mohr et al. 2006). It is uncertain whether occupational exposure to carbon black is related to cancer risk (Morfeld and McCunney 2007; Puntoni et al. 2001; Ramanakumar et al. 2008; Sorahan and Harrington 2007), but carbon black has been classified by the International Agency for Research on Cancer as possibly carcinogenic to humans (Baan et al. 2006).

2.2 Inhalation and instillation exposure

In occupational settings, exposure to nanoparticles primarily occurs via inhalation (Maynard and Kuempel E.D. 2005). The dustiness of nanoparticles is in order of magnitude larger than the dustiness of fine particles of similar chemical composition (Schneider and Jensen 2008). Aerosolization of particles for inhalation exposure is therefore the preferred exposure regimen in toxicological studies of nanoparticles (Figure 3). For this route of exposure, there are a number of OECD guidelines addressing sub-chronic inhalation exposure, and short-term particle inhalation protocols are being developed (Grassian et al. 2007; van Ravenzwaay et al. 2008).



A) B)

Figure 3. Exposure apparatus.
A) Inhalation chamber with steel-cage.
B) Mice instilled by intratracheal instillation. (Photo from Kenneth Klingenberg Barfod).

However, it is not always possible to perform inhalation studies, e.g. when: 1) there is a potential health risk associated with occupational exposure during management of particles in the inhalation chamber or on animals after exposure (e.g. carbon nanotubes); 2) only small amounts of nanoparticles are available; and 3) inhalation equipment is not available. Particle instillation in suspension is an alternative method of exposure for pulmonary administration in particle toxicology (Figure 3) (Oberdöster et al. 1992; Park et al. 2009). Particles are suspended in a liquid and are subsequently injected as a solution into the lumen of the trachea while the animals are under general anaesthesia. Particle instillation is a widely used and accepted procedure, even though inhalation and instillation methods may not be fully comparable

(Driscoll et al. 2000; Osier and Oberdöster 1997). Generally, instilled particles tend to induce stronger lung inflammation compared to inhaled particles (Jacobsen et al. 2009). Instilled particles are forced into the alveoli, resulting in lesser deposition in the bronchia and bronchioles, leading to slower clearance. The instillation procedure in the pregnant animal does not seem to affect gestation or lactation; and it does not induce significant lung inflammation or lung and liver DNA damage (Jackson et al. 2011a).

3. DEVELOPMENTAL TOXICITY TESTING OF NANOPARTICLES

Until now, relatively little attention has been given to the potential adverse effects of prenatal exposure to nanoparticles. In USEPA's nanomaterial research strategy, *in vivo* toxicity testing in animals also includes reproductive and developmental toxicity (USEPA 2009). One of the anticipated outcomes of this strategy is an identification of testing methods to predict *in vivo* toxicity of nanomaterials. The EU chemical legislature, REACH, requires reproductive and developmental studies from producers or importers of chemicals with tonnage above the ten-ton limit (Scialli 2008). It is currently discussed whether a precautionary principle should be applied in the EU regulation of nanoparticles; possibly nanoparticles should be exempt from the tonnage rule and be fully tested at much lower tonnage limits. Developmental toxicity testing would then be one of the expected requirements.

3.1 Animal model

Pregnancy is a complicated biologic process involving many developmental stages of the fetus. Chemical exposure can negatively interfere with the course of pregnancy, depending on the timing. We exposed mice during gestation on gestation day (GD) 7(8)-18. Exposure was timed after fertilization, implantation and after initiation of organ development. This exposure roughly corresponds to the first two trimesters of human pregnancy, where fetal organs are formed and developed. Exposure terminated two days before expected delivery to avoid stressing the animals during birth preparations and negative birth outcomes (premature birth and offspring loss). Exposure was designed to provide information about the effects of prenatal exposure on the pregnant female and on the developing organism.

The maternal toxicity was assessed at two time points in the time-mated mice. Time-mated females that did not give birth, or had only a few offspring, were used to assess the inflammation timed soon after birth (3-5 days after exposure). Pregnancy is reported to alter inflammatory response (Fedulov et al. 2008; Lamoureux et al. 2010), thus, different background levels were anticipated. Littering dams were assessed at the end of lactation after weaning (23-27 days after exposure). Offspring toxicity was assessed at birth, weaning, adolescents, and selected adults were assessed for neurotoxicity.

The chosen animal model was the mouse, specifically C57BL/6BomTac, because this species and strain were used in our laboratory previously and thus direct comparisons could be made. The mouse is generally considered a suitable species for studying particle toxicity (Bermudez et al. 2004; Elder et al. 2005). C57BL/6BomTac mice are a robust strain and these mice tolerate transport after mating without major loss of offspring due to stress. They have a weaker bond to the offspring, however and they are prone to suffer 'Spontaneous acute intestinal pseudo-obstruction' during lactation, described in (Percy D.H. and Barthold S.W. 2001).

3.2 Mechanisms of nanoparticle toxicity during fetal development

It has been suggested that the fetus could be affected either: 1) directly by particle and/or impurities translocating through the placenta; 2) by altered placental function; or 3) indirectly by circulating cytokines or other secondary messengers from an inflammatory process in the mother (Hougaard et al. 2011).

To affect the offspring directly, nanoparticles have to translocate through the placenta and enter the embryo/fetus, which is summarized in Table 2and Table 3 . Several studies investigated placental transfer of nanoparticles *in vivo* in mice and rats (Kennison et al. 1971; Sadauskas et al. 2007; Takahashi and Matsuoka 1981), as well as *ex vivo* in perfused human placentas collected by caesarean sections (Myllynen et al. 2008; Wick et al. 2010), and *in vitro* in a placenta trophoblast model (BeWo choriocarcinoma cells) (Myllynen et al. 2008). In addition, *in vitro* translocation and the effects on embryonic tissue were also assessed (Bosman et al. 2005; Tian et al. 2009). Nanoparticle translocation depends strongly on particle size, concentration, surface modifications and other properties, as well as maternal exposure type and duration, and exposure timing in pregnancy. Translocation of nanoparticles from the maternal lung into circulation is considered to be slow, and probably only a fraction of a percent gets beyond the lung cavity, regional lymph nodes or over the gastrointestinal mucosa (discussed earlier). After pulmonary exposure, once in circulation, the distribution across the placenta and into the fetus may also be small (Sadauskas et al. 2007). It is however likely that impurities and surface modifications could dissociate from nanoparticles. Once in the blood stream, some nanoparticles can cross the placenta, as reported for polycyclic aromatic hydrocarbons leached from coal fly ash administered intratracheally to pregnant rats on gestation day 18 and 19 (Srivastava et al. 1986).

Nanoparticles can also affect the placenta function. Nanoparticles may accumulate at the maternal side and be internalized by placental cells, when attempting to cross the placenta (Myllynen et al. 2008; Wick et al. 2010). There are currently no published reports identifying alterations in exposed placentas, though it can be expected that placenta function, and maybe even morphology, could be changed by such a intracellular particle burden. Transport across the placenta may also be affected by inflammation, which is common during nanoparticle exposure. (Saunders 2009) suggested leakage across endothelial tight junctions, potentially allowing greater passage of nanoparticles.

Nanoparticles may also affect the fetus indirectly. The general effects of pulmonary exposure to nanoparticles include persistent lung inflammation and genotoxicity. The mother is the route of exposure for the offspring exposed to xenobiotics *in utero*. Generally, maternal inflammation is reported to affect the immune and nervous system of the offspring exposed *in utero* (Hodyl et al. 2007; Lasala and Zhou 2007; Surriga et al. 2009; Graciarena et al. 2010; Mark et al. 2009) and transplacental genotoxins are reported to induce damage to the DNA in the offspring (Brunborg et al. 1996; Tripathi et al. 2008).

Particle (NP)	Vehicle	Physical and chemical characteristics	Model	Exposure				Results		Ref.
				Route	Time	Tissues collected	Dose	In placenta	In fetus/embryo	
In vivo										
Styrene divinylbenzene	PBS	6-14 µm	A/JAX & C-57 Mice	Transcardiac inj.	Various	1/2h after exposure (Offspring PND 4-9)	10^8/mL	-	None (Particles in lungs & livers when pretreated with hyaluronidase)	1
$_{198}$Gold	Saline	2 nm 40 nm	C57BL/6 Mice	IV	GD 16-18	24h after exposure	12.13 µg/animal ($15x10^{13}$/mL) 58.21 µg/animal ($9x10^{10}$/mL)	None None	None None	2
$_{198}$Gold	Saline	5 nm 30 nm	Wistar rats	IV	GD 19	24h after exposure	20 µg/animal	0.361% 0.118% (Placenta and fetal membrane)	0.018% passed 0.005% passed	3
Ex vivo										
Gold (coated with Polyethylene glycol)	Saline	15 nm 30 nm 10 nm 35 nm	Human placenta	Open perfusion Recirculation perfusion	-	3min intervals 6h perfusion	$7.9x10^{11}$/mL $7.8x10^{11}$/mL $9.1x10^9$/mL $2.0x10^9$/mL	None NP in trophoblastic layer	None None	4
Polystyrene	PBS 15 min vortexed	50 nm 80 nm 240 nm 500 nm	Human placenta	Recirculation perfusion	-	3h perfusion	25 µg/mL 10^6-10^9/mL	Placenta function was not affected by NP	35% passed 30% passed 9% passed 1% passed	5

Table 2. Placenta transfer of nanoparticles *in vivo* in mice and rats, and in *ex vivo* in perfused human placenta models.
1) Kennison et al. 1971; 2) Sadauskas et al. 2007; 3) Takahashi & Matsuoka 1981; 4) Myllynen et al. 2008; 5) Wick et al. 2010.

Particle (NP)	Vehicle	Physical and chemical characteristics	Model	Exposure				Results		Ref.
				Route	Time	Tissues collected	Dose	In placenta	In fetus/embryo	
In vitro										
Gold (coated with Polyethylene glycol)	Saline	10 nm	BeWo	-	-	6, 24 & 48 h	$3.6x10^{10}$/mL	NP in cells	-	6
Polystyrene (with carboxyl groups)	50nM Na_3PO_4/NaCl	20 nm 100 nm 500 nm	Mouse embryo tissue GD 7.5	Injection to extraembryonic tissues	-	12h	0.5%w	-	Translocation None None	7
(with amine groups)		200 nm							Translocation	
Polystyrene	G1.2 medium	40-120 nm	2-cell embryos	-	-	72h	$11x10^{9}$/mL	-	No effect: Development Hatching Implantation Degeneration Not embryo-toxic	8

Table 3. Placenta transfer and effects of nanoparticles *in vitro* in embryonic tissue and in a placenta cell line human choriocarcinoma cell line (BeWo).
6) Myllynen et al. 2008; 7) Tian et al. 2009; 8) Bosman et al. 2005

3.3 Maternal effects of pulmonary exposure to nanoparticles

To confirm particle effects in the mother, we evaluated maternal lung inflammation by cellular response in bronchoalveolar lavage, gene expression in lunge and liver, and genotoxicity in lung and liver.

3.3.2 Maternal lung inflammatory response by cellular profile in BAL fluid

Maternal lung inflammation was evaluated by analysis of the cellular profile in bronchoalveolar lavage fluid (BAL) (Hunninghake et al. 1979).

The response to nanoparticle exposure is characterized by activation of alveolar macrophages and recruitment of polymorphonuclear neutrophils, as a sign of ongoing lung injury. Neutrophil infiltration is usually observed during the acute phase response; however, patients with lung fibrosis or asbestosis exhibit persistent accumulation of high proportions of neutrophils (Hunninghake et al. 1979), supporting the validity of this biomarker for particle toxicology screening. Cellular infiltration into the alveolar spaces is associated with the release of acute inflammatory mediators, which may ultimately lead to a systemic inflammation state.

Bronchoalveolar lavage fluid was collected from mice under Hypnorm-dormicum anaesthesia by washing lungs four times with 0.8 mL 0.9% sterile saline through the trachea. The BAL was immediately stored on ice until the BAL fluid and BAL cells were separated by centrifugation. The BAL cells were re-suspended in a cell medium and the number of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were determined by differential count, as described (Jackson et al. 2011b). Counts were presented relative to the total cell number in the BAL fluid. The total number of cells was determined in a NucleoCounter, following the standard kit procedure (Chemometec, Denmark).

Macrophage Neutrophil Lymphocyte Eosinophil Epithelia

Figure 4. Cells present in the bronchoalveolar lavage. (Modified from Terese Winslow).

3.3.3 Maternal lung and liver gene expression

Maternal lung and liver global gene expression was analyzed by using DNA microarrays. Microarray analysis and gene expression profiling was performed at Mutagenesis Section at Health Canada, by Sabina Halappanavar and her colleagues. Selected significant genes were confirmed by real-time polymerase chain reaction (RT-PCR).

Where the classical toxicological testing approach generates information about adverse effects of tested substance based on observations in tested animals and subsequent extrapolations to expected human responses at lower doses, the current advances in molecular biology allow exploring toxicity pathways and predicting the molecular mechanisms of toxicity. Recent developments in the field of toxicology envision replacing animal testing with high throughput *in vitro* testing in human cells and cell lines, supported by computational toxicology and systems biology. Chemical risk assessment would be based on identifying pathway perturbations and

identifying direct human risks associated with chemical exposure (Andersen and Krewski 2009; Firestone et al. 2010). The new strategy will require a validation process comparing the effects *in vivo* to the effects *in vitro*.

To follow the current developments in toxicology and to obtain a deeper understanding of mechanisms of nanoparticle toxicity, we have analyzed the entire transcriptome with microarray analysis to identify the changes in pathways to be able to predict the health outcomes of exposure. Microarray analysis was performed in total ribonucleic acid (RNA) purified from examined tissue (Figure 5). Individual total RNA samples and universal reference total RNA were used to synthesize double-stranded cDNA and cyanine labelled cRNA. Labelled cRNA targets were *in vitro* transcribed and hybridized to Agilent mouse 4 x 44 oligonucleotide microarrays at 60ºC overnight. Arrays were washed and scanned on an Agilent G2505B scanner. Data were acquired using Agilent Feature Extraction software version 9.5.3.1. A reference design (Kerr 2003; Kerr and Churchill 2007) was used to analyse gene expression microarray data. Complete microarray data were submitted to Gene Expression Omnibus (GSE) at The National Center for Biotechnology Information (NCBI). The most pronounced statistically significant changes to genes were validated by RT-PCR. RNA isolation and cDNA synthesis were performed as described (Bornholdt et al. 2007; Saber et al. 2009). Briefly, total RNA was isolated from tissue lysates, the RNA was treated with DNAse kit (QIAGEN) to remove extra DNA, cDNA was synthesized and quantitative PCR was performed. The gene analysis was performed on ABI PRISM® 7500 sequence detector as described (Saber et al. 2009). Gene expression analysis is a sensitive method quantifying pathway perturbations and therefore it identifies processes induced by the chemical exposure. Changes to gene expression do not always lead to protein changes in the exposed organism. Protein analysis may therefore not identify early or marginal effects, which may have significant biological significance.

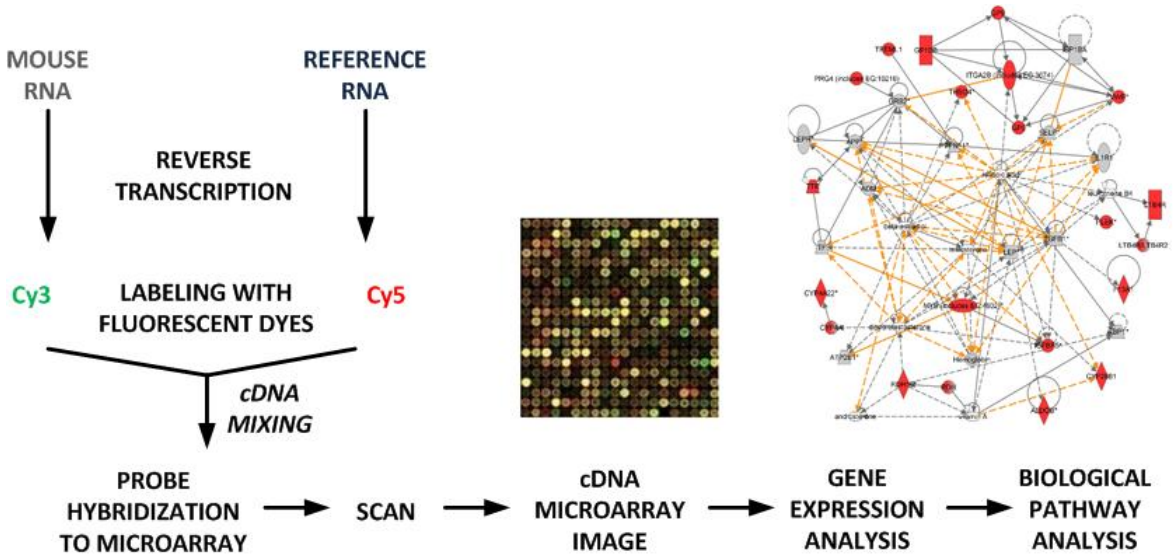


Figure 5. Work flow for DNA microarray gene expression analysis.
RNA is isolated from the tested tissues, followed by reverse transcription, labelled with fluorescent dyes to differentiate the tested and reference genes, cDNA is mixed, samples are applied to the array chip, the chips are scanned, cDNA image is analyzed for gene expression data, followed by gene ontology analysis assigning expressed genes to functional categories.

3.3.4 Maternal levels of DNA strand breaks

Maternal lung and liver levels of DNA strand breaks were analyzed by the comet assay. I have implemented a high throughput protocol at our NRCWE laboratory, allowing the procession of a large number of samples with reduced variation, developed at the Norwegian Institute of Public Health (Gunnar Brunborg and Kristine Bjerve Gutzkow) within the COMICS EU project. I have also improved the preparation of frozen tissues, reducing the result background and improving the assay sensitivity.

Particularly concerning about nanoparticle exposure is that some types of nanoparticles have the ability to induce genotoxicity (Borm et al. 2004; Knaapen et al. 2004; Schins and Knaapen 2007; Brauner et al. 2007; Jacobsen et al. 2009; Møller et al. 2010a). The primary genotoxicity of nanoparticles is believed to be related to their ability to induce reactive oxygen species (ROS) (Jacobsen et al. 2008b), or to form DNA adducts due to surface-bound organic compounds (Jacobsen et al. 2008a), or both. Particle inflammation can also mediate secondary genotoxicity (Knaapen et al. 2004).

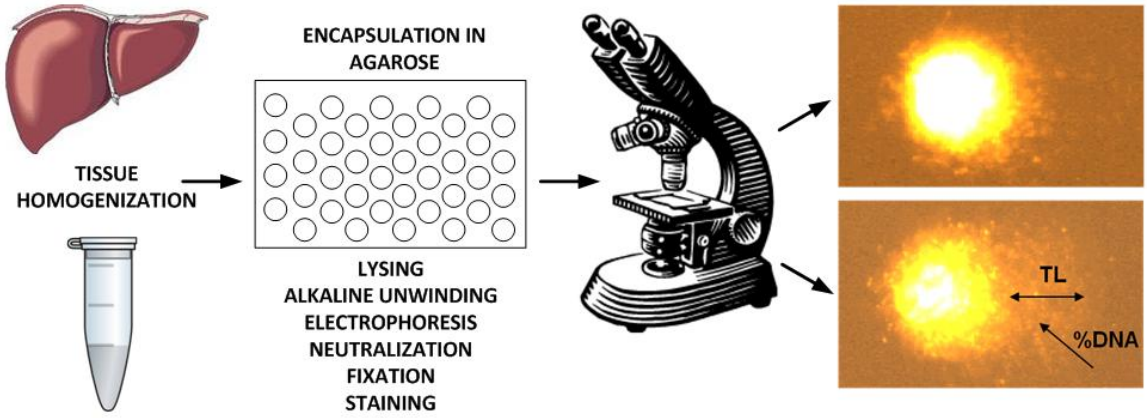


Figure 6. Work flow for comet assay.
Tissue is homogenized, encapsulated in agarose (small agarose drops on the GelBond film), cell membranes are lysed, samples are alkaline treated to unwind DNA and subjected to alkaline electrophoresis to separate DNA strand breaks from the nucleus. Following electrophoresis the samples are neutralized, fixed, stained and randomly selected comets are scored by fluorescent microscopy. (Comet images from Peter Møller).

Various approaches can be used for testing of nanoparticle genotoxicity (Trouiller et al. 2009). One of the approaches, also available at our NRCWE institute, is the alkaline comet assay (McNamee et al. 2000), which is an analysis of the level of DNA strand breaks in the exposed tissue (Figure 6). The comet assay is becoming a common screening strategy used in nanotoxicology, reviewed by (Karlsson 2010). The strand breaks measured by the assay represent a mixture of direct strand breaks, alkaline labile sites and transient breaks in the DNA due to repair processes (Collins 2009). DNA strand breaks are a temporary endpoint, because repair mechanisms can rejoin DNA breaks shortly after exposure (Bornholdt et al. 2002). Comet assay is based on a relatively simple protocol by which cell suspensions are embedded in agarose, cells are lysed and subjected to alkaline electrophoresis where DNA fragments migrate away from the nuclei and form 'comets'. The comets are microscopically analyzed and reported as mean comet tail length, %DNA in tail or tail moment per sample. Data can be recalculated to the number of lesions per million base pair (Forchhammer et al. 2010). The protocol is being

internationally validated to achieve standardized and reliable results (Johansson et al. 2010; Moller 2006; Møller et al. 2010b).

3.4 Developmental endpoints

The main focus of nanotoxicology research is on effects and their mechanisms in adults. The effects of exposure to nanoparticles during pregnancy, prenatal and postnatal development are poorly understood. We assessed maternal toxicity during gestation and lactation, fetotoxicity, as well as developmental toxicity. Gene expression and genotoxicity was also evaluated in offspring liver, to characterize the offspring exposure response.

3.4.1 Gestation and lactation

Endpoints to assess toxicity during gestation and lactation included:

- Maternal weight gain during gestation and lactation, and offspring weight at birth, during lactation and maturation. Growth is a classical toxicological parameter, and changes in growth pattern can indicate acute toxicity in the exposed animals.
- Gestation length. All females were time-mated (mated at the same time-period, confirmed by plug on GD 1) and therefore they should all give birth on the same day. Deviation from the standard indicates changes in gestation length. Since particles are linked to prematurity in epidemiologic studies (Shah and Balkhair 2010), this time point is highly relevant to particle toxicology.
- Number of pregnant animals, number of implantations and litter size. Assuming that all females were equally fertilized at mating, change in percentage of pregnant animals, confirmed by number of uterine implantations, can indicate fetotoxicity.
- Postnatal viability. Maternal exposure or effects of exposure extending into lactation can influence viability after birth.
- Gender ratio. Skewed gender ratio can be an indication of effect on a specific sex of exposed animals. It can be altered by either interference during prenatal sexual development or by selective mortality in one sex.

3.4.2 Sexual maturation

Endpoints to assess sexual differentiation during maturation included:

- Anogenital-distance. AGD is the distance from the anus to the genital bud. It is androgen dependent and it is normally approximately two times longer in male than in female mice. AGD is significantly correlated to body weight. Therefore, we calculated the relative anogenital distance as AGD/cube root of body weight, since the body increases in three dimensions and AGD is a linear measure as suggested by (Gallavan, Jr. et al. 1999). AGD was measured at weaning, based on the study by (Andrade et al. 2006). Significant changes in AGD are recognized as an adverse effect on androgen availability and are related to masculinisation or feminization effects of a chemical.
- Onset of puberty (vaginal opening in females and preputial separation in males). Puberty is a final maturation of the sexual phenotype and it is started by increased secretion of gonadal steroids, which is driven by increased secretion of gonadotropins. Based on a study by (Nelson et al. 1990), we recorded the onset of puberty three times a week for one male and

one female, in litters were possible, between PND 26-40. Changes in the onset of puberty are related to altered neuro-endocrine mechanisms.

3.4.3 Offspring liver gene expression

Offspring liver global gene expression was analyzed by using DNA microarrays by method described earlier for mothers. The focus was on the effects in the newborn offspring, supported by analysis of effects in the exposed mothers.

Fetal liver receives maternal blood directly after placenta passage and it is reported to receive the majority of nanoparticles received by the fetus (Challier et al. 1973). Liver plays an important role in various processes including detoxification, regulation of metabolic homeostasis, and endocrine function. These functions may be compromised during liver injury. Prenatal exposure to toxicants may therefore affect growth, development and developmental programming, causing susceptibility to diseases.

3.4.4 Offspring levels of DNA strand breaks

Offspring liver levels of DNA strand breaks were analyzed by the comet assay by method described earlier for mothers.

During development, frequent cell divisions allow only short time for repair of DNA damage and the immune system is not fully functional. Maternal exposure to air pollution during pregnancy has been associated with increased levels of bulky DNA adducts and micronuclei in umbilical blood of newborns (Pedersen et al. 2009). Moreover, children born and raised in areas with heavy air pollution have systemic inflammation and increased levels of urinary 8-oxodeoxyguanosine, a marker of oxidative damage to DNA (Calderon-Garciduenas et al. 2008; Švecová et al. 2009). Early-life exposure might therefore predispose to cancer and other diseases later in life (Barton et al. 2005).

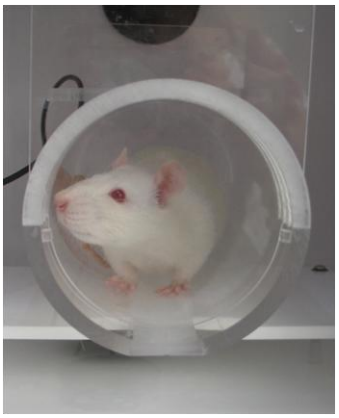
3.4.5 Developmental neurotoxicity

It is becoming more evident that prenatal exposure to chemicals can influence the developing nervous system. Developmental neurotoxicity testing is therefore one of the additional endpoints included in the new 'extended one-generation reproductive toxicity study' developed under REACH (Spielmann 2009). Maternal exposure during pregnancy to industrial or traffic-related air pollution was associated with adverse effects on cognitive development of their children (Freire et al. 2010; Tang et al. 2008). Changes in the brain and neurofunction are reported in animal studies with diesel exhaust exposure (Masao Sugamata 2006a; Masao Sugamata 2006b; Suzuki et al. 2010; Takeda et al. 2004; Yokota et al. 2009) and titanium dioxide exposure (Shimizu et al. 2009; Takahashi et al. 2010; Takeda et al. 2009). On one hand, the developing brain *in utero* may not be exposed to high doses of nanoparticles directly, due to restricted transport through several membrane barriers; though some nanoparticles have been found in the brain of offspring weeks after prenatal exposure (Gao et al. 2011; Takeda et al. 2009). On the other hand, the developing brain may be especially vulnerable to nanoparticle induced oxidative stress, because induced developmental mismatch in the sequential antioxidant enzyme cascade would contribute to the free radical toxicity (or reduced anti-oxidant capacity) in immature cerebral white matter, as suggested in (Hougaard et al. 2011). Therefore, it is possible that uncontrolled dispersion of engineered nanoparticles will cause developmental neurotoxicity by neurobehavioral testing.

Methods used to test developmental neurotoxicity:

- Acoustic startle test (Figure 7). A test of sensorimotor processes. Animals placed in an acoustic chamber were exposed to a series of startle stimuli (sounds), which were designed to release the startle reflex (response to unexpected stimuli, measured by the magnitude of movement). The startle reflex follows basic auditory pathway and ends by contraction of muscles and movement away from the stimuli. Startle stimuli were sounds with a combination of different intensity, supplemented by pre-pulse stimuli (warning mechanism). Habituation to sound, amplitude of startle response and reaction after pre-pulse stimulation were analyzed.

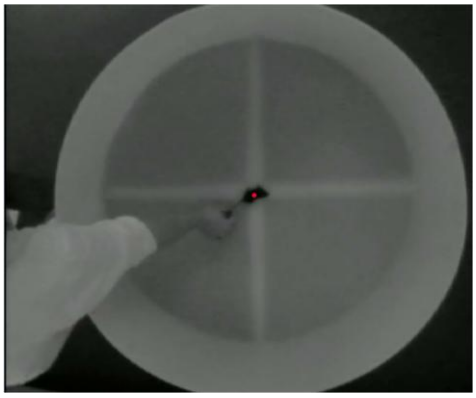
A)



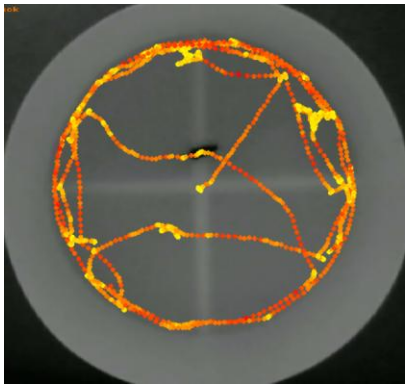
B)

Figure 7. Acoustic startle test.
A) Startle chamber
B) Rodent ready for an acoustic startle test. (Photo from Sanna Lemming Kjær)

- Open field test (Figure 8). A test that evaluates temperament and emotionality exhibited by locomotor activity. It is used as a measure of anxiety. Animals were placed in a brightly illuminated field and exploratory behaviour was recorded. Animal activity as well as duration in pre-defined zones was analyzed.



A)

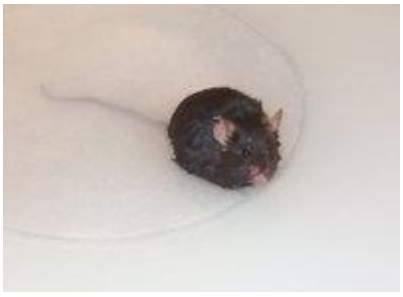


B)

Figure 8. Open field test.
A) Start
B) End after three minute exploratory behavior. (Images from Karin S. Hougaard)

- Morris water maze (Figure 9). A test of place-learning and working memory (located in hippocampus). Animals were placed in a water pool with an invisible escape platform placed in the water surrounded by visual landmarks. The animal swam around the pool in search of an exit (platform). In following trials (with the same spatial setup and with altered platform positions), the memory and learning were analyzed.

A)



B)

Figure 9. Morris water maze.
A) Water maze with under water platform. B) Mouse on the escape platform. (Images from www.scholarpedia.org).

4. EXPERIMENTAL DESIGN

Four developmental studies were included in this work, summarized in Figure 10 and Table 4. Two studies assessed maternal inhalation exposure to titanium dioxide (UV-Titan) and carbon black (Printex 90). Printex 90 was also assessed by dose-response study with maternal exposure by intratracheal instillation. Prior to initiation of Printex 90 dose-response study, the intratracheal instillation protocol with short-term isoflurane anaesthesia was evaluated for effects in the mothers and their offspring.

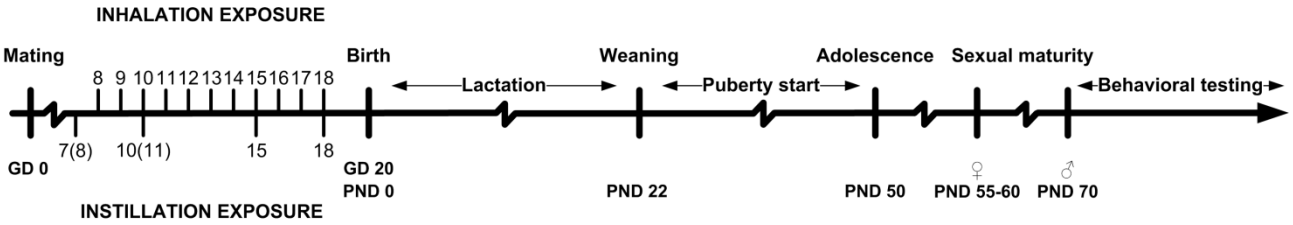


Figure 10. Overview of experimental design.

	Tissues collected (PND)					Sexual maturation testing (PND)		Neurobehavioral testing (PND)		
	Females	Newborns	Offspring at weaning	Dams	Adoles-cents	AGD	Puberty start	Water maze	OF	Startle
4.1	3	2	23-24	24-25	-	-	-	♂ 71 &99 (11, 15 wk) ♀ 78 &106 (12, 16 wk)	94 (14 wk)	♂ 122 (18 wk) ♀ 129 (19 wk)
4.2	1	2	22	23	-	22	26-43	-	-	-
4.3	3	2	22-23	22-23	50	-	-	-	-	-
4.4	1-2	2	23	24-25	47	23	26-40	-	72-73 (11 wk)	74-75 (11wk)

Table 4. Overview of timing of tissue collection, sexual maturation testing and neurobehavioral testing.
Gestation day (GD), Post natal day (PND, days after birth), PND 0 = day of birth, and two days after the last exposure for time-mated mice (GD 20), Anogenital distance (AGD), Open field test (OF).

4.1 Titanium dioxide inhalation study (Paper I, II and III)

We have investigated the effect of maternal pulmonary exposure to a classical model compound in nanotoxicology (titanium dioxide, UV-Titan specifically) on the offspring exposed *in utero*. Time-mated mice (C57BL/6BomTac) were exposed by inhalation for 1 h/day to 42 mg UV-titan/m^3 aerosolized powder or filtered air during gestation days (GD) 8-18. Endpoints included cell composition in bronchoalveolar lavage (BAL, a marker of maternal lung inflammation) early after exposure (5 days after exposure) and at weaning (26-27 days after exposure), UV-titan deposition and distribution in maternal and offspring tissues, classical gestation and lactation parameters, offspring neurofunction and reproduction (PAPER I), toxicogenomic study of early effects in the non-pregnant time-mated females (5 days after exposure) (PAPER II), toxicogenomic study in offspring (2 days after birth), and maternal and offspring genotoxicity evaluated as level of DNA strand breaks in BAL and liver cells (PAPER III).

4.2 Pilot instillation study (Paper IV)

In order to establish a protocol for studying the effects of maternal pulmonary exposure to nanoparticles in developmental toxicity studies, we evaluated the effects of multiple intratracheal sham instillations under short-term isoflurane anaesthesia. Time-mated mice (C57BL/6BomTac) were anesthetized with isoflurane and intratracheally instilled with saline containing 10% bronchoalveolar lavage (BAL) on gestation days (GD) 8, 11, 15 and 18. In addition, the early effect of the procedure was assessed in naïve female mice. Control animals were not handled. Dams were followed until weaning and the offspring were observed from birth through sexual maturation. The cell composition of BAL was examined in the females early after treatment (3 days) and in the dams at weaning (25 days). Maternal and offspring genotoxicity was evaluated as a level of DNA strand breaks in BAL and liver cells.

4.3 Carbon black inhalation study (Paper V)

Carbon black (Printex 90) was evaluated for effects of maternal pulmonary exposure on the offspring exposed *in utero*. Similar to the titanium dioxide study, time-mated mice (C57BL/6BomTac) were exposed by inhalation for 1 h/day to 42 mg Printex 90/m^3 aerosolized powder or filtered air during GD 8-18. Endpoints included cell composition in BAL early after exposure (5 days after exposure) and at weaning (24-25 days after exposure), classical gestation and lactation parameters, and genotoxicity was evaluated as a level of DNA strand breaks in BAL and liver cells.

4.4 Carbon black instillation study (Paper V and VI)

After assessing the effects of maternal inhalation exposure to carbon black (Printex 90) in the exposed mothers and offspring, a study was initiated to characterize the dose-response relationship. Time-mated C57BL/6BomTac mice were exposed by four intratracheal instillations on GD 7, 10, 15 and 18, with total doses of 11, 54 and 268 µg Printex 90/animal. The highest dose was chosen to be comparable to the estimated deposited dose in the pulmonary region in mice from the inhalation study. Endpoints included cell composition in BAL early after exposure (3-4 days after exposure) and at weaning (26-27 days after exposure), classical gestation and lactation parameters, and genotoxicity was evaluated as a level of DNA strand breaks in BAL and liver cells (PAPER V), and offspring sexual maturation and neurofunction (PAPER VI).

5. RESULTS

The following sections present the results of the four studies. The results are summarized as papers as well as overview tables of common results.

Paper I and II

Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice.
Hougaard KS, Jackson P, Jensen KA, Sloth JJ, Loschner K, Larsen EH, Birkedal RK, Vibenholt A, Boisen AM, Wallin H & Vogel U.
Particle and Fibre Toxicology, 2010, 7:16.

Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: A toxicogenomic study.
Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard KS, Vogel U, Yauk CL & Wallin H.
Environmental and Molecular Mutagenesis, 52(6):425-39.

Inhalation exposure to surface-coated nanosized titanium dioxide (42.4 ± 2.9 mg UV-Titan/m^3 for 1hr/day on gestation days 8-18) induced persistent maternal inflammation measured by large neutrophil influx in BAL in the exposed females and in dams. Inflammation persisted 27 days after exposure termination (non-pregnant females 5 days after exposure: 19-fold increase; dams 25-27 days after exposure: 3-fold increase), which was the last tested time point (Hougaard et al. 2010).

Moreover, the analysis of total gene expression in the lungs of exposed non-pregnant females, 5 days after exposure termination, revealed increased levels of lung mRNA for acute phase serum amyloid A-1 and serum amyloid A-3, and higher levels of several lung C-X-C and C-C motif chemokines and miRNAs (Halappanavar et al. 2011).

Despite the inflammation and acute phase response in the lung, gene expression in the female liver was relatively unchanged (Halappanavar et al. 2011).

Based on work of others, translocation of UV-titan from the lung into circulation was expected to be low, only a fraction of a percent would get beyond the lung cavity or pass over the gastrointestinal mucosa, and therefore the distribution to the fetus would be minimal. Thirty- to thirty-four percent of the predicted pulmonary-deposited UV-titan remained in the maternal lung, determined by the titanium content in the tissue. Titanium content was similar to the control levels in the maternal liver, offspring liver and milk content in the offspring stomach (values were below detection limit) (Hougaard et al. 2010).

Maternal pulmonary exposure to UV-titan did not significantly affect gestation, lactation and offspring development (endpoints: maternal weight gain during gestation and lactation, gestation length, offspring weight at birth, during lactation and maturation, litter size, gender ratio, number of implantations, and postnatal viability) (Hougaard et al. 2010).

Exposed offspring however exhibited minor behavioural changes after reaching adulthood (exposed offspring tended to avoid the central zone in open field test and exposed female

offspring displayed enhanced pre-pulse inhibition in an acoustic startle test and exposed male offspring had slightly delayed reproductive ability (time-to-first litter) (Hougaard et al. 2010).

Paper III

Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan): Effects in prenatally exposed offspring on hepatic DNA damage and gene expression.
Jackson P, Halappanavar S, Hougaard KS, Yauk C, Williams A, Lamson JS, Andersen O, Wallin H and Vogel U.
Nanotoxicology, 2011, submitted.

The effect of maternal pulmonary exposure to titanium dioxide (UV-Titan designed for use in the paint and lacquer industry) on prenatally exposed offspring were evaluated. Time-mated mice (C57BL/6BomTac) were exposed by inhalation for 1 h/day to 42 mg UV-titan/m^3 aerosolized powder or filtered air during gestation days (GD) 8-18, previously presented by (Hougaard et al. 2010). Levels of DNA strand breaks were evaluated using comet assay, quantified in bronchoalveolar lavage (BAL) cells and liver cells of the time-mated mice, and in liver cells of the offspring. In parallel, changes in gene expression in the liver of offspring using DNA microarrays were analyzed. We demonstrate that UV-Titan did not induce DNA strand breaks in the time-mated mice or their offspring. Transcriptional profiling of newborn liver tissue revealed changes in the expression of genes related to the retinoic acid (RA) signalling pathway in the females, while gene expression in male offspring was relatively unaffected by exposure.

Paper IV

An Experimental Protocol for Maternal Pulmonary Exposure in Developmental Toxicology.
Jackson P, Lund SP, Kristiansen G, Andersen O, Vogel U, Wallin H & Hougaard KS.
Basic & Clinical Pharmacology & Toxicology, 2011, 108: 202-207.

The intratracheal sham instillation under short-term isoflurane anaesthesia procedure did not affect gestation or offspring viability, growth and sexual maturation of the offspring. Lung markers of inflammation and DNA damage were comparable in control and treated time-mated mice. There was no induction of DNA damage in livers of the anesthetized and instilled females, dams and their offspring. Intratracheal instillation under isoflurane anaesthesia did not induce observable effects in pregnant mice or their offspring. This procedure was found suitable for use in studies of developmental toxicity.

Paper V

Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: Effects on liver DNA strand breaks in dams and offspring.
Jackson P, Hougaard KS, Boisen AMZ, Jacobsen NR, Jensen KA, Møller P, Brunborg G, Gutzkow KB, Andersen O, Loft S, Vogel U and Wallin H.
Nanotoxicology, 2011, DOI: 10.3109/17435390.2011.587902.

Effects of inhalation exposure to nanosized carbon black (42.4 ± 2.9 mg Printex 90/m^3 for 1hr/day on gestation days 8-18), and effects in a follow-up study with exposure by 4 intratracheal instillations of Printex 90 dispersed in Millipore water (on GD 7, 10, 15 and 18, with total doses of 11, 54 and 268 μg/animal) were evaluated. The protocol described in (Jackson et al. 2011a) was used. Exposures induced persistent maternal inflammation measured by large neutrophil influx in BAL in the exposed females and in dams. Inflammation persisted 24-26 days

after exposure termination, which was the latest tested time point. The lung inflammation in the inhalation exposed mice was 2 to 6-fold smaller compared to similar instilled pulmonary doses (inhalation females 5 days after exposure: 11.4-fold increase and dams 24 days after exposure: 11.6-fold increase; instillation females 3 days after exposure: 28.7-fold increase and dams 26 days after exposure: 60.9-fold increase). This result corresponds with results reported previously; instillation induces stronger lung inflammation compared to inhalation. Maternal inhalation exposure to Printex 90 induced DNA strand breaks in the liver of time-mated mice and in the offspring even weeks after end of exposure. There were no changes in the levels of DNA strand breaks in mice intratracheally instilled with similar pulmonary doses. Despite this, we did not observe any gestational or developmental toxicity in the offspring.

Paper VI

Prenatal exposure to carbon black (Printex 90):
Effects on sexual development and neurofunction.
Jackson P, Vogel U, Wallin H and Hougaard KS.
Basic & Clinical Pharmacology & Toxicology, 2011, DOI: 10.1111/j.1742-7843.2011.00745.x.

Effects of maternal pulmonary exposure to Printex 90 (intratracheal instillations of Printex 90 dispersed in Millipore water on GD 7, 10, 15 and 18, with total doses of 11, 54 and 268 µg/animal) on offspring sexual maturation and neurodevelopment were evaluated. Anogenital distance measured on all offspring at weaning did not significantly differ between offspring prenatally exposed to Printex 90 and their controls. Female offspring prenatally exposed to 11 µg Printex 90/animal entered puberty significantly earlier compared to controls (time of vaginal opening), while male offspring entered puberty at a similar time in all exposed groups and in the controls (time of preputial separation). Since the data did not indicate a dose response, this may be a chance finding. The female offspring prenatally exposed to 268 µg Printex 90/animal displayed altered habituation pattern during the Open field test, whereas no effects on acoustic startle were observed.

Study	Maternal		Gestation & Lactation	Offspring			Ref.
	Females (3-5 days after exposure)	Dams (24-27 days after exposure)		Newborn	At weaning	Adolescents	
1)	*Lung inflammation:* 19-fold ↑ PMN *Ti content in tissues:* 38±6 mg/kg Ti in lung <0.5 mg/kg Ti in liver *Gene expression:* Lungs - Inflammation, immune response, acute phase response Liver - Minor changes *Genotoxicity:* BAL - none Liver - none	*Lung inflammation:* 3-fold ↑ PMN *Ti content in tissues:* 33±18mg/kg Ti/lung 0.5±0.3 mg/kg Ti/liver *Genotoxicity:* BAL - none Liver - none	No effect	*Ti content in tissues:* <0.4 mg/kg Ti/liver <1 mg/kg Ti/milk *Genotoxicity:* Liver – none (high background) *Gene expression:* *Liver:* Changes in RA signalling (♀)	*Ti content in tissues:* <0.4 mg/kg Ti/liver *Genotoxicity:* Liver – none	*Behavioral changes:* Activity Sensorimotor processes	I II III
2)	*Lung inflammation:* No effect *Genotoxicity:* No effect	*Lung inflammation:* No effect *Genotoxicity:* No effect	No effect	*Genotoxicity:* Liver – none	*Genotoxicity:* Liver - none	-	IV
3)	*Lung inflammation:* 11-fold ↑ PMN *Genotoxicity:* BAL - none Liver - 1.3-fold ↑	*Lung inflammation:* 12-fold ↑ PMN *Genotoxicity:* BAL - none Liver - 1.6-fold ↑	No effect	*Genotoxicity:* Liver – none (high background)	*Genotoxicity:* Liver - 1.4-fold ↑	*Genotoxicity:* Liver - 1.5-fold ↑	V
4)	*Lung inflammation:* 29-fold ↑ PMN *Genotoxicity:* BAL - none Liver – none	*Lung inflammation:* 61-fold ↑ PMN *Genotoxicity:* BAL - none Liver – none	No effect	*Genotoxicity:* Liver – none (high background)	*Genotoxicity:* Liver - none	*Genotoxicity:* Liver – none Behavioral changes: Habituation Sexual maturation: Minor effect	V VI

Table 5. Selected result overview.

Polymorphonuclear neutrophil (PMN), Gestation & Lactation (weight gain during gestation and lactation, gestation length, offspring weight at birth, during lactation and maturation, litter size, gender ratio, number of implantations, and postnatal viability), Retinoic acid (RA), Genotoxicity (levels of DNA strand breaks by comet assay)

1) TiO2 inhalation study (42 mg/m^3 UV-titan 1h/day on GD 8-18, 1.7*106 n/cm^3; peak-size 97 nm)

2) Pilot instillation study (intratracheal sham instillations under short-term isoflurane anaesthesia on GD 8, 11, 15 &18)

3) Carbon black inhalation study (42 mg/m^3 Printex 90 on GD 8-18, 4.1*106 n/cm^3; peak-size 45 nm)

4) Carbon black instillation study (11, 54, 268 µg/animal Printex 90 on GD 7, 10, 15 & 18, zeta-size 140 nm; peak-size 55 nm)

	Inhalation		Inhalation		Instillation			
	Control	UV-Titan 42 mg/m^3 1h/dayx11days[a]	Control	Printex 90 42 mg/m^3 1h/dayx11days[b]	Control	Printex 90 11 µg/animal[c]	Printex 90 54 µg/animal[c]	Printex 90 268 µg/animal[c]
				(x 10^{-3})				
Females 3-5 days after exposure								
Total count	166.5±13.6	202.0±18.1	66.1±6.0	96.9±10.7*	168.0±45.0	175.5±15.6	200.4±19.2	292.0±54.2
Dead cells	14.0±2.2	18.7±2.0	1.1±0.4	0.8±0.3	14.0±7.2	12.5±4.3	11.2±5.2	18.7±5.9
Macrophages	145.0±12.1	97.7±10.2**	56.4±4.8	69.2±6.7	139.7±42.3	128.8±12.0	154.5±20.5	115.1±22.6
Neutrophils	4.6±2.9	88.4±11.8***	1.0±0.6	11.5±3.1**	3.7±2.1	5.5±1.7	9.3±4.8	105.8±26.2***
Lymphocytes	0.9±0.3	5.6±1.4**	0.8±0.2	2.6±0.6*	3.5±1.0	7.2±3.1	7.2±1.5	27.0±9.7**
Eosinophils	0.1±0.1	0.7±0.3	0.5±0.3	4.0±2.6	5.9±3.7	22.2±10.0	11.4±6.4	33.0±9.1
Epithelial cells	16.0±2.6	9.7±1.0	7.5±1.3	9.6±1.6	15.2±4.2	11.8±2.7	18.0±3.4	10.7±3.8
Dams at weaning 24-26 days after exposure								
Total count	171.6±19.7	177.0±14.3	44.1±3.4	54.8±4.3(*)	137.3±5.6	93.4±8.3	154.5±10.8	361.3±43.4***
Dead cells	14.0±10.6	25.9±3.14**	0.7±0.1	0.5±0.1	6.9±1.9	3.4±2.3	4.5±2.2	19.4±2.8***
Macrophages	134.5±14.7	102.9±8.2	31.9±3.3	37.7±3.2	113.7±4.9	83.0±7.5	128.8±9.5	131.8±16.4
Neutrophils	15.4±10.8	50.5±7.8*	0.4±0.1	4.8±1.1***	2.9±0.9	0.9±0.2	5.2±1.7	173.3±22.7***
Lymphocytes	3.7±1.1	6.2±1.1	1.4±0.3	2.0±0.5	7.7±2.1	3.4±0.7	6.9±1.1	34.1±8.4***
Eosinophils	1.8±0.8	1.7±0.8	0.4±0.3	0.7±0.5	2.3±1.0	0.3±0.2	2.8±2.3	1.3±0.4
Epithelial cells	16.2±1.7	15.7±3.0	10.0±0.9	9.6±0.8	10.8±1.8	5.8±1.2	10.7±1.1	20.7±4.5

Table 6. Bronchoalveolar lavage cell composition of females or dams exposed to Printex 90 by inhalation or instillation and control mice.
The total cell count in BAL samples from Printex 90 inhalation study is manually counted in hemocytometer with Trypan blue dye, while the total cell count in BAL samples from UV-Titan inhalation study and Printex 90 instillation study is determined in a NucleoCounter by standard kit procedure. Cell composition was determined by differentially counting 200 cells in samples stained with May-Grünwald-Giemsa stain.
Data presented as mean cell number x 10^3 in BAL ± SEM.
(*)p~0.05, *p<0.05, **p<0.01, ***p<0.001
a Total inhaled dose 840 µg/animal; estimated pulmonary deposition 73 µg/animal
b Total inhaled dose 826 µg/animal; estimated pulmonary deposition 287 µg/animal
c Final instilled dose

	Inhalation		Inhalation		Instillation			
	Control	UV-Titan 42mg/m^3 1h/dayx11days[a]	Control	Printex 90 42 mg/m^3 1h/dayx11days[a]	Control	Printex 90 11 µg/animal[c]	Printex 90 54 µg/animal[c]	Printex 90 268 µg/animal[c]
Females 3-5 days after exposure								
BAL	0.45±0.06	0.57±0.03	0.55±0.06	0.70±0.10	0.90±0.04	0.91±0.09	0.78±0.07	0.72±0.07
Liver	0.43±0.07	0.41±0.08	2.57±0.19	3.25±0.21*	0.66±0.06	0.55±0.07	0.57±0.07	0.52±0.07
Dams at weaning 24-27 days after exposure								
BAL	0.60±0.05	0.61±0.05	0.58±0.05	0.60±0.04	0.62±0.02	0.69±0.06	0.58±0.01	0.50±0.03**
Liver	0.61±0.10	0.66±0.08	1.24±0.07	1.94±0.11***	0.52±0.05	0.55±0.09	0.48±0.02	0.58±0.04
Offspring								
Liver newborns PND 2	0.68±0.11	0.62±0.14	3.50±0.28	3.64±0.37	0.66±0.02	0.65±0.03	0.73±0.02	0.64±0.03
Liver offspring at weaning PND 22-23	0.39±0.05	0.39±0.04	1.13±0.09	1.56±0.08***	0.39±0.03	0.41±0.04	0.42±0.04	0.42±0.03
Liver adolescents PND 50 (47)	-	-	1.10±0.16	1.69±0.14**	0.54±0.04	0.48±0.04	0.47±0.04	0.52±0.03

Table 7. Level of DNA strand breaks for females, dams or offspring exposed to Printex 90 by inhalation or intratracheal instillation and control mice.
Bronchoalveolar lavage (BAL); Post natal day (PND, days after birth).
Data are presented as mean number of lesions per 106 base pair ± SEM (calculated from %DNA results); offspring data are calculated as litter average, when sibling liver tissues were analyzed at the same collection point.
***p<0.05, ** p<0.01, *** p<0.001**
a Total inhaled dose 840 µg/animal; estimated pulmonary deposition 73 µg/animal
b Total inhaled dose 826 µg/animal; estimated pulmonary deposition 287 µg/animal
c Final instilled dose

End point	Inhalation		Inhalation		Instillation			
	Control	UV-Titan 42 mg/m^3 1h/dayx11days[a]	Control	Printex 90 42 mg/m^3 1h/dayx11days[b]	Control	Printex 90 11 µg/animal[c]	Printex 90 54 µg/animal[c]	Printex 90 268 µg/animal[c]
Time mated	22	23	22	22	24	17	17	22
Number of litters PND1	13	14	18	17	20 (-1[†])	10 (-4[†])	11 (-2[†])	20
Dam gestation weight gain, exposure days (g)	10.5±0.5	10.1±0.5	11.3±0.6	10.6±0.7	11.9±0.3	12.0±0.7	11.0±0.6	10.8±0.4
Dam lactation weight gain, during full lactation (g)	5.1±0.4	5.7±0.3	4.3±0.3	4.3±0.4	4.4±0.4	4.4±0.8	4.9±0.6	4.9±0.5
Gestation length (days)	20.2±0.1	20.0±0.0	19.9±0.1	20.1±0.1	20.0±0.0	20.0±0.0	20.1±0.1	20.1±0.1
Implantations	8.3±0.6	8.6±0.3	7.4±0.6	7.6±0.5	9.3±0.2	9.3±0.6	8.1±0.6	8.3±0.5
Implantation loss (%)	20.0±4.7	22.0±3.3	14.6±3.9	17.0±4.6	21.5±2.5	30.7±8.4	25.6±7.8	24.2±3.7
Live pups per litter PND 1	6.2±0.4	6.5±0.4	5.1±0.9	5.0±0.7	7.3±0.3	7.0±0.7	6.8±0.5	6.2±0.3
Offspring dead during lactation (%)	5.1±4.0	10.7±4.5	4.1±2.0	1.7±1.2	5.5±2.1	10.0±10.0	1.3±1.3	3.3±1.9
Birth weight females (g)	1.3±0.04	1.3±0.03	1.4±0.04	1.41±0.05	1.3±0.03	1.3±0.03	1.3±0.04	1.3±0.03
Birth weight males (g)	1.3±0.04	1.3±0.03	1.4±0.03	1.42±0.05	1.4±0.02	1.4±0.04	1.4±0.04	1.4±0.04
Weight gain females, from birth - weaning (g)	7.1±0.3	7.0±0.3	8.0±0.2	7.7±0.3	6.4±0.3	7.3±0.5	7.0±0.5	7.5±0.3
Weight gain males, from birth - weaning (g)	7.4±0.3	7.4±0.3	8.3±0.2	7.9±0.4	7.1±0.3	7.3±0.6	7.9±0.6	7.9±0.2
Sex ratio [§]	0.5±0.04	0.5±0.03	0.4±0.06	0.5±0.06	0.5±0.04	0.7±0.06	0.6±0.06	0.5±0.04
AGD at weaning females (mm) (n=17, 8, 8 and 14)					4.2±0.1	4.8±0.3	4.2±0.2	4.4±0.1
AGD at weaning males (mm) (n=17, 8, 8 and 14)					6.7±0.2	7.2±0.4	7.2±0.2	6.9±0.1
Relative AGD at weaning females					2.12±0.1	2.3±0.1	2.1±0.1	2.1±0.0
Relative AGD at weaning males					3.3±0.1	3.5±0.1	3.4±0.1	3.3±0.0
Vaginal opening (n=8, 8, 7, 4)					37.7±2.3	35.4±0.8**	37.6±1.1	37.0±1.8
Preputial separation (n=10, 4, 7, 7)					34.7±1.1	33.0±0.0	33.8±0.9	35.8±0.6

Table 8. Gestation, lactation and developmental parameters of nanoparticle exposed dams or offspring, and their respective controls.
Dams were allowed to deliver their pups on gestation day (GD) 20, equal to post natal day (PND) 0. Weights of dams and individual offspring were recorded on PND 1 (2), and offspring were counted and sex determined. Time-mated mice were examined for the number of implantation sites, allowing for calculation of implantation loss. Females that did not give birth or had small litters were killed on PND 3 (1) and the dams on PND 22 (24). Data are expressed as mean ± SEM.
†Died during instillation
§Females in litter (%)
a Total inhaled dose 840 µg/animal; estimated pulmonary deposition 73 µg/animal
b Total inhaled dose 826 µg/animal; estimated pulmonary deposition 287 µg/animal
c Final instilled dose

6. DISCUSSION

The purpose of this PhD work was to assess the effects of pulmonary exposure to nanoparticles during pregnancy on the mother/dam and her offspring. Classical gestational, lactational and developmental parameters were evaluated, supported by assessment of maternal inflammation, maternal and offspring gene expression, maternal and offspring levels of DNA strand breaks, and offspring neurotoxicity.

6.1 Exposure characterization

Two different nanoparticles were evaluated: titanium dioxide (UV-Titan) and carbon black (Printex 90). Both UV-Titan and Printex 90 were assessed in similar whole-body inhalation exposures 42 mg/m^3 for 1hr/day on gestation days 8-18. Inhalation exposures were followed by a dose response study, achieved by instilling specific doses of nanoparticles suspended in a liquid solution into the lumen of the airways, while the animal was under general anaesthesia. Three doses of Printex 90 were intratracheally instilled on gestation days 7, 10, 15 and 18. The final, highest instilled dose was designed to be comparable to the expected inhaled pulmonary dose in the Printex 90 inhalation study. This method could be expected as more stressful to the pregnant mice; however, it did not induce observable effects in pregnant mice or their offspring (Jackson et al. 2011a). Because there are virtually no data on developmental toxicity studies, the chosen exposures were relatively high. Still, the daily exposures corresponded to approximately one-and-a-half day exposure that Danish workers would experience at the current 8-hr time weighed average occupational exposure limit (6 mg Titanium dioxide/m^3 and 3.5 mg carbon black/m^3, The Danish Working Environment Authority 2007).

It is important to include careful physico-chemical characterization of the exposure to establish the background for toxicity assessment, because nanoparticles, similarly to other chemicals, are different from each other, even if they share similar characteristics. In our studies, the gravimetric doses of UV-Titan and Printex 90 were very similar and the total inhaled masses were estimated to be comparable in both studies (840 and 826 µg/animal UV-Titan and Printex 90, respectively). However, the exposures differed in particle size. As displayed in Figure 11 and Figure 13, and summarized in Table 9 and Table 10 (bellow), UV-Titan dispersion contained large agglomerates, while Printex 90 air dispersion was more in the nanosize. The mean particle size distribution in number of aerosolized UV-Titan was 97 nm, while Printex 90 was 45 nm. Less than one percent of mass size distribution of UV-Titan was below 100 nm, while it was five percent of Printex 90. Seventy-five percent of UV-Titan agglomerates were greater than 1600 nm, while Printex 90 agglomerates were 200 nm. This distribution naturally affected the particle number concentration, and there were half the amount of nanoparticles in the UV-Titan aerosol compared to Printex 90.

Particle size affects the deposition in the lung. Particles with size above 10 µm are deposited in nose and throat, particles between 2 and 10 µm are deposited in the throat, and particles bellow 200 nm are deposited in the pulmonary region of the alveoli (Figure 2). There is a limited transport up from the pulmonary region. Because of inefficient clearance by alveolar macrophages, nanoparticles persist in the deep lung for prolonged periods (Oberdöster et al. 2005). Particles from the extra-pulmonary region are transported up by the mucociliary escalator and swallowed. When we have calculated the particle deposition in the body, based on

the model revised from (Jacobsen et al. 2009), a lower pulmonary deposition was achieved with UV-Titan compared to Printex 90 (73 compared to 287 µg/animal, for UV-Titan and Printex 90, respectively). The estimated intragastric deposition of UV-Titan was higher compared to Printex 90 (365 compared to 137 µg/animal, for UV-Titan and Printex 90, respectively). Because the time-mated mice were exposed by whole-body inhalation exposure to avoid restraint stress during pregnancy, it can be expected that the received dose in the gastrointestinal tract was even greater due to fur grooming. However, based on previously published work, we assume that the agglomerates likely passed though the digestive system without major translocation to the liver or the rest of the body. Microparticles or nanoparticles administered to rats by oral gavage were mostly excreted by faecal excretion; only a fraction was taken up by epithelial tissues of small intestine and transported to lymph nodes (Carr et al. 1996; Kreyling et al. 2002).

To make inhalation and instillation exposure to Printex 90 comparable, we estimated the dose deposited in the pulmonary region by inhalation, and intratracheally instilled a similar dose (268 µg/animal) as the greatest of the three instilled doses. Therefore, the received maternal dose was smaller, compared to the total inhaled mass 826 µg/animal. When comparing the actual particle size during the two exposures, the instilled Printex 90 nanoparticles dispersed in Milipore water resembled the aerosolized Printex 90. The hydrodynamic number size-distribution peaked between 50-60 nm in the instilled solution (Figure 12), which was comparable to the particle size distribution number 45 nm measured in the exposure atmosphere. Thus, the particle effects in maternal lung could be expected to be similar. Printex 90 instillation exposure probably also resulted in small intragastric deposition, however smaller compared to the inhalation exposure.

A fraction of nanoparticles would translocate from the maternal lung, based on the particle size and properties (Kreyling et al. 2010; Kreyling et al. 2002; Kreyling et al. 2009; Sadauskas et al. 2009; van Ravenzwaay et al. 2008). To evaluate the post inhalation particle translocation in the maternal and offspring body, we have analyzed titanium (Ti) content in maternal lung and liver, in offspring liver, and in milk content in newborn stomachs. Titanium was determined days and weeks after exposure by quadrupole-based inductively coupled plasma mass spectrometer (ICPMS) (Hougaard et al. 2010). We have determined that maternal lung tissue contained 38 and 33 mg Ti/kg lung (90 and 78 mg UV-Titan/kg lung, given that 100 mg UV-Titan contains 42.44 mg Ti), 5 and 26-27 days after exposure, respectively. The estimated pulmonary deposition based on the model described by (Jacobsen et al. 2009) was calculated to 112 mg Ti/kg lung (264 mg UV-Titan/kg lung, assuming that lung weight was 274 mg). Thus, approximately 34 and 33% of the predicted pulmonary titanium (34 and 30% UV-Titan) was accounted for 5 and 26-27 days after exposure, respectively. We have further determined that maternal liver tissue contained less titanium than was detectable by the analytical method (0.5 mg Ti/kg). Assuming that liver tissue weight is between 1-2 g (difference between non-pregnant and pregnant mice), the minimum amount of titanium we could detect would be 0.5-1.0 µg/mouse liver. We estimated that 72.5 µg UV-Titan/animal would deposit in the pulmonary region and could translocate further. Estimated by (van Ravenzwaay et al. 2008), pulmonary translocation and accumulation in the liver would occur at 0.025%, thus the titanium content in the liver would be low (<1.8 ng/animal). Taking in account the detection limit 0.5-1.0 µg/mouse liver, 0.7-1.4% of nanoparticles would have to translocate from the maternal lung to be detected in the maternal liver. Thus, the low titanium content was not possible to detect in the liver of

time-mated mice exposed to UV-Titan by inhalation. Similar for detecting the titanium content in the offspring liver, the detection limit was high (0.4 Ti/kg liver). Assuming that the nanoparticles in the maternal circulation would have to also cross the placenta barrier to reach the offspring liver, the detection limit was too high. We had no estimates how many particles could be found in the maternal milk; however, the detection limit of 1 mg Ti/kg tissue made it impossible to detect the small content of titanium possibly present in the milk.

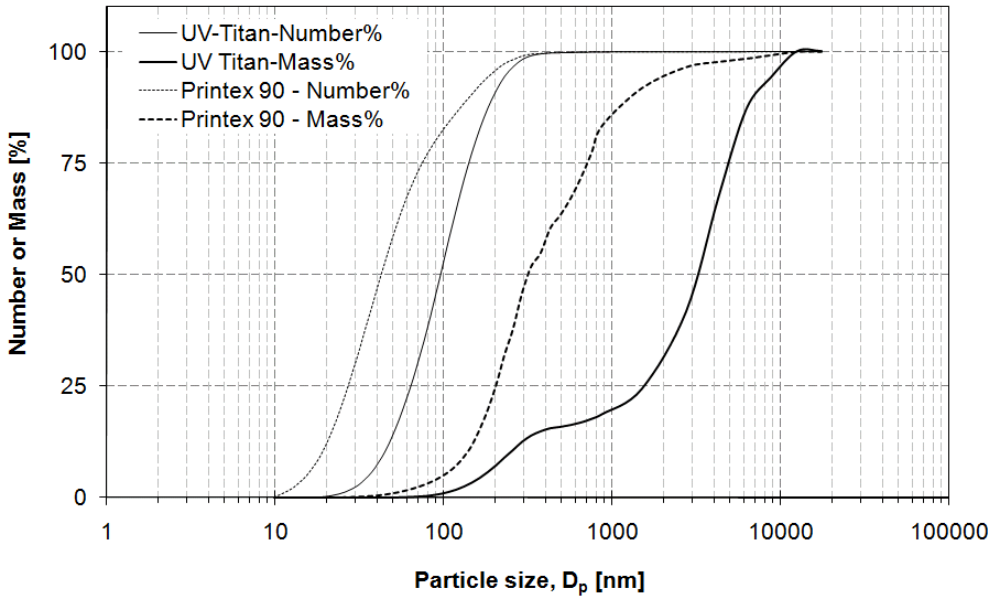


Figure 11. Characteristics of the exposure atmosphere for UV-Titan and Printex 90 inhalation exposures.
Accumulated number and mass concentration of particle concentrations in the exposure chamber. It is assumed that the optical mobility particle sizes can be directly compare and data gap is filled be linear interpolation.

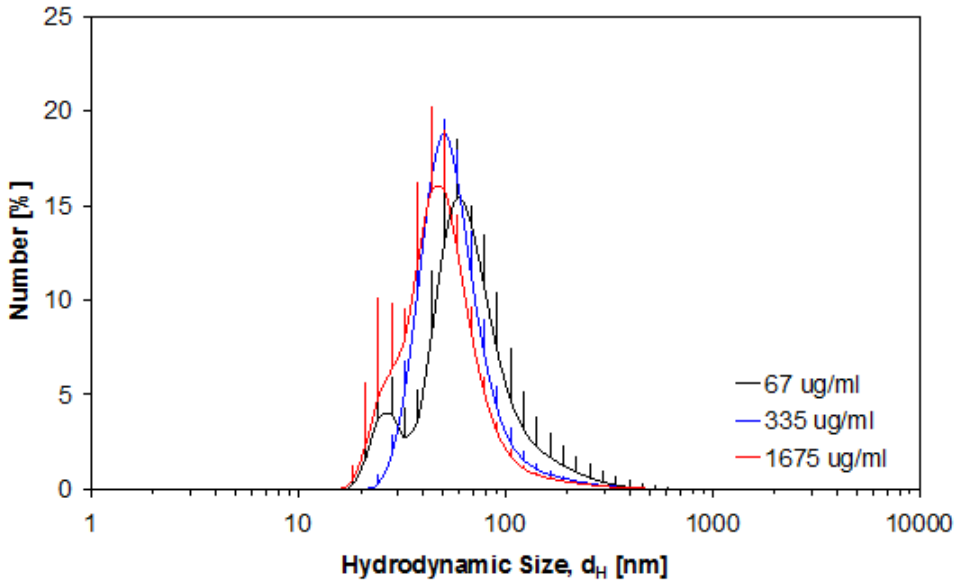
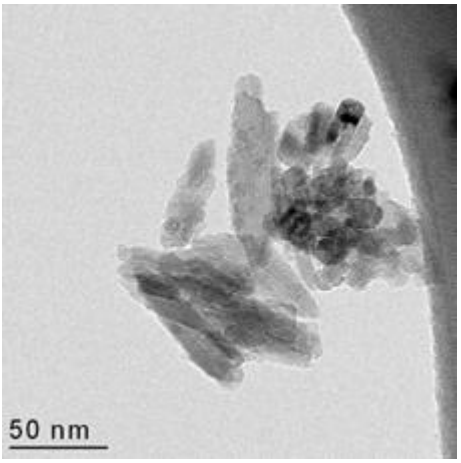
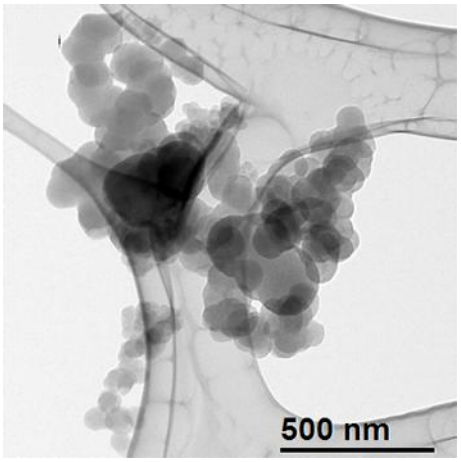


Figure 12. Hydrodynamic size distribution of the three intratracheally instilled Printex 90 dispersions, measured by Dynamic Light Scattering.
Error bars indicate the standard deviation of six measurements.



A)



B)

Figure 13. Transmission electron microscopy (TEM) pictures.
A) UV-Titan
B) Printex 90.
(Images by Keld A. Jensen)

UV-Titan particle characteristics:		
Declared particle size	17 nm	Kemira
Phase	Rutile	Kemira Hougaard et al. (2010)
Crystallite size	20.6±0.3 nm (14.4-15.5 [100]; 38.4 [001])	(Hougaard et al. 2010)
Surface area (BET)	70 m^2/g 107.7 m^2/g	Kemira (Hougaard et al. 2010)
Chemical composition	Na_2O 0.60 wt% SiO_2 12.01 wt% Al_2O_3 4.58 wt% ZrO_2 1.17 wt%	(Saber et al. 2011)
Loss of ignition	5.19 wt%	(Hougaard et al. 2010)
Thermogravimetric analysis (N_2 atmosphere, 40-800˚C, 10˚C/min)	6.1±0.4 wt%	(Hougaard et al. 2010)
Inhalation exposure:		
Particle size distribution in number	Aggregates and agglomerates were equidimensional to needle-shaped TiO2 crystallites with diameters from less than 10 nm to more than 100 nm along the shortest and longest axis, 50%<97 nm.	(Hougaard et al. 2010)
Particle number concentration	$1.70\pm0.20x10^6/cm^3$	(Hougaard et al. 2010)
Geometric mean size	97 nm	(Hougaard et al. 2010)
Mass-size distribution	75%>1600 nm and <1%<100 nm	(Hougaard et al. 2010)
Total inhaled dose	840 µg/animal	(Hougaard et al. 2010)
Estimated deposition	73 µg/animal in pulmonary region 315 µg/animal in extra-pulmonary region 365 µg/animal in gastro-intestinal tract	(Hougaard et al. 2010)

Table 9. Key physico-chemical characteristics of titanium dioxide (UV-Titan L181).

Printex 90 particle characteristics		
Declared particle size	14 nm	Degussa-Hüls
Geometric mean size	65 nm (carbon spheres)	(Saber et al. 2005)
Surface area (BET)	295-338 m^2/g	(Saber et al. 2005) (Jacobsen et al. 2008b)
Pycnometric particle density	2.1 g/cm^3	(Saber et al. 2005)
Chemical composition	99% C, 0.8% N and 0.01% H_2	(Jacobsen et al. 2008b)
The total PAH content (Carbon black extract - Soxhlet)	0.0742 µg/g	(Jacobsen et al. 2007)
The total PAH content (DEP extract - NIST SRM 1650)	216 µg/g	(Jacobsen et al. 2008a)
Inhalation exposure:		
Particle size distribution in number	Average distribution was multimodal and highly dominated by sub-100 nm particles, aggregates were most commonly 41 nm and that was also the average size, 50%<45 nm.	(Jackson et al. 2011b)
Particle number concentration	$4.09 \pm 0.03 \times 10^6/cm^3$	(Jackson et al. 2011b)
Mass-size distribution	310 nm (bimodal; 290 and 1500 nm) 75%>200nm and 5%<100 nm	(Jackson et al. 2011b)
Total inhaled dose	826 µg/animal	(Jackson et al. 2011b)
Estimated deposition	287 µg/animal in pulmonary region 166 µg/animal in extra-pulmonary region 137 µg/animal in gastro-intestinal tract	(Jackson et al. 2011b)
Instillation exposure:		
Morphology	The agglomerates consisted of spherical to sub-spherical carbonaceous particles as well as minor amounts of free single primary spheres.	(Jackson et al. 2011b)
Average zeta-size	140 nm	(Jackson et al. 2011b)
Hydrodynamic number	50-60 nm	(Jackson et al. 2011b)
Volume distributions	Peaks 50-60 nm and 200-400 nm	(Jackson et al. 2011b)

Table 10. Key physico-chemical characteristics of carbon black (Printex 90).

6.2 Maternal effects of pulmonary exposure to nanoparticles

We have assessed the particle effects in the mothers such as maternal lung inflammation and genotoxicity, to fully characterize the offspring exposure.

6.2.1 Maternal lung inflammatory response by cellular profile in BAL fluid

Maternal pulmonary response to nanoparticle exposure was evaluated by the analysis of cellular inflammatory profile in bronchoalveolar lavage fluid (BAL). Dams were assessed at weaning. Females that did not give birth, or had only a few offspring, were assessed 3-5 days after the end of exposure. To make best use of all animals, these females were used to characterize the lung inflammation in the 'mothers' at the time around birth. In the UV-Titan study, all these females had zero implantations, thus they were not pregnant. In the Printex 90 studies, the females 5 days after exposure were a mixed group of non-pregnant females and females that had implantations or even a few offspring. It was suggested that pregnancy induces natural inflammation (Fedulov et al. 2008; Lamoureux et al. 2010). In the UV-Titan study, it appears that the inflammatory response in the air exposed non-pregnant females 5 days after exposure and air exposed dams 26-27 days after exposure differs in magnitude, supporting that pregnancy induces natural inflammation. In Printex 90 female air exposed groups, this analysis was not possible and data are used for exposure verification only.

Inhalation exposure to nanoparticles (UV-Titan and Printex 90) induced potent and persistent lung inflammation (by polymorphonuclear neutrophil infiltration) in the exposed females and dams, observed as late as 24-27 days after the end of exposure. Thus, extensive inflammation persisted throughout lactation. Differences in the magnitude of the inflammatory response of the two particles were difficult to compare because of slightly different timing of tissue collection and change in BAL counting procedure between the studies. However, it appears that UV-Titan induced lung inflammation was slowly declining; while Printex 90 induced inflammation remained unchanged until weaning.

When comparing inhalation and instillation exposures (the Printex 90 studies), both exposures induced a massive influx of neutrophils in the BAL fluid of exposed females and dams, which persisted for 24-27 days after the end of exposure. After inhalation of Printex 90, the pulmonary inflammation was similar in magnitude as in the mice instilled with the medium dose. The instilled medium dose was one fifth of the instilled highest dose, which was estimated to be equal to the pulmonary deposition in the inhalation study. We and others have previously found that instilled particles induce stronger inflammatory responses in the lung compared to inhaled particles (Driscoll et al. 2000; Jacobsen et al. 2009; Osier and Oberdöster 1997). This may be because a greater fraction of the instilled particles is deposited deeper into the lung and, consequently, is cleared more slowly.

6.2.2 Maternal lung and liver gene expression

Global gene expression in lungs and livers of exposed time-mated females was analyzed by using DNA microarrays. Gene expression was analyzed in UV-Titan exposed lungs of non-pregnant females five days after exposure.

Gene profiling revealed increased levels of several genes involved in inflammation, acute phase response and immune response along with significant changes in expression of several miRNAs (Halappanavar et al. 2011). Significantly upregulated genes included several chemokines

involved in chemotaxis, infiltration of neutrophils, and epithelial proliferation. Exposure to UV-Titan also increased levels of genes belonging to the acute phase response pathway and several acute phase proteins. Collectively, these molecules act in host defence by promoting phagocytosis and inflammation. In addition, the expression of genes involved in mucosal immune defence, antimicrobial defence- and proinflammatory genes were changed, confirming the strong activation of pulmonary immune response. The changes in miRNAs in response to UV-Titan may indicate their involvement in the inflammatory response, possibly by modulation of the inflammatory process, as suggested previously by (Jardim et al. 2009). The expressed genes five days after exposure sustain persistent pulmonary inflammation and initiate secondary lung response. A genomic study with pregnant mice intranasally exposed to micro-sized 50 µg/ mouse TiO2 (2 mg/kg) demonstrated that pregnant mice had enhanced lung inflammation compared to non-pregnant mice (Lamoureux et al. 2010). This suggests that the observed response in the pregnant mice was likely even stronger. Similarly, gene expression analysis followed by protein analysis indicated that the lungs of time-mated females exposed to Printex 90 by inhalation or instillation also undergo chronic inflammation and acute phase response (Petra Jackson, Sabina Halappanavar and Anne T. Saber, manuscripts in preparation).

Interestingly, the gene expression in the liver was relatively unaffected in UV-Titan exposed non-pregnant females 5 days after exposure (Halappanavar et al. 2011). These findings agree with a previous report, where few changes in global hepatic transcriptome were observed after inhalation exposure to carbon black or diesel exhaust particle (20 mg/m^3 for 90 min/day for 4 consecutive days), while pulmonary inflammation was present (Saber et al. 2009). The gene expression in the liver of Printex 90 exposed time-mated mice is in progress (Petra Jackson, Sabina Halappanavar and Anne T. Saber, manuscripts in preparation). Preliminary results suggest that inhalation exposure of Printex 90 induced liver response in the exposed time-mated mice.

6.2.3 Maternal levels of DNA strand breaks

We quantified DNA damage in BAL cells and liver cells in the time-mated mice exposed by inhalation to UV-Titan, or Printex 90 by inhalation or intratracheal instillation. The method used was the alkaline comet assay (McNamee et al. 2000), an analysis of the level of DNA strand breaks in the exposed tissue. The primary genotoxicity of nanoparticles is related to their ability to induce reactive oxygen species (ROS) (Jacobsen et al. 2008b), and nanoparticles can induce secondary genotoxicity mediated by inflammation (Knaapen et al. 2004). It has been suggested that significant neutrophilic inflammation was associated with DNA damage (Driscoll et al. 1997).

Such a relationship between lung inflammation and DNA damage in BAL cells was not found in other *in vivo* studies by (Bornholdt et al. 2002; Madsen et al. 2008; Saber et al. 2005; Saber et al. 2009). In one study, mice were exposed to four intratracheal instillations to 54 µg/animal of highly inflamagenic biofuel dust. No DNA strand breaks were observed in BAL cells one hour after the last instillation (Madsen et al. 2008). In another study, mice were exposed by single intratracheal instillation to 54 µg/animal to different nanoparticles (UV-Titan and Printex 90 among others) designed to be used by the paint and lacquer industry, among others UV-Titan and Printex 90. Even though UV-Titan induced only a third of neutrophil influx compared to that of Printex 90, it increased levels of DNA strand breaks in BAL cells 24 hours after instillation, while Printex 90 levels of DNA strand breaks were similar to the controls (Saber et al. 2011).

(Saber et al. 2005) observed that nose-only inhalation exposure to 20 mg/m^3 for 90 min/day on four consecutive days induced DNA strand breaks in BAL cells one hour after the last exposure, while inflammation was evident as cytokine influx in the lung without neutrophil influx. Similarly, it was observed (Bornholdt et al. 2002) that levels of DNA strand breaks peaked before induction of proinflammatory cytokines and were repaired subsequently. Thus, DNA strand breaks detected by the comet assay do not link strongly to lung inflammation. Moreover, DNA strand breaks last for only a short-term, probably because the DNA damage is rapidly repaired by DNA repair enzymes (Bornholdt et al. 2002). In our studies, we did not observed DNA strand breaks in BAL cells after inhalation exposure to UV-Titan or Printex 90 at the time of analysis, 5 and 26-27 days after the last exposure. We assume that it is possible that induced DNA damage was repaired at the time.

In the liver cells, DNA strand breaks were increased after inhalation exposure to Printex 90 even weeks after exposure, while no genotoxicity was found after inhalation exposure to UV-Titan or instillation exposure to Printex 90. All three studies induced a strong and persistent inflammatory response in the exposed lung, so it is unlikely that the DNA strand breaks were affected by circulating cytokines. Similar to our studies, pulmonary exposure to Printex 90 (nose-only inhalation exposure to 20 mg/m^3 for 90 min/day on 4 consecutive days) resulted in pulmonary production of cytokines (Saber et al. 2005), but no liver inflammation or acute phase response was found in liver after to Printex 90 (Saber et al. 2009). Thus, it is unlikely that the observed DNA strand breaks were caused by liver inflammation induced by pulmonary exposure.

The exposure procedure is a key determinant for particle size-distribution and consequently for deposition and uptake (Landsiedel et al. 2008), which probably determined genotoxicity of UV-Titan and Printex 90. The DNA damage observed in the liver of Printex 90 inhalation exposed mice may be a result of the inhalation-associated gastrointestinal exposure rather than from exposure in the lungs. It has been reported that an intra-gastric exposure to 0.64 mg/kg Printex 90 induced DNA damage in the liver of rats 24 hours after exposure, whereas the same dose administered by intratracheal instillation caused no DNA damage in the liver or lung (Danielsen et al. 2010). Similarly, intra-gastric administration of other carbonaceous nanoparticles (such as single wall carbon nanotubes, C60 fullerenes and diesel exhaust particles) at the same or even lower doses, caused DNA base oxidation damage in the liver and lung of rats (Danielsen et al. 2008; Folkmann et al. 2009).

UV-Titan and Printex 90 inhalation exposures resulted in a large immediate exposure to the gastrointestinal tract, because inhaled nanoparticles deposited in the extra-pulmonary region were transported up by mucociliary transport and swallowed. However, even though the intragastric deposition of UV-Titan was greater compared to Printex 90, we assume that the large UV-Titan agglomerates likely passed through the digestive system without translocation to liver or rest of the body. Printex 90 instillation exposure also resulted in small intragastric deposition; however, a smaller instilled dose would end in less intragastric dose. It is possible that most Printex 90 particles that would reach the circulation were expected to accumulate in the liver. Nanoparticles would persist in the Kupffer cells of the liver for months (Oberdöster et al. 2002; Sadauskas et al. 2007; Sadauskas et al. 2009). Consequently, few liver cells would be directly exposed to ROS generated from Printex 90. ROS production and oxidative stress are linked to damage to the DNA (Borm et al. 2006; Møller et al. 2010a), Thus it is likely that

observed DNA damage in Printex 90 exposed liver cells is caused by ROS-induced primary genotoxicity. Comparing the ability of UV-Titan and Printex 90 to induce reactive oxygen species, Printex 90 is an efficient generator of reactive oxygen species (Jacobsen et al. 2008b) and induces more oxidative stress compared to UV-Titan (Saber et al. 2011). Thus even if UV-Titan nanoparticles were translocated to liver, less damage could be expected compared to Printex 90 nanoparticles.

6.3 Developmental toxicity of nanoparticles

As discussed previously, we expect that only a small fraction of nanoparticles translocate outside the lung cavity or the gastrointestinal mucosa. Therefore, only few particles would reach the placental barrier, and even fewer would reach the fetus and affect the fetus directly. Analysis of UV-Titan in the maternal lung, liver and offspring liver confirmed our expectation, and significant amounts of UV-Titan were restricted to the maternal lung (Hougaard et al. 2010). However, small amounts of nanoparticles would not be detected due to a high analytical detection limit for titanium. The UV-Titan was coated with Zr, Si, Al, Na as well complex polyalcohols. It is possible that impurities leached from the particle coating reached the blood stream and crossed the placenta and affected the offspring. Printex 90 contained minute amounts of polycyclic aromatic hydrocarbons (PAH) that are also reported to leach from the particle and cause effects in the prenatally exposed offspring (Srivastava et al. 1986). However, we assumed that the concentration of PAHs in Printex 90 was negligible and effects of Printex 90 were not caused by leached impurities.

Pulmonary exposure to nanoparticles induced inflammation and acute phase response in the lungs of exposed time-mated mice (Halappanavar et al. 2011; Hougaard et al. 2010; Jackson et al. 2011b). The inflammatory cytokines and acute phase proteins induced in the maternal lungs may have crossed the placenta and induced effects in the offspring. The inflammatory cytokines interleukin-1b (IL-1b), interleukin-6 (IL-6) and tumor necrosis factor (TNF-a) are important for fetal development. IL-1b regulates embryogenesis and fetal development, and inflammatory cytokines increase as a signal for the onset of labour (similarly to 'transplant rejection') (Protonotariou et al. 1999). However, excessive inflammation during pregnancy negatively affects the offspring. Maternal inflammation during pregnancy, induced by exposure to lipopolysaccharide (LPS), resulted in reduced activity of the hypothalamic pituitary adrenal axis and altered the immune response in offspring later in life (Hodyl et al. 2007; Hodyl et al. 2008; Lasala and Zhou 2007; Surriga et al. 2009). Pro-inflammatory insult during gestation may also affect neuronal development and memory (Gilmore et al. 2004; Graciarena et al. 2010; Lasala and Zhou 2007; Marx et al. 2001). Based on these results, it can be hypothesised that nanoparticle induced inflammatory signals from the mother could interfere with development of the offspring.

6.3.1 Gestation and lactation

Despite the persistent inflammation and acute phase response in the maternal lung, inhalation exposure to UV–Titan and Printex 90, and instillation exposure to Printex 90, did not affect gestational and developmental parameters. The pregnancy length and maternal weight gain during gestation and lactation were not affected by the nanoparticle exposure. Number and loss of implantations, litter size, offspring birth weight, survival success, and weight gain during lactation and maturation were similar to the controls. Sex ratio was also unaffected. To our

knowledge, only one other study reported gestational parameters. Time-mated mice were intratracheally instilled with carbon black dispersed in saline solution with 0.05% Tween 80 (0.2 mg/animal total dose) on GD 7 and 14. Similar to our studies, no effects on gestation were observed (Yoshida et al. 2010). In conclusion, pulmonary exposure to nanoparticles repeatedly did not induce maternal toxicity or fetotoxicity and developmental toxicity.

6.3.2 Sexual maturation

Chemicals can possess properties to alter sexual differentiation and sexual maturation. Nanoparticles in the ambient air are reported to alter reproductive function and therefore we included sexual maturation as one of the tested parameters.

Female offspring exposed to Printex 90 after maternal intratracheal instillation had unaffected anogenital distance at weaning, however females exposed to the lowest exposure dose entered puberty earlier (time of vaginal opening). Since data did not indicate dose response, this may be a chance finding. UV-Titan exposed female offspring by maternal inhalation exposure had gene expression changes related to retinoic acid signalling pathway (discussed later). Retinoic acid is a key factor in germ cell development and survival (Morita and Tilly 1999). One of the most significantly expressed genes, *Cyp26b1*, affects the timing when germ cells enter meiosis. Wrong timing can further lead to altered maturation of gonads *in utero* or even infertility (Niederreither and Dolle 2008). No published studies addressing nanoparticle effect on female reproductive system are available.

Male offspring exposed to Printex 90 after maternal intratracheal instillation had also unaffected anogenital distance at weaning and entered puberty similarly to their controls (time of preputial separation). Moreover, male offspring exposed prenatally to UV-Titan had slightly prolonged time-to-first litter (insignificantly though). Others report that prenatal exposure to titanium dioxide and carbon black nanoparticles affected male testicular tissue and sperm production (Takeda et al. 2009; Yoshida et al. 2010). The results together imply that maternal pulmonary exposure to nanoparticles may affect offspring reproductive function, both female and male.

6.3.3 Offspring liver gene expression

Global gene expression in the liver of newborn offspring exposed prenatally to nanoparticles was analyzed by using DNA microarrays to evaluate the liver response to the nanoparticle exposure.

Changes in gene expression in the liver of newborn offspring exposed prenatally to UV-Titan were analyzed. Hepatic gene expression profiling indicated differential expression of genes relative to control animals: thirty-five in females and seventeen in males (p-value < 0.05). Twenty-eight genes for females and four genes for males had fold changes higher than 1.3. The results suggest that male offspring were less responsive to prenatal UV-Titan exposure than females, and their response was substantially different. The differentially expressed genes in male offspring belonged to phase 1 xenobiotic metabolism, gluconeogenesis and amino acid metabolism.

The altered genes in female offspring were implicated in the basic biological pathway for retinoic acid (RA) receptor signalling, which plays an important role in embryonic growth, fetal development, neurogenesis, limb growth, organogenesis and regulation of fertility (Mark et al. 2009; Niederreither and Dolle 2008). Inflammation has been closely linked to RA mediated

signalling. It has been reported that lipopolysaccharide induced inflammation in rats can potentially disrupt the balance of RA metabolism and maintenance of RA homeostasis by regulating genes involved in RA degradation, which may further affect the expression of other RA regulated genes (Zolfaghari et al. 2007). It is possible that the prenatal exposure to UV-Titan and accompanying pulmonary inflammation and acute phase response induces a response similar to the one induced by LPS.

Proinflammatory cytokines are reported to activate the RA pathway signalling in a vascular smooth muscle, which in turn may modulate the inflammatory response in the vascular wall (Gidlof et al. 2001). As a possible feed-back mechanism, Il-1β down-regulates retinoic acid binding proteins and receptors, as reported in mice lung tissue (Bry and Lappalainen 2006). RA directly reduces synthesis of Il-1β and response to inflammatory stimuli human monocytes (Gross et al. 1993). RA activates toll-like receptor 4 (TLR4, activator of innate immunity) gene expression and decreases nuclear factor kappa-B (NF-κB, regulator of immune response to infection) binding activity in LPS exposed mammary tissue, which is a possible mechanism suggested by (Gu et al. 2010). Similar processes are possible in the fetal tissue, where maternal inflammatory mediators could increase RA pathway signalling in the fetus, to attenuate the inflammatory response in the offspring. While disturbance in RA pathway signalling could trigger a variety of effects in developing offspring, the results are not complete to draw such conclusions.

The effects of prenatal exposure to Printex 90 by maternal inhalation and instillation are further evaluated (Petra Jackson and Sabina Halappanavar, manuscripts in preparation). Preliminary results indicate that the exposure induced inflammatory response in the offspring. Thus, maternal exposure to nanoparticles induces gene expression changes in the offspring. Further studies are necessary to fully explore the consequences of such perturbation in adult life.

6.3.4 Offspring levels of DNA strand breaks

To assess genotoxicity, we evaluated liver DNA damage in the offspring prenatally exposed to UV-Titan or Printex 90 at birth, weaning and during adolescence. DNA damage was quantified by the alkaline comet assay, an analysis of the level of DNA strand breaks.

The background level of DNA strand breaks was higher in liver cells from newborns compared to older siblings in all three studies. These DNA strand breaks might be related to a high proliferation rate during tissue maturation and/or the naturally occurring high level of oxidative stress at birth (Cindrova-Davies et al. 2007; McArt et al. 2010; Randerath et al. 1996). This may have reduced the sensitivity of the comet assay to detect differences between the exposure groups at this time point.

Prenatal exposure to Printex 90 after maternal inhalation exposure increased levels of DNA strand breaks even weeks after birth, while no effects were found after exposure to UV-Titan or exposure to Printex 90 after maternal instillation exposure. A few molecular genotoxins have been demonstrated to pass from the mother to the fetus and generate DNA damage in fetal tissues (Brunborg et al. 1996; Tripathi et al. 2008). However, we expect that only a small fraction of Printex 90 particles translocated from the lungs of the mothers to the fetuses because the particles would have to pass two compartmental barriers, i.e. in the lung and placenta. The observed effects of prenatal exposure were therefore more likely due to changes

in signaling cascades. It is possible that inflammatory molecules were transferred from the maternal to the fetal compartment (Jonakait 2007) and affect the fetus. Thus, the increased levels of DNA strand breaks in liver tissue of the offspring may be caused by maternally induced inflammatory mediators after Printex 90 inhalation exposure.

DNA strand breaks in offspring liver of the inhalation-exposed dams were still evident in fifty-day old offspring. At this time, the offspring were independently fed and had no contact with the dams. Therefore, it is unlikely that secondary genotoxicity caused by inflammatory signaling from the dams caused the observed DNA strand breaks in the older offspring. Further work is needed to establish the basis of this extended damage to the DNA in the Printex 90 prenatally exposed offspring.

6.3.5 Developmental neurotoxicity

Prenatal exposure to airborne particulates is reported to affect neurodevelopment. Offspring prenatally exposed to UV-Titan (after maternal inhalation exposure) exhibited changes in activity and in sensorimotor processes (tested in the Open field and the Startle test, respectively); no changes in learning and memory tested in the Morris water maze were observed. Prenatal exposure to Printex 90 (after maternal intratracheal instillation exposure) induced small changes in habituation assessed in the Open field, whereas no effects on acoustic startle were observed.

A few other studies have assessed the effects of prenatal exposure to nanoparticles on the nervous system. Titanium dioxide nanoparticles (100 µL of 1 mg/ml) were injected subcutaneously (SC) into pregnant mice on GD 3, 7, 10, and 14. Nanoparticles were found in the brain, and they induced apoptotic changes in the olfactory bulb in the prenatally exposed male offspring six weeks after the prenatal exposure (Takeda et al. 2009). A comparable exposure (100 µl SC of 1 mg/ml on GD 6, 9, 12, 15, 18) increased levels of dopamine in the offspring brain (Takahashi et al. 2010). Another exposure (100 µl SC of 1 mg TiO2/ml on GD 6, 9, 12, 15) changed gene expression in the offspring brain related to development and faction of the central nervous system. However, it has to be noted that only one offspring per group was analysed, which undermines the statistical validity of these results (Shimizu et al. 2009). After an oral dose (100 mg/kg BW per day) administered to rats during gestation (GD 2-21) or during lactation (PND 2-21), titanium was increased in the hippocampus after both exposures (~4.9 and 8.4 mg/g hippocampus after gestation and lactation, respectively). Lactational exposure attenuated synaptic plasticity in the hippocampus (associated with learning and memory), while a lesser effect was observed in the prenatally exposed offspring (only the paired pulse function appeared reduced) (Gao et al. 2011).

We have reported previously that female offspring prenatally exposed to diesel exhaust particles (dams inhaled 19 mg DEP/m^3 1 hr/day on GD 8-18) exhibited increased activity in the Open field (Hougaard et al. 2009). In conclusion, prenatal exposure to nanoparticles have been repeatedly associated with changes in behaviour related to activity level and pathological changes in the brain, suggesting that nanoparticles may impact development of the nervous system.

7. CONCLUSIONS

Although it seems that there is a limited uptake of insoluble nanoparticles over epithelial barriers in lung and intestines, particles that reach the blood stream may reach the placenta and the fetus. Furthermore, the well established effects of nanoparticles, inflammation and genotoxicity, may be especially damaging to the developing fetus. There is a lack of information of particle effects during pregnancy, and the consequences of prenatal exposure later in the life.

Prenatal exposure to titanium dioxide (UV-Titan) and carbon black (Printex 90) nanoparticles was evaluated in similar whole-body inhalation exposures, and Printex 90 was also evaluated by a comparable dose response study, achieved by intratracheal instillation. The chosen exposures were relatively high, where the daily exposures corresponded approximately to the 8-hr time weighed average occupational exposure limit (TWA 6 mg titanium dioxide/m^3 and 3.5 mg carbon black/m^3, The Danish Working Environment Authority 2007). The UV-Titan exposure atmosphere contained more agglomerates, compared to the Printex 90 exposures. This affected the particle number in the inhaled air, as well as the estimated pulmonary and intragastric exposure. All exposures induced persistent maternal pulmonary inflammation, lasting for weeks after the end of exposure (Hougaard et al. 2010; Jackson et al. 2011b), an effect not observed in our laboratory before. Maternal lungs also exhibited signs of acute phase response (Halappanavar et al. 2011) and (Petra Jackson, Sabina Halappanavar and Anne T. Saber, manuscripts in preparation). Based on observed effects, it can be argued that the current TWA is actually too high and a lower limit should be implemented for exposure to nanoparticles to protect the worker health.

To identify the changes in gene pathways to be able to predict the molecular mechanisms of nanoparticle toxicity and health outcomes of the exposure, we have analyzed the entire transcriptome with microarray analysis. Prenatal exposure to nanoparticles induced gene expression changes in the newborn offspring liver related to inflammatory response. Female offspring seemed to be more responsive to exposure compared to male offspring (paper III and Petra Jackson and Sabina Halappanavar, manuscripts in preparation). Maternal inflammatory cytokines likely crossed the placenta and induced inflammation in the offspring. Prenatal inflammation is reported to affect offspring immune function and neurofunction. Further work is needed to fully explore the consequences of changes in gene expression after prenatal exposure to nanoparticles in adult life.

Higher DNA damage due to chemical exposure is associated with an increased rate of mutations and ultimately increased risk of cancer. Especially the fast developing fetus may be sensitive to DNA damage, because frequent cell divisions may not allow sufficient DNA repair and thus exposure may be predisposition to cancer. In our study, the nanoparticle-induced genotoxicity was particle and exposure specific. Inhalation exposure to Printex 90 induced DNA strand breaks in the liver of exposed time-mated females and their offspring lasting weeks after the end of exposure (Jackson et al. 2011b). Genotoxicity was not observed in time-mated mice or their offspring exposed by UV-Titan by inhalation, or to Printex 90 by intratracheal instillation (Jackson et al. 2011b; PAPER III). Differences in genotoxicity were likely caused by different particle deposition, uptake and reactivity.

The developing brain may be much more sensitive to chemical exposure compared to the adult brain. Despite this, developmental neurotoxicity is not a commonly tested endpoint included in chemical testing. We report that prenatal exposure to nanoparticles induced developmental neurotoxicity in the exposed offspring after reaching adulthood. While exposure to UV-Titan induced changes in activity and sensorimotor processes (Hougaard et al. 2010), exposure to Printex 90 affected offspring activity in a form of habituation pattern changes (PAPER VI). Our results combined with results reported by others (Gao et al. 2011; Hougaard et al. 2008; Shimizu et al. 2009; Takahashi et al. 2010; Takeda et al. 2009) suggest that prenatal exposure to nanoparticles may affect the development of the nervous system.

Despite the observed effects, maternal exposure to nanoparticles did not affect gestation, lactation and prenatally exposed offspring survived and developed normally. Minor changes were observed in exposed female offspring sexual maturation. The results suggest that the traditional endpoints in the existing guidelines may not fully evaluate nanoparticle effects, because nanoparticles do not seem to be fetotoxic, as such. Nanoparticle effect seem be more subtle, such as altering functional domains of the offspring that require testing beyond the traditional scope.

8. FURTHER RESEARCH

Many scientific questions regarding prenatal exposure to nanoparticles have been answered in the presented work, and more questions are waiting to be answered, suggesting the complexity of the exposure assessment.

The nanoparticles used in the current work were relatively low toxic particles and the question remains whether more toxic nanoparticles, e.g. carbon nanotubes or different metal oxides, would affect gestational and developmental parameters. Moreover, the exposure regime used in the presented work excluded early pregnancy and thus did not assess effects on fertilization, early embryonic development and implantation. It remains to be answered whether parental (maternal or paternal) exposure to nanoparticles before fertilization would affect parental reproductive function, gestation, lactation and offspring development.

Moreover, our results and that of others suggest that prenatal exposure to nanoparticles may affect the outcome reproductive function (Takeda et al. 2009; Yoshida et al. 2010) (PAPER VI) and neurodevelopment (Gao et al. 2011; Shimizu et al. 2009; Takahashi et al. 2010; Takeda et al. 2009) (PAPER VI) of the offspring. More research addressing both female and male offspring reproduction, and mechanisms of observed neurotoxicity is necessary to answer this question.

Some new research is emerging on assessing nanoparticle transport across placenta and effects on the placental function. Placental transport ranges from a negligible fraction to a relatively large portion of particles translocated into the fetal tissue. More systematic work addressing different nanoparticle characteristics and their relationship to placenta transfer is needed. This would aid explaining whether the effects observed after nanoparticle exposure are due to direct particle exposure *in utero*, indirect particle effect due to exposure to maternal inflammation, or both.

We have begun a gene expression profiling of gene changes in newborn offspring liver. We are currently analyzing the pathways perturbated by the exposure and we will interpret the results based on their biologic significance. We will only assume the possible effects later in life. However, further analysis in exposed offspring later in life could confirm our assumptions about consequences of perturbated pathways in the adult organism. It may be possible to link changes in gene expression with previously observed effects on offspring's DNA damage, and changes in immune-, reproductive- and neurofunction.

It has been suggested that the fetus makes physiological adaptations in response to changes in its environment to prepare itself for postnatal life: 'developmental origins of disease – Baker hypothesis'. In particular, links are well established between reduced birth weight and increased risk of coronary heart disease, diabetes, hypertension and stroke in adulthood (de Boo and Harding 2006). The most widely accepted reasons for changes on fetal programming are exposure to undernourished environment or excess exposure to glucocorticoids (Meaney et al. 2007). These changes may include epigenetic modification of gene expression. Several environmental chemicals are reported to induce epigenetic changes, reviewed by (Baccarelli and Bollati 2009). The question remains, does prenatal exposure to nanoparticles lead to epigenetic alterations and are the epigenetic alterations transmitted transgenerationaly?

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PAPER I

Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice.

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RESEARCH Open Access

Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice

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Abstract

Background: Engineered nanoparticles are smaller than 100 nm and designed to improve or achieve new physico-chemical properties. Consequently, also toxicological properties may change compared to the parent compound. We examined developmental and neurobehavioral effects following maternal exposure to a nanoparticulate UV-filter (UV-titan L181).

Methods: Time-mated mice (C57BL/6BomTac) were exposed by inhalation 1h/day to 42 mg/m^3 aerosolized powder ($1.7 \cdot 10^6$ n/cm^3; peak-size: 97 nm) on gestation days 8-18. Endpoints included: maternal lung inflammation; gestational and litter parameters; offspring neurofunction and fertility. Physicochemical particle properties were determined to provide information on specific exposure and deposition.

Results: Particles consisted of mainly elongated rutile titanium dioxide (TiO_2) with an average crystallite size of 21 nm, modified with Al, Si and Zr, and coated with polyalcohols. In exposed adult mice, 38 mg Ti/kg was detected in the lungs on day 5 and differential cell counts of bronchoalveolar lavage fluid revealed lung inflammation 5 and 26-27 days following exposure termination, relative to control mice. As young adults, prenatally exposed offspring tended to avoid the central zone of the open field and exposed female offspring displayed enhanced prepulse inhibition. Cognitive function was unaffected (Morris water maze test).

Conclusion: Inhalation exposure to nano-sized UV Titan dusts induced long term lung inflammation in time-mated adult female mice. Gestationally exposed offspring displayed moderate neurobehavioral alterations. The results are discussed in the light of the observed particle size distribution in the exposure atmosphere and the potential pathways by which nanoparticles may impart changes in fetal development.

Background

Nanomaterial research and development is proceeding at a rapid pace and many new nanotechnology products are becoming commercially available [1]. Nanoparticles are usually defined as particles with a primary particle size between 1 and 100 nm along at least one axis. Engineered nanoparticles (ENPs) normally possess new or enhanced physico-chemical properties compared to that of the bulk material due to inherent quantum size effects, a large surface to volume ratio, and controlled particle shape and surface coating. Consequently, toxicological properties of ENPs may differ from that of their larger counterparts [2]. This highlights the need for toxicological assessment of ENPs early in material development. Free nanoparticles may behave more like a gas than solid matter because of their small size. However, most primary particles in powders are firmly agglomerated and/or aggregated.

Although primary ENPs may be emitted during production and de-agglomeration occurs during generation of dust in powder handling, subsequent re-agglomeration may still result from coagulation and scavenging when particles are aerosolized (reviewed in [3]). Consequently, it is impossible to predict the size-distribution and aerosol behavior of ENPs or their potential de-agglomeration

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during airway deposition. Therefore, experimental work is urgently required to assess these parameters as well as to determine the resulting biological effects *in vivo*. When inhaled, a considerable fraction of sub-μm size particles may deposit in the deeper airways. Once deposited in the lung, material may be retained for a long time [4]. Nanoparticles can also translocate across the lung epithelium, although the rate of distribution to other organs varies [3,5-7]. Airborne particles released during production or handling of ENPs are therefore of particular concern.

The toxicological properties of nanosized particles are generally poorly understood, although knowledge in some areas (especially inflammation and particle translocation) is rapidly growing. Reproductive and developmental toxicity is integrated into the nanomaterials research strategy of the U.S. Environmental Protection Agency [8] and recommended by the Reproductive Health Research Team under the National Occupational Research Agenda of the U.S. National Institute of Occupational Safety and Health [9]. Nanomaterials may affect the developing fetus either directly or indirectly. Direct effects might occur after translocation of particles from maternal lung to blood and then across the placenta. By the indirect pathway, maternal pulmonary inflammation orchestrates release of signaling molecules which potentially affect both mother and fetus. Preliminary work suggests that the fetal nervous system is specifically sensitive to maternal particulate exposure during pregnancy [10,11]. Today very little is known on developmental toxicity of nanomaterials.

Titanium dioxide (TiO_2) has previously been used as a generic model compound to illustrate potential toxic effects of exposure to relatively inert nanoparticles. However, TiO_2 is also a widely-used industrial nanomaterial (e.g., sunscreens and lacquers with "invisible" UV-filters, and paints with photocatalytic-induced self-cleaning properties). Thus, the exposure of consumers and factory workers who handle TiO_2 nanomaterials and nanomaterial-based products must be considered. Increasing evidence suggests that the toxicity of TiO_2 not only depends on size, but also varies with crystalline polymorph, particle shape, surface coating and functionalization (reviewed in [12]). Thus silica-coated TiO_2 increased lung inflammation significantly compared to pure TiO_2 and pure silica in the mouse [4].

The present study investigated developmental neurotoxicity in offspring of mice that inhaled TiO_2 (UV-titan L181, a coated and chemically modified rutile) during pregnancy, in parallel with maternal inflammatory response. Effects on the nervous system were evaluated by use of a neurobehavioral test battery. Furthermore, particle physicochemical properties and exposure were characterized in detail.

Materials and methods

Animals

Time-mated, nulliparous mice (C57BL/6BomTac, Taconic Europe, Ejby, Denmark) arrived at gestation day (GD) 3 and were randomly grouped 5 or 6 in polypropylene cages with bedding and enrichment (removed during nursing). Animals were housed under controlled environmental conditions, with 12 hour light from 6.00 a.m. and access to food (Altromin 1324) and tap water ad libitum (further information in Additional file 1). On GD4, animals were weighed and assigned to two groups of 22 and 23 animals, respectively, with similar weight distributions. For cross-over mating, naïve CBA/J mice (Charles River Wiga, Sulzfeld, Germany) were supplied at nine weeks of age. Procedures complied with EC Directive 86/609/EEC and Danish regulations on experiments with animals (Permission 2006/561-1123).

Material characterization

This study used UV-titan L181 (Kemira, Pori, Finland), a rutile modified with unspecified amounts of zirconium (Zr), silicon (Si), aluminum (Al) and coated with polyalcohols.

Physical particle size, morphology and general state of agglomeration/aggregation were determined by analysis of particles suspended on holey carbon-coated Cu TEM-grids using a 200 kV Transmission Electron Microscope (TEM) (Tecnai G20, FEI Company, Hillsboro, Oregon, USA). Sample preparation for TEM analysis is described in Additional file 1.

Crystalline phases and crystallite sizes were determined by powder X-ray diffraction (XRD) with a Bruker D8 Advance diffractometer equipped with a Lynxeye CCD detector (Bruker AXS Inc., Madison, WI 53711-5373, USA), using monochromated $Cu_{K\alpha 1}$ (1.540598 Å) rays. Results were obtained by Rietveld refinement of the X-ray diffractograms using Bruker TOPAS V4.1 software. Elongation was determined by analysis of reflections from principal crystallographical axis using the Scherrer equation.

Specific surface area was determined on a Quantachrome Autosorp-1 (Quantachrome GmbH & Co. KG, Odelzhausen, Germany) using multipoint Brunauer, Emmett, and Teller (BET) nitrogen adsorption method after 1 h degassing at 300°C. Analysis was completed according to DIN ISO 9277 as a commercial service by Quantachrome GmbH & Co. KG.

Elemental composition was analyzed by X-ray Fluorescence analysis on a Philips PW-2400 spectrometer as a commercial service by the Department of Earth Sciences,

University of Aarhus, Denmark. Elemental concentrations were determined using their standard protocol using rock standards for calibration.

The organic coating of the UV-titan particles was extracted with methanol by Pressurized Liquid Extraction (PLE) at 2000 psi and 200°C, followed by centrifugation at 4000 rpm (3310 g) for 10 min. Chemical composition of the supernatant was analyzed by laser desorption ionization and time of flight MS (MALDI-TOF without matrix) on a stainless steel ground target with a Bruker AutoFlex II (Bruker Daltonics, Inc., Bremen Germany). Acurate mass determination (1 ppm) was performed with electrospray-MS (ESI-MS) on a Bruker microQ-TOF (Bruker Daltonics, Inc., Bremen Germany) with direct injection. The masses are reported as mass to charge ratios (*m/z*) of the protonated compounds ($[M+H]^+$).

Exposure

Mice were exposed to filtered clean air or a target concentration of 40 mg UV-Titan/m^3 on GD8-18, one hr/day as described [13,14]. Airflow in the exposure chamber was dynamic (20 L/min) with evenly distributed exposure atmosphere. A microfeeder aerosolized powder particles through a dispersion nozzle at a pressure of 5 bar (Fraunhofer Institute für Toxicologie und Aerosolforschung, Hannover, Germany). The dose from one hour exposure to 40 mg TiO_2/m^3 corresponds to the 8-hr time weighted average (TWA) occupational exposure limit according to Danish Regulations [15]. Animals were placed separately in rooms of a "twelve-room-pie"; a cylindrical wire mesh cage (? 29 cm, height 9 cm) with radical partitions. Females were observed for signs of toxicity and returned to cages less than 5 min after exposure. Body weight was recorded before exposure on GD9, 11, 14, and 18.

Exposure monitoring

Mass-concentrations of total suspended dust was controlled periodically by filter sampling and adjusted to maintain a concentration of ~40 mg/m^3. Exposure air was sampled on pre-weighed Millipore Fluoropore filters (? 2.5 cm; pore size 0.45 μm) at an airflow of 2 L/min using Millipore cassettes, for 10 min. Filters were weighed immediately on a Sartorius Microscale (Type M3P 000V001). Final gravimetric data were obtained on acclimatized filters (50%RH and 20°C).

Particle number and size distribution in the exposure atmosphere were monitored using a GRIMM Sequential (Stepping) Mobility Particle Sizer (SMPS) system for sub-μm particles (12.8 to 486 nm; based on the rutile density of 4.25 g/cm^3 [16]) and a GRIMM Dustmonitor (Model 1.106) for coarse particles (0.75 to > 15 μm). The SMPS consisted of a Long Electrostatic Classifier (Model No. 5.521) and a GRIMM Condensation Particle Counter (Model 5.400). The time resolution was 218 and 6 s for the SMPS and Dustmonitor data, respectively (see Additional file 1 for further explanation on the on-line particle exposure monitoring).

Parturition and lactation

After exposure on GD18, females were singly housed. Delivery was expected on GD20, and designated postnatal day (PND) 0. Pups were counted and sexed on PND1. Dams and individual pups were weighed at PND1, 8, 11, 16, 19, and 22. On PND2, one pup from litters with at least 5 pups, and on PND23-24 one male and one female per litter, were sacrificed by decapitation. Lungs, liver, heart, brain, and on PND2, stomachs containing milk, were dissected, weighed, snap frozen in liquid N_2 and stored at -80°C. At weaning (PND22), one male and female per litter were randomly chosen for behavioral testing and housed as described.

Non-pregnant time-mated females without implantations ("NP females") were euthanized on PND3 (i.e. 5 days post exposure) and subjected to bronchoalveolar lavage (BAL), as were dams with litters at PND24-25 ("P females"; 26-27 days post exposure). Females were anaesthetized with Hypnorm and Dormicum and sacrificed by withdrawal of heart blood (stabilized in 0.17 mol/l K_2EDTA). BAL was performed as described below, followed by determination of uterine implantation sites and dissection of organs as described for offspring.

Titanium in tissue and milk

Approximately 25-75 mg tissue (lung and liver for adults, liver for offspring) and 110-140 mg (milk) were weighed and analyzed for content of titanium (Ti). For PND2 pups, milk and liver samples were pooled from 4-5 animals. Maternal lung was included to determine remaining TiO_2 and liver to assess systemic distribution in adults [17,18] and fetal animals [19]. Tissues were digested in concentrated nitric acid (PlasmaPure, SCP Science, Quebec, Canada) in a microwave oven (Multiwave, Anton Paar, Graz, Austria), and Ti content determined by quadrupole-based inductively coupled plasma mass spectrometer (ICPMS 7500ce, Agilent Technologies, Tokyo, Japan) equipped with a collision/reaction cell (CRC). The CRC was pressurized with helium as collision gas to reduce polyatomic interferences on Ti isotopes. Settings for ICPMS measurements are given in (Additional file 1, Table S1). Sulphur-containing polyatomics (e.g. $^{32}S^{16}O^+$) strongly interfered with the most abundant Ti isotope, ^{48}Ti (abundance 73.8%). There was less interference with ^{49}Ti and ^{50}Ti (abundance 5.5 and 5.4%, respectively), which were selected for quantitative analysis. The limit of detection (LOD) for Ti in tissues, based on three times the standard deviation of repeated blank measurements,

was estimated to be 0.2-5 mg/kg depending on sample intake and dilution.

BAL preparation and analyses

We used BAL cell composition and neutrophil influx to indicate lung inflammation. This has proven to be a relevant and sensitive marker of pulmonary inflammation (e.g. [20,21]). BAL was performed four times with 0.8 ml 0.9% sterile saline ([20]; further information in Additional file 1). The total number of cells and of dead cells in BAL samples was determined in cell suspension B by Nucleo-Counter. Differential counts of macrophages, neutrophils, lymphocytes, eosinophils, and epithelial cells were determined by counting 200 cells in cell supernatant fixed with 96% ethanol and stained with May-Grünwald-Giemsa stain. All slides from both time points were randomized, blinded and scored on the same day. Total number of cells was calculated by combining data from differential cell counts with the total number of cells in BAL.

Behavioral testing

Investigations were performed during the light period. Exposed and control animals were tested alternately. Animals were transferred to the experimental room 1 hr before the first test. Observers were blinded to exposure status of the animals, and the same observer was used throughout any specific test.

Learning and memory was tested in the Morris water maze at age 11 and 15 weeks (males), and 12 and 16 weeks (females) as described [13] with minor modifications. A stable, invisible platform was submerged 1 cm below the water surface in a circular plastic pool (? 100 cm). Animals were tested in four daily trials. Mice were placed at the designated starting position and completed the trial when climbing onto the platform. When failing to locate the platform within 60 s, animals were led to the platform. All animals spent 15 s on the platform before returning to the cage. The following scheme was used: *Learning:* Test for 5 consecutive days with platform in center of the southeastern quadrant. *Memory:* Three weeks later, test with platform in south-eastern quadrant, for 3 days. *Reversal learning:* The following day, test with platform in north-western quadrant for 4 trials. *New learning:* The following day, test with platform in center of pool for 4 trials. Noldus Ethovision (Version 5, Noldus Information Technology, Wageningen, The Netherlands) was used to register latency and path length, and calculated swimming velocity and relative occupancy in the each of the quadrants.

Activity was assessed for 3 min at 14 weeks of age in an open field using the dry water maze pool. Trials commenced in the center of the field and the location of the animal was registered by Noldus Ethovision XT version 5. The tracking device calculated total ambulation, which was subsequently split into three time-bins of 1 min to test for habituation. Duration in the central and the outer 9 cm peripheral zone of the field, as well as the number of crossings from the outer to the central zone were extracted.

Acoustic startle reaction (ASR) and prepulse inhibition (PPI) were tested at 4 months as described [22] in two chambers (San Diego Instruments, San Diego, USA) with 70 dB(A) white background noise. A piezoelectric accelerometer transduced displacement of test tubes (? 3.6 cm) in response to movements of the animal. Animals were acclimatized for 5 min in the tube before sessions started and ended with 5 startle trials of 40 ms 120 dB(A) bursts of white noise. In between, 35 trials were delivered in semi-randomized order (10 trials of 120 dB(A); 5 each of 4 prepulse + startle trials (prepulses of 72, 74, 78, and 86 dB(A)); 5 trials with only background noise). Tube movements were averaged over 100 ms following onset of the startle stimulus (AVG). The five AVGs for each prepulse intensity were averaged and used to calculate PPI, which was expressed as percent reduction in AVG compared to the average of the 10 middle startle trials: %PPI = 100*((AVG at prepulse+startle trial)/(AVG at startle trial))*100%.

Time-to-first F2 litter

At 19 weeks of age, control and exposed offspring were cross-mated to naïve CBA/J mice (12 weeks old) and time-to-first-delivery of F2 litter, litter size, and gender ratio were recorded.

Statistics

Litter was considered the statistical unit. Gestational parameters were analyzed by Mann-Whitney *U*-test, and time-to-first-delivery by log rank test (separately by gender). ANOVAs were applied to the remaining data when relevant with repeated measures in trials, days, or time-bins. In the analysis of weight gain in adult females, Ti in adult tissues, and BAL cell counts, the factor "Pregnancy" was added to distinguish (barren) NP females from (littering) P females. Since these groups of adult females differed with regard to both time after exposure and pregnancy, only pairwise comparisons related to exposure were explored. ANCOVA controlled for litter size in the analyses of weight gain during exposure, birth weights, and pre-weaning pup weights. Behavioral data were analyzed by two-way ANOVA, with Prenatal exposure and Gender as factors, apart from startle data, where PPI was analyzed separately for each prepulse intensity [22]. Pairwise comparisons were performed by T-test or Mann Whitney U-test ($p < 0.1$). Analyses were performed in SYSTAT Software Package 9, MINITAB 14, and SAS 9.1.

Results

Particle characteristics

Physicochemical characteristics of the UV-titan sample are summarized in Table 1. Rutile was the only crystalline phase in the sample and TiO_2 accounted for 70.8 wt%. Residual mass was composed of Zr, Si, Al, and a little sodium (Na) as well as 5.2 wt% volatiles (loss on ignition). Stochiometric calculations show that the modifier elements partly occurred in oxides, but presence of native metals or non-stochiometric amorphous compounds are also possible. BET measurements show that the specific surface area was ~38 m^2/g higher (i.e. 107.7 m^2/g) than reported by the manufacturer (ca. 70 m^2/g). This difference in specific surface area may arise due to out-gassing of the powder at 300°C for 1 h before analysis. This may have volatilized the organic coating, thereby increasing the accessible surface area.

By TEM, we mainly observed aggregates and agglomerates of equidimensional to needle-shaped TiO_2 crystallites with diameters ranging from less than 10 nm to more than 100 nm along the shortest and longest axis, respectively (Figure 1). The average crystallite size was determined to be 20.6 ± 0.3 nm, in reasonable agreement with product data (Table 1). However, calculation of the average crystallite sizes in specific crystallographic directions indicated that the size along the c-axis (38.4 nm) was about 2.5 times the average size along the × and y (short) axes (14.4-15.5 nm). This is supported by the TEM analysis (Figure 1).

Table 1: Physico-chemical characteristics of UV-Titan L181 particles.

	This study	Product data sheet
Phases	Rutile	Rutile
Average XRD-size [nm]	20.6 ± 0.3	Approx. 17
XRD-size [100][a]	14.4-15.5	-
XRD-size [001][a]	38.4	-
Specific surface area [m^2/g]	107.7	Approx. 70
Elemental concentrations	[wt%]	
Silicon	5.61	-
Titanium	42.44	-
Aluminum	2.42	-
Zirconium	8.65	-
Sodium	0.45	-
Oxygen[b]	35.24	-
LOI	5.19	-
TGA	6.1 ± 0.4	-

LOI, loss on ignition; TGA, thermogravimetric analysis (N_2 atmosphere, 40 - 800°C, 10°C/min). [a] Estimate of the average crystallite size along the shortest and longest crystallographic direction. [b]Calculated by difference from 100 wt%.

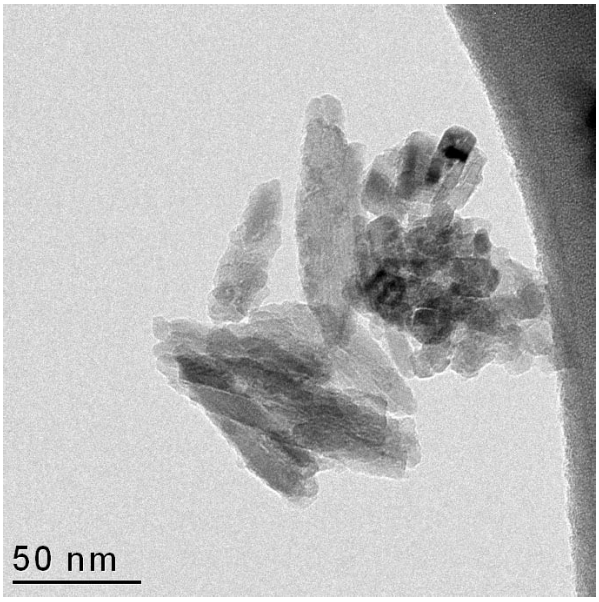

Figure 1 TEM image of TiO_2 crystallites. Transmission electron microscopy image showing the typical equidimensional to elongated morphology of the TiO_2 crystallites in UV-Titan L181. Bar = 50 nm.

The organic coating was analyzed by MALDI-MS. The identity of positive (protonated) molecules was deduced from the *m/z* values. The observed molecular formulas of the tentatively identified compounds are summarized in Table 2. The *m/z* values occurred in 4 series (as indicated in the MALDI-TOF spectrum in Additional file 1, Figure S1). Within each series, compounds were spaced by an *m/z* of 16, corresponding to oxygen, as shown in Table 2. This indicates that within each series, similar structures only differ by an OH-group. Thus, compounds with m/z = 104, 113, 115 and 173 contain at least two OH-groups. Furthermore, compounds in group 1 contain one c-c double bond, in group 2 three double bonds, in group 3 two double bonds and in group 4 three double bonds or carbonyl groups. In ESI-MS, only one peak was observed at *m/z* = 157 and this is the only peak for which the molecular formula has been determined via accurate mass determination. Our mass spectrometric analyses suggest that a fraction of the UV Titan L181 consists of polyalcohols with a chain length of 4, 6 or 8 carbons. However, these polyalcohols appear to be of a complex nature.

Exposure characteristics

Filter measurements demonstrated that animals were exposed to a mean total suspended particle mass concentration of 42.4 ± 2.9 (SEM) mg/m^3 UV-Titan. The particle number concentration in the exposure atmosphere was

Table 2: Observed m/z values and tentative molecular formulas

	m/z $[M+H]^+$	Tentative molecular formular
1	72	C_4H_7N
	88	C_4H_7NO
	104	C4H7NO2
2	81	C_6H_8
	97	C_6H_8O
	113	$C_6H_8O_2$
3	83	C_6H_{10}
	99	$C_6H_{10}O$
	115	$C_6H_{10}O_2$
4	141	$C_8H_{13}O_2$
	157*	$C_8H_{13}O_3$
	173	$C_8H_{13}O_4$

* Molecular formula determined from exact mass measurement (mass accuracy 1 ppm)

1.70 ± 0.20·10^6/cm^3. The major particle size-mode was ~100 nm (geometric mean number diameter 97 nm), with a coarser size mode at ~4 μm (Figure 2A). Smaller size modes were observed at ~20 nm and 1 μm. By number, 80% of the particles were between 40 and 200 nm and no particles were coarser than 12.5 μm detected (Figure 2B). The mass-size distribution was strongly dominated by μm-size particles (geometric mean 3.2 μm) and 75% of the mass were represented by particles larger than 1.6 μm (Figure 2B). The fraction of sub-100-nm-size particles amounted to 1% of the mass.

Ti concentration in tissues and milk

Ti concentration in tissue and milk samples is shown in Table 3. Lungs from exposed females contained 38 mg Ti/kg on day 5 after the exposure and 33 mg Ti/kg on days 26-27. No Ti was detected in unexposed female lungs (p = 0.0002). Values were similar between control and exposed animals for all other samples.

Maternal and litter parameters

Similar numbers of control and exposed females delivered litters, and none of the time-mated females without litters displayed implantations. Gestational and litter parameters were similar, apart from a slight decrease in pup viability in TiO_2 litters (p = 0.083, c.f. Table B, Additional file 1, Table S2l). Only maternal lung weight showed overall statistical significant variation with exposure, in both absolute (p = 0.04) and relative (p = 0.05) measures (data not shown). Pairwise comparisons showed both measures to be marginally increased in only in P females ($0.05 < p < 0.1$). No effects related to exposure were detected for offspring organ weights.

Lung inflammation in time-mated females

Lung inflammation was evaluated by cell counts of BAL fluid (Table 4 and Figure 3). Overall, more neutrophils were present in BAL in TiO_2 exposed compared to unexposed females (p < 0.001), with significant exposure-pregnancy interaction (p = 0.02). BAL from exposed NP females contained 19 times more neutrophils in BAL than did unexposed NP females (5 days after exposure, p < 0.001). The exposed P females displayed 3-fold more neutrophils compared to unexposed P females (26-27 days after exposure, p = 0.02). The exposure also resulted in overall change in macrophages (p = 0.002) and lymphocytes (p = 0.007) compared to unexposed P females. In NP females, pairwise comparisons revealed fewer macrophages (p = 0.009) but more lymphocytes (p = 0.008) in exposed compared to UNexposed NP females. No cell type showed significant change in exposed P females compared to respective controls. Overall, a statistically significant increase in the total number of dead cells in BAL fluid (p = 0.03) was observed in BAL from the exposed P females (p = 0.004) but not in BAL from exposed NP females. Total cell counts, total number of eosinophils, and epithelial cells in BAL were did not vary with exposure.

Behavioral data

In the Morris water maze, no change was observed in performance as a result of prenatal TiO_2 exposure in either male or female offspring (data not shown).

In the open field, ambulation differed by gender (p < 0.001) but not exposure, as females moved approximately 50% longer than males (Figure 4A). Prenatally exposed animals spent significantly less time than controls in the central zone of the field (p = 0.009), and visited the central zone less frequently (Exposure: p = 0.056; Gender: p = 0.003). Exposed males entered the central zone significantly less frequently than unexposed males (Figure 4B, p = 0.021) and exposed females spent less time in the central zone than did unexposed females (Figure 4C, p = 0.009).

Analysis of acoustic startle demonstrated that exposed male offspring startled less than control males and were less inhibited by prepulse, whereas the opposite pattern was apparent for female offspring (Additional file 1, Figure S3). Statistical analysis substantiated a stronger PPI in prenatally exposed females at the highest and lowest prepulse compared to control offspring (Figure 5B; p = 0.041 and p = 0.089, respectively).

Time-to-first F2 litter

At termination of behavioral testing, control and exposed C57BL offspring were cross-mated to naïve CBA/J mice.

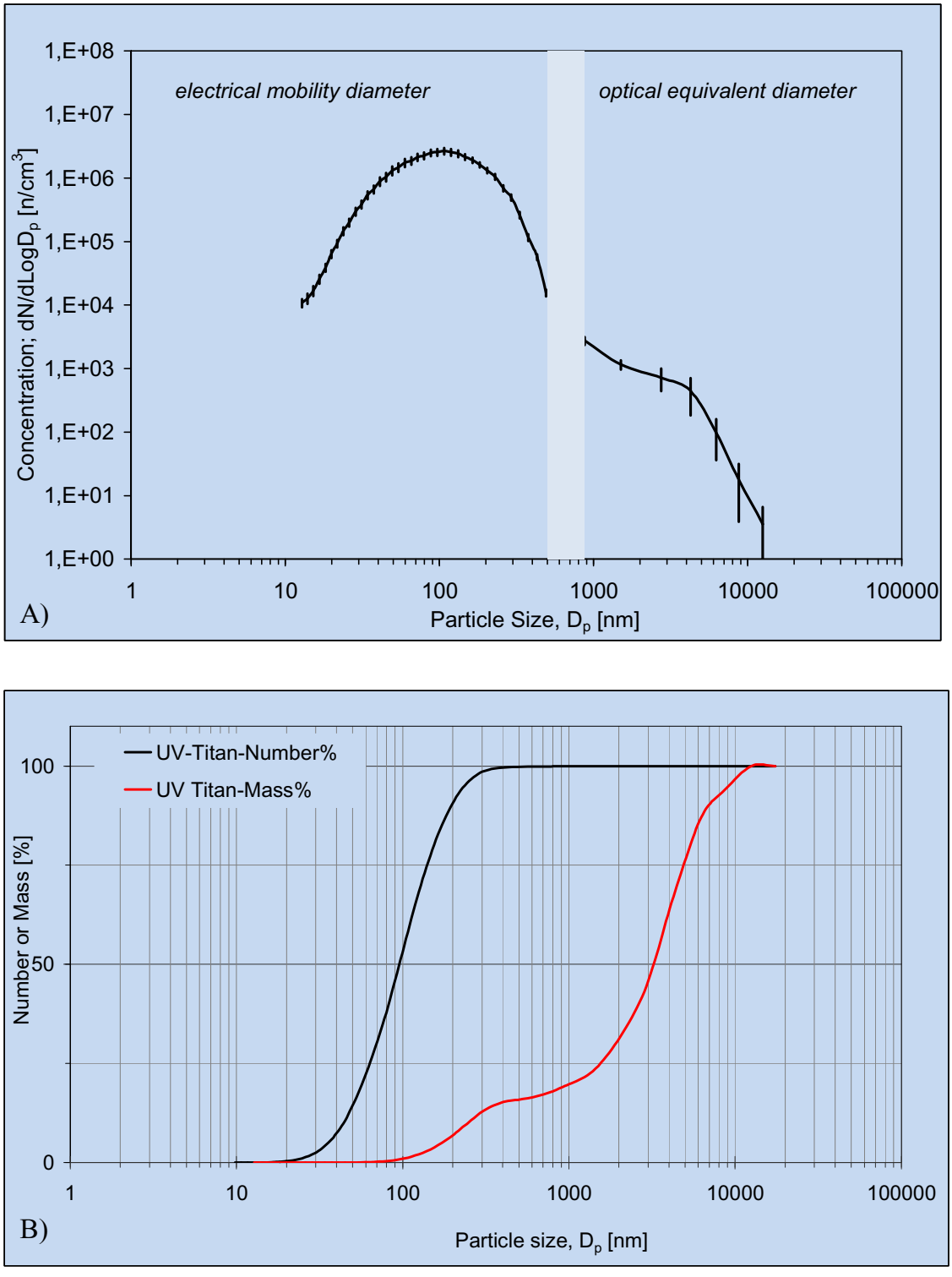

Figure 2 Characteristics of the exposure atmosphere. A) Particle number size distribution of the UV-Titan L181 in the exposure chamber. Data are based on nine one-hour exposure measurements. Mean ± SD. B) Accumulated number and mass concentration of particle concentrations in the exposure chamber. It is assumed that the optical and mobility particle sizes can be directly compared and data gap is filled by linear interpolation.

Table 3: Titanium concentration in livers, lungs and milk.

Origin	Tissue	Treatment	N	Time after exposure (days)	Ti (mg/kg)
Adult females	Lungs	Exposed	3	5	38 ± 6
		Controls	3	5	< 5
		Exposed	3	26-27	33 ± 18
		Controls	3	26-27	< 0.7
	Livers	Exposed	3	5	< 0.5
		Controls	3	5	< 0.5
		Exposed	3	26-27	0.5 ± 0.3
		Controls	3	26-27	< 0.2
Pups	Livers	Exposed	2[a]	5	< 0.4
		Controls	2[a]	5	0.4 ± 0.1
		Exposed	3	26-27	< 0.4
		Controls	3	26-27	< 0.4
	Milk	Exposed	2[b]	5	< 1
		Controls	2[b]	5	< 1

Mean ± SD corresponding to the two detected Ti isotopes. Pooled sample from 5[a] and 4[b] animals.

Time-to-first-delivery of F2 litter was similar in control and exposed female offspring, but was extended in exposed male compared to control male offspring (32.9 ± 3.1 (SD) and 25.2 ± 16.8 (SD) days, respectively; Figure 6). However, this result did not reach statistical significance (p = 0.12). Litter size was similar in control and exposed F2 litters.

Discussion

The effects of maternal inhalation of the UV Titan on offspring development were investigated. Eleven days of inhalation was associated with Ti deposition in pulmonary tissues and lung inflammation in adult females. High amounts of Ti and lung inflammation persisted in lungs 26-27 days following the last exposure. In addition, male and female mice exposed during fetal life displayed neurobehavioral alterations in adulthood. These observations occurred after a relevant route of exposure (inhalation) and dose (the 8-hour TWA for Danish Regulations). Thus, the results warrant careful scrutiny.

Our findings support previous evidence demonstrating long-term pulmonary inflammation following inhalation of TiO_2 nanoparticles in both mice and rats [18,23-25]. Inflammation characterized by increased recruitment of neutrophils after inhalation of mixed anatase and rutile TiO_2 nanoparticles (100 mg/m^3 for 6 hr/day for 5 days) was evident after two weeks in male rats, with slight signs of recovery [18,25]. A similar exposure carried out over 13 weeks also resulted in neutrophilic infiltration at 10 mg/m^3, but not at 0.5 and 2.0 mg/m^3. Altered cytological profiles persisted for 26 weeks in female rats and mice [24]. Interestingly it has been reported that the inflammatory response differs between the pregnant and the non-pregnant state. For example, pregnant mice displayed enhanced inflammation based on cell counts and inflam-

Table 4: Total cell counts after bronchioalveolar lavage

Treatment	Days after exposure	Total live cell count	Dead cell count
Control	5	166500 ± 13642	14000 ± 2236 (9%)
Exposed	5	202000 ± 18083	18667 ± 2014 (10%)
Control	26-27	171600 ± 19724	13600 ± 3748 (9%)**
Exposed	26-27	177000 ± 14325	25857 ± 3141 (15%)

Cells were counted in bronchoalveolar lavage fluid from time-mated mice that had not achieved pregnancy 5 days after termination of exposure (NP; n = 8-9) and in littering time-mated dams after weaning, 26-27 days after exposure (P; n = 13-14). Mean ± SEM. **p < 0.01 vs. control P.

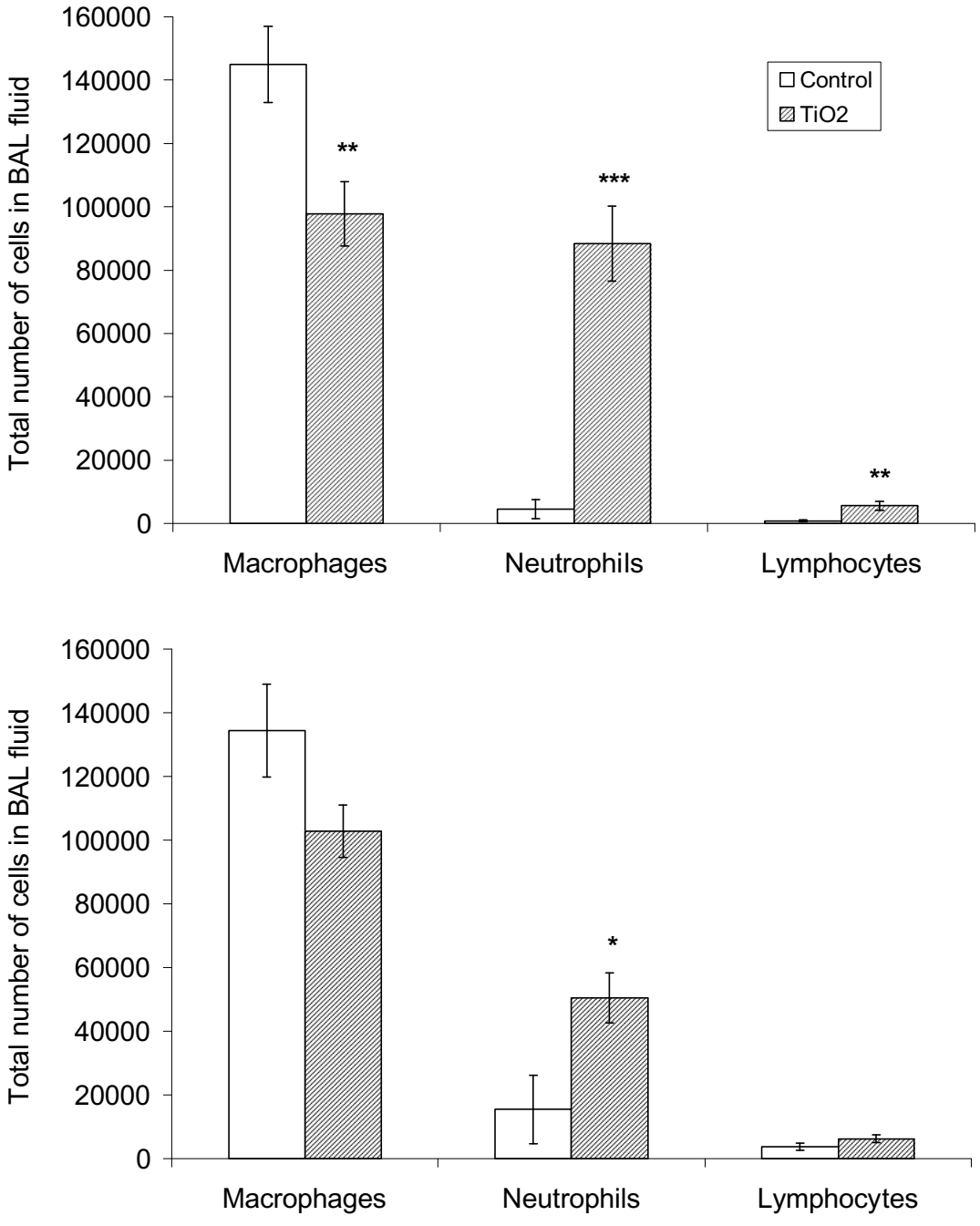

Figure 3 Differential cell count in bronchoalveolar lavage fluid. The total number of cells in BAL subdivided by cell type. A: time-mated mice that had not achieved pregnancy, 5 days after termination of exposure (n = 8-9). B: littering time-mated dams after weaning, 26-27 days after exposure (n = 10-14). Mean ±SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. controls.

matory cytokines in BAL fluids as compared to non-pregnant mice [26]. However, the present study design did not allow determination of the relative contribution of pregnancy and time after exposure.

Inhalation of nanoparticles during pregnancy may affect fetal development, through direct or indirect mechanisms. Once in the airways, the majority of nano-sized particles are predicted to deposit in the lung [7,18]. However, in the present study, despite the high number of 100 nm-size particles, the mass of airborne particles was strongly dominated by μm-size particles. Using the deposition model described in [21] and assuming electrical

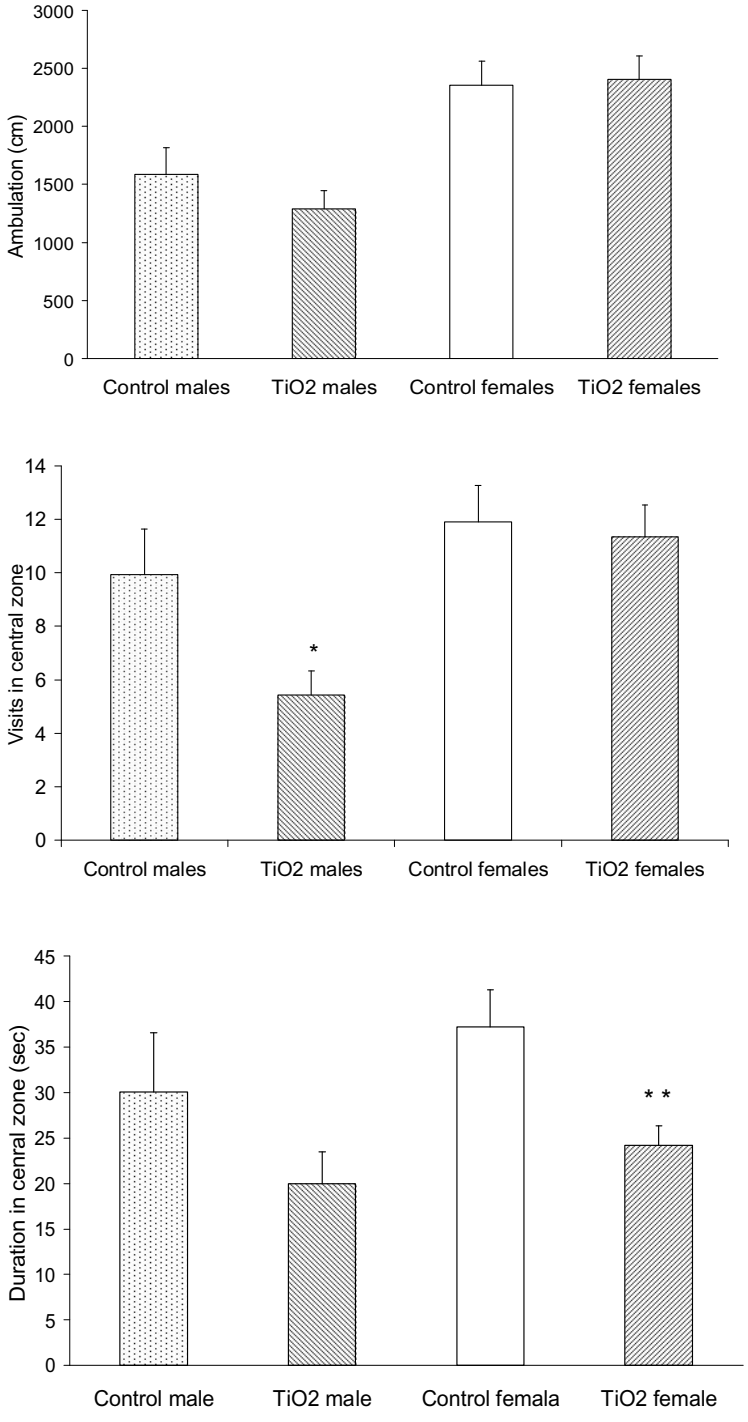

Figure 4 Open field. Open field performance during a 3-min observation period in male and female offspring from dams exposed to ambient air or TiO_2 during gestation. (A) Ambulation. (B) Visits to the central zone of the open field. Time spent in central zone of the open field (C). Mean ± SEM, n = 12-14. *$p < 0.05$, **$p < 0.01$, vs. same gender controls.

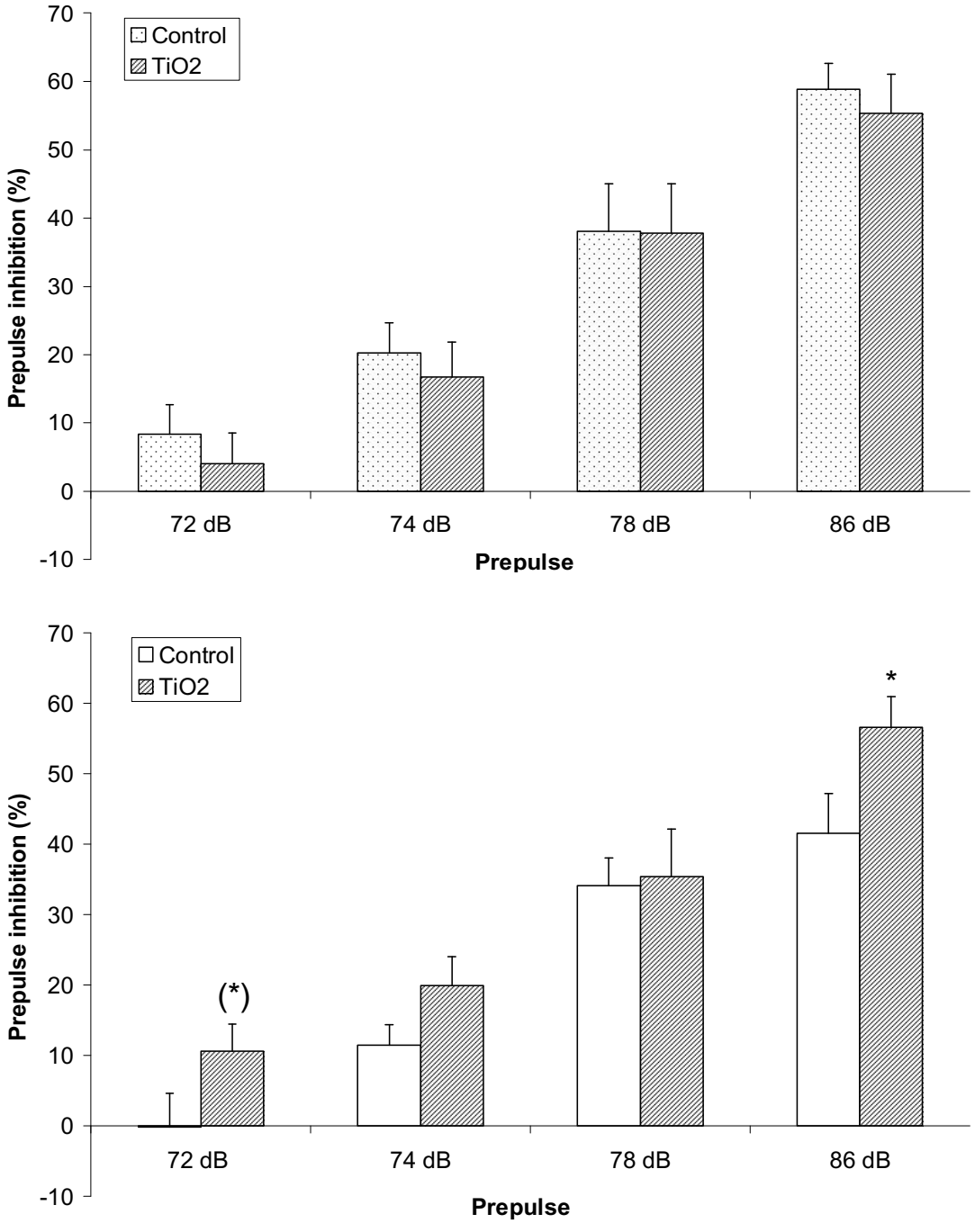

Figure 5 Prepulse inhibition. Prepulse inhibition in male (A) and female (B) offspring from dams exposed to ambient air or TiO_2 during gestation, at four different levels of prepulse. Mean ± SEM, n = 10-14. (*) $0.05 < p < 0.1$; *$p < 0.05$ vs. controls at same level of prepulse.

and optical equivalent sizes compare to the aerodynamic diameters, only 8.6% and 5.8% of the inhaled mass of airborne UV-Titan were predicted to deposit in pulmonary and tracheobronchial regions, respectively (Additional file 1, Figure S2A). Most of the UV-Titan mass is predicted to deposit in the upper airways (42.5%) and the gastrointestinal tract (42.5%). Correspondingly, the model suggests that 56.1% of the particle number would deposit in pulmonary and 18.4% in the tracheobronchial regions (Additional file 1, Figure S2B). Only 4.1% of the particle numbers are estimated to end up in the upper airways and 4.5% in the gastro-intestinal tract.

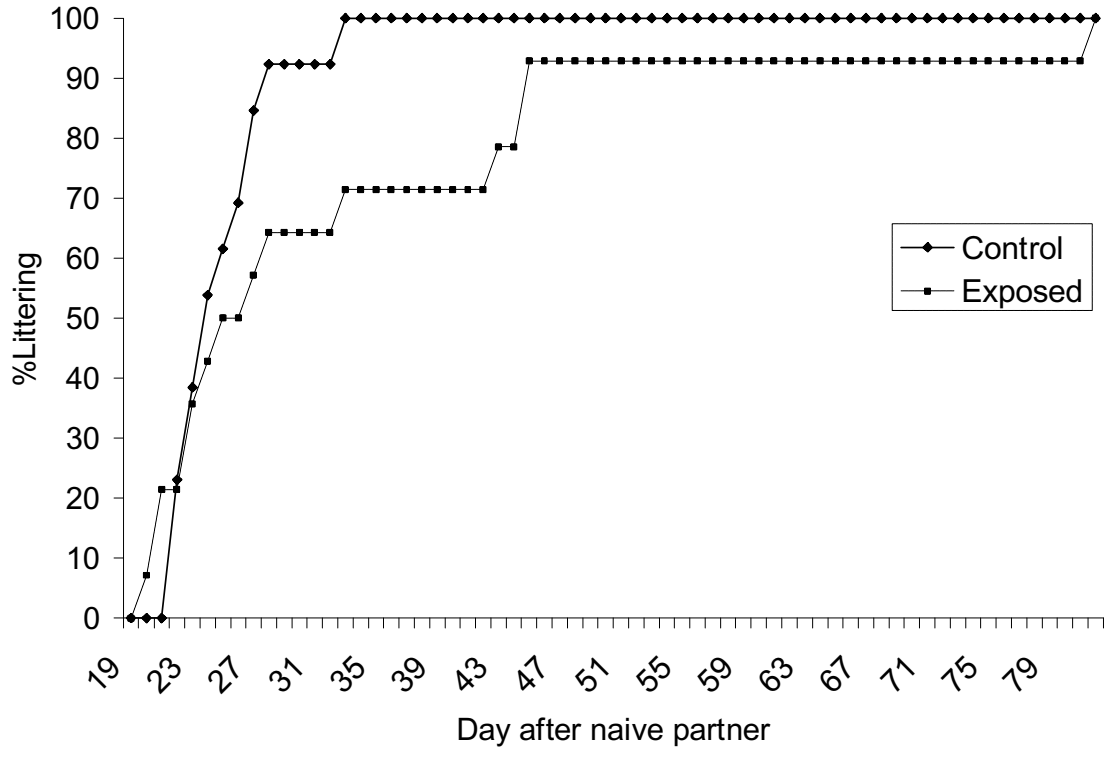

Figure 6 Time-to-first F2 litter. Littering curves for male offspring of control and UV-titan exposed pregnant mice. As adults, male C57BL offspring were mated to naïve CBA/J mice and time-to-first-delivery of F2 litter was recorded.

Assuming each animal inhaled 1.8 L/hr with a particle concentration of 42.4 mg/m^3 through 11 exposure sessions, each animal inhaled a total of 840 μg. Applying the deposition estimated above and ignoring clearance and potential translocation, we expected a deposition of 72.5 μg in the pulmonary and 48 μg in the tracheobronchial region. The majority of the mass was expected to deposit in the gastrointestinal tract (356 μg) and skull (267 μg). Hence, with an average lung weight of 274 mg, the estimated deposited pulmonary dose amounts to 112-159 mg UV Titan/kg lung depending on whether pulmonary or bronchopulmonary regions are considered. This corresponds to 48-67 mg Ti/kg after adjusting for Ti concentration in the sample (Table 1). The lungs of females contained 38 and 33 mg Ti/kg at 5 and 26-27 days post-exposure, respectively. Thus, approximately 60-80% of predicted pulmonary UV-Titan deposition could be accounted for. Clearance from the airways is the most plausible explanation for the observed discrepancy. In a recent study, 60% of the deposited 10 × 40 nm-size Si-coated rutile were cleared from lungs of mice inhaling 10 mg/m^3 for a total of 32 h over a 4 week period [4], which is in line with the present findings.

A small fraction of inhaled particles may translocate from the lungs to maternal body compartments [5,18]. Results from systemic exposure by intravenous injection suggest that most nano-size TiO_2 is distributed to the liver in rodents [17,18,27]. However, less than 0.25% of inhaled 20-30 nm mixed anatase/rutile (20 hr inhalation of 100 mg/m^3) was detected in liver up to 19 days after exposure, although some deposition in mediastinal lymph nodes was noted [18]. The low hepatic Ti-concentrations in the present study also suggest negligible translocation to the liver. Considering the observed particle size of the UV-Titan sample (Figure 1 and 2), translocation may only be relevant for a small fraction of the particles. About 30% of the particle number, but only 0.75% of the weight of the inhaled UV-Titan particles smaller than 100 nm, were estimated to deposit in the pulmonary region. If translocation and accumulation in the liver occur at an efficiency of 0.25‰, then the Ti concentration in the liver would be very low (< 375 ng) even though the number could be on the order of 100 particles. As expected, the Ti content in the offspring liver tissue was below the limit of detection even a few days after birth.

It has been demonstrated that a very limited fraction of particles in maternal blood is expected to transfer to the fetal compartment, although translocation may be higher for smaller compared to larger nanoparticles [19,28,29]. However, recent data from human placental perfusion

models showed that nearly 30% of polystyrene beads in the maternal circuit were transferred to the fetal compartment [30]. Takeda et al. (2009) also observed TiO_2 aggregates of particles in offspring testicle and brain tissue as long as six weeks after birth when pregnant mice were exposed subcutaneously to 25-70 nm particles at a total dose of 16 mg/kg [10]. Thus, since nano-sized particles may reach fetal tissues, direct exposure of the fetus to particles is possible.

Direct exposure of the fetus in the present study is expected to be low. However, indirect mechanisms could lead to fetal effects. Indeed, developmental effects have been observed even following limited maternal exposure. For example, the offspring of mothers exposed intranasally to a single dose of 50 μg nano-sized particles (TiO_2, carbon black, or diesel exhaust particles) during gestation display a more pronounced asthmatic phenotype. The underlying mechanism for this outcome remains unknown [26].

Engineered nanoparticles are often coated and/or organically functionalized. In this study, rutile is modified by Zr, Si, Al, and Na and coated with complex polyalcohols. Degradation or release of such coatings followed by placental transfer presents an additional mechanism by which nanoparticles may influence fetal development. Also, metals leached or dissolved from the nanomaterial may speciate into mobile ions and traverse the placenta [31,32]. For future studies it would be interesting to investigate the effect of pure and coated particles to elucidate the role of the particle surface in toxicity.

Thus, the literature, as well as our results, suggests that signaling cascades may be responsible for effects in animals exposed *in utero*. This is corroborated by observations of widespread changes in the expression of genes associated with acute phase, inflammation and immune response in NP females in the present study (Halappanavar S, personal communication). In the present experiment, maternal lung-inflammation following inhalation of UV-Titan may have resulted in cross-placental transfer of inflammatory cytokines [33]. Also diesel exhaust has been shown to increase placental mRNA levels of inflammatory cytokines in pregnant mice [34]. It is well established that maternal inflammation may adversely interfere with fetal neurodevelopment. Thus activation of the maternal immune system (in absence of pathogens) during gestation may induce significant changes in the nervous system and behavior of the offspring, and administration of exogenous pro-inflammatory cytokines may induce structural and functional abnormalities in the adult offspring (reviewed in [33,35]). Particle-induced inflammation may therefore represent yet another pathway for interference with fetal development. Finally, postnatal transfer could potentially take place through maternal milk [32], although we detected no Ti in milk a few days after delivery.

Offspring were evaluated in a neurobehavioral test battery. Exposed offspring tended to avoid the central zone of the open field. Furthermore, exposed female offspring displayed enhanced prepulse inhibition. To our knowledge this is the first study of prenatal (inhalation) exposure to nano-TiO_2 to assess nervous system function after birth. As described above, one study of prenatal exposure to pure 20-70 nm anatase TiO_2 reported particle aggregates in offspring brain tissue six weeks after birth. In addition, nervous tissue (olfactory bulb) showed some indications of increased apoptosis [10]. Another study, with an almost similar prenatal exposure regimen, reported gene expression changes related to apoptosis, development, and central neural system function in whole brain homogenate [36]. Two older studies assessed function of the central nervous system after prenatal exposure, but to trace amounts of dissolved Ti rather than particles. Exposed male offspring displayed some signs of delayed reflex emergency and decreased ambulation in the open field test, whereas female offspring showed increased number of errors in a maze learning test [37,38]. However, limited information of study designs for all three studies renders interpretation of these findings difficult. The minimal database on neurodevelopment following prenatal exposure to nanoparticles does not provide a background on which gender specificity of effects can be discussed. However, it is a common observation in neurodevelopmental studies that male and female offspring display differential phenotypes after prenatal insults (e.g. [39,40]), as is also reflected in the present study.

In a previous study, prenatal exposure to 20-70 nm anatase TiO_2 particles were observed in Leydig and Sertoli cells and in spermatids, 4 days and 6 weeks after birth. Furthermore, daily sperm production was significantly lower in exposed compared to control offspring [10]. Also exposure of pregnant mice to 14 nm carbon black particles by intratracheal instillation has been associated with significantly decreased daily sperm production and seminiferous tubule damage in the male offspring [41]. Following the behavioral testing, fecundity was therefore assessed by mating offspring to unexposed mice and recording time-to-first-litter. Male offspring that had been exposed to particulate TiO_2 during fetal life displayed a (non-significant) delay in time-to-first-litter. With this endpoint we would recommend to increase statistical power by increasing the number of breeding pairs.

Conclusions

Inhalation of nano-sized coated TiO_2 induced long-term lung inflammation in time-mated adult mice, and their

gestationally exposed offspring displayed neurobehavioral alterations. Exposure was conducted at an exposure level approximating the 8-hour TWA in Denmark. Future assessments of TiO_2 toxicity would benefit from adding more dose levels to aid risk assessment. Careful analysis of physicochemical characteristics of the nanomaterial and monitoring of the exposure atmosphere made estimation of actual dose possible. Although direct fetal exposure to UV-Titan was probably low, both direct and indirect pathways resulting from the exposure may interfere with fetal development and it is likely that several pathways operate to determine the outcome. In future studies, mapping changes in e.g. the molecular pathways that are altered in the brains of the descendents would help to shed light on the biological basis for the altered behavior. This would also reveal the molecular targets of the exposure and open up for understanding the potential relevance to human health.

Abbreviations and definitions

?: Diameter; ANCOVA: analysis of covariance; ANOVA: analysis of variance; ASR: acoustic startle reaction; AVG: average of tube movements for 100 ms following onset of startle stimulus; BAL: bronchoalveolar lavage; BET: Brunauer, Emmett, and Teller; CRC: collision/reaction cell; dB(A): decibel, A-weighted; EC: European Commission; ESI-MS: electrospray-MS; ENP: engineered nanoparticles; F2 litter: second generation litter; GD: gestation day; ICPMS: inductively coupled plasma mass spectrometer; LDI-TOF: laser desorption ionization time of flight mass spectrometry (MALDI-TOF without matrix assistance); LOD: limit of detection; *m/z* : mass to charge ratios MBq, megabecquerel; MS: mass spectrometry; nm: nanometer; NP females: non-pregnant time-mated females without implantations; P females: time-mated females with litters; PND: postnatal day; PPI: prepulse inhibition; SD: standard deviation; SEM: standard error of the mean; SMPS: sequential (stepping) mobility particle sizer; TEM: transmission electron microscopy; TGA: thermogravimetric analysis; TiO_2: titanium dioxide; TWA: time weighted average; UV: ultraviolet; XRD: X-ray diffraction.

Additional material

Additional file 1 PDF-file, containing additional description of methods, two tables and three figures. - Housing of animals. - BAL preparation and analysis. -Sample preparation for TEM analysis. - On-line particle exposure monitoring. Table S1: Settings for the ICPMS measurements. Table S2: Pregnancy and litter data. Figure S1: MALDI-TOF spectrum of methanol extract of UV-titan 181. Figure S2: Estimated deposition curves in the airways of UV-Titan in exposed mice. (A) Estimated accumulated mass deposition curves in the airways of UV-Titan in exposed mice. (B) Estimated accumulated particle number deposition curves in the airways of UV-Titan in exposed mice. Figure S3. Basal startle reaction, in male (A) and female (B) offspring from dams exposed to ambient air or TiO_2 during gestation.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KSH was substantially involved in design of the study, acquisition and analysis of gestational and behavioral data, statistical analyses, interpretation of results, and drafted the manuscript. PJA was substantially involved in designing the study, acquisition of gestational data, and drafting of the manuscript regarding BAL data and revised the manuscript critically. KAJ made substantial contribution to particle analysis, drafting of the manuscript regarding exposure characterization and discussion of data, and revised the manuscript critically. JJS, EHL and KAL analyzed Ti in tissues, and drafted the manuscript regarding this endpoint. RKB carried out particulate X-ray diffraction and drafted the manuscript regarding this endpoint. AV characterized the organic coating of the UV-titan and drafted the manuscript regarding this endpoint. HW contributed substantially to the study design, set up the particulate exposure and the exposure protocol, and revised the manuscript critically. AMB carried out postnatal breeding and contributed to the manuscript regarding this endpoint. UBV contributed substantially to the study design, drafting and interpretation of BAL data, and revised the manuscript critically. All authors have read and approved the final manuscript.

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PARTICLE AND FIBRE TOXICOLOGY

CORRECTION Open Access

Correction: Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice

Karin S Hougaard[1*], Petra Jackson[1,4], Keld A Jensen[1], Jens J Sloth[2], Katrin Löschner[2], Erik H Larsen[2], Renie K Birkedal[1], Anni Vibenholt[1], Anne-Mette Z Boisen[1,2], Håkan Wallin[1,3] and Ulla Vogel[1,2,4]

Correction

Some statements in our paper [1] are incorrect, below find corrections.

Table one, Zirkonium

The content of Zirkonium in the UV Titan was miscalculated by a factor 10. The correct number is therefore 0.86% (and not 8.65%).

P. 12, 1st column (In discussion)

For calculation of the retained amount of titanium, some inconsistencies appear in the text regarding titanium vs. the studied particle UV Titan, leading to overestimation of the retained dose. The correct text and numbers are given below:

Assuming each animal inhaled 1.8 L/hr with a particle concentration of 42.4 mg/m^3 through 11 exposure sessions, each animal inhaled a total of 840 μg. Applying the deposition estimated above and ignoring clearance and potential translocation, we expected a deposition of 72.5 μg in the pulmonary and 48 μg in the tracheobronchial region. The majority of the mass was expected to deposit in the gastrointestinal tract (356 μg) and skull (267 μg). Hence, with an average lung weight of 274 mg, the estimated deposited pulmonary dose amounts to 263-440 mg UV Titan/kg lung depending on whether pulmonary or bronchopulmonary regions are considered. Adjusting for Ti in the sample, this corresponds to 112-159 mg Ti/kg lung (Table one). The lungs of females contained 38 and 33 mg Ti/kg at 5 and 26-27 days post-exposure, respectively. Thus, approximately 21-24% of predicted pulmonary UV-Titan deposition could be accounted for."

Page 5, 2nd column (last lines of "Behavioral testing")

Final paragraph should read "The average of the 10 middle startle trials %PPI = 100-((AVG at prepulse+startle trial)/(AVG at startle trial))*100%".

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1. Hougaard KS, Jackson P, Jensen KA, Sloth JJ, Löschner K, Larsen EH, Birkedal RK, Vibenholt A, Boisen AM, Wallin H, Vogel U: **Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice.** *Part Fibre Toxicol* 2010, **7**:16.

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PAPER II

Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: A toxicogenomic study.

Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard KS, Vogel U, Yauk CL & Wallin H.

Environmental and Molecular Mutagenesis, 52(6):425-39.

Environmental and Molecular Mutagenesis 52:425–439 (2011)

Research Article

Pulmonary Response to Surface-Coated Nanotitanium Dioxide Particles Includes Induction of Acute Phase Response Genes, Inflammatory Cascades, and Changes in MicroRNAs: A Toxicogenomic Study

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Titanium dioxide nanoparticles (nanoTiO_2) are used in various applications including in paints. NanoTiO_2 inhalation may induce pulmonary toxicity and systemic effects. However, the underlying molecular mechanisms are poorly understood. In this study, the effects of inhaled surface-coated nanoTiO_2 on pulmonary global messenger RNA (mRNA) and microRNA (miRNA) expression in mouse were characterized to provide insight into the molecular response. Female C57BL/6BomTac mice were exposed for 1 hr daily to 42.4 ± 2.9 (SEM) mg surface-coated nanoTiO_2/m^3 for 11 consecutive days by inhalation and were sacrificed 5 days following the last exposure. Physicochemical properties of the particles were determined. Pulmonary response to nanoTiO_2 was characterized using DNA microarrays and pathway-specific PCR arrays and related to data on pulmonary inflammation from bronchial lavages. NanoTiO_2 exposure resulted in increased levels of mRNA for acute phase markers *serum amyloid A-1 (Saa1)* and *serum amyloid A-3 (Saa3*), several C-X-C and C-C motif chemokines, and cytokine *tumor necrosis factor* genes. Protein analysis of Saa1 and 3 showed selective upregulation of Saa3 in lung tissues. Sixteen miRNAs were induced by more than 1.2-fold (adjusted *P*-value < 0.05) following exposure. Real time polymerase chain reaction confirmed the upregulation of miR-1, miR-449a and revealed dramatic induction of miR-135b (60-fold). Thus, inhalation of surface-coated nanoTiO_2 results in changes in the expression of genes associated with acute phase, inflammation and immune response 5 days post exposure with concomitant changes in several miRNAs. The role of these miRNAs in pulmonary response to inhaled particles is unknown and warrants further research. Environ. Mol. Mutagen. 52:425–439, 2011.

Key words: nanotitanium dioxide; gene expression; microRNA; inflammation

Additional Supporting Information may be found in the online version of this article.

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BACKGROUND

Rapid developments in nanotechnology are resulting in increased use and potential release of a variety of engineered nanomaterials into the workplace and environment. Nanoparticles (NPs) are particles with a diameter less than 100 nm along at least one dimension and exhibit new or enhanced size-associated properties compared with larger particles of the same material. The large surface area, unique surface chemistry, and reactivity of NPs pose a unique challenge in assessing their effects on biological systems. Consequently, there is little knowledge of the molecular mechanisms leading to toxic effects following exposure to the diverse types of NPs, and there is no accepted framework for risk assessment of NP exposure [Oberdorster et al., 2005a,b; Borm et al., 2006].

Titanium dioxide nano particles (nanoTiO_2) are poorly soluble and were originally considered to be physiologically inert, posing little risk to human health. However, the International Agency for Research on Cancer has recently classified pigment-grade titanium dioxide as a group 2B carcinogen [IARC, Feb. 2006]. NanoTiO_2 has also been listed as a high priority NP by the Organization for Economic Cooperation and Development steering group for test guidelines. NanoTiO_2 are widely used in paints, paper, plastics, ceramics, and cosmetic sunscreens. Thus, there is potential for human exposure to nanoTiO_2 occupationally or through release from consumer products. As a result, there is an urgent need for experimental investigations on the biological effects of various types of NPs to increase our understanding of the mechanisms of NP action and establish safe regulatory guidelines for human exposure.

Studies have demonstrated that nanoTiO_2 causes cellular damage in exposed rodents. For example, chronic exposure to high doses of nanoTiO_2 by inhalation causes bronchoalveolar hyperplasia, metaplasia, pulmonary fibrosis, and tumor formation in rats [Lee et al., 1985; Heinrich et al., 1995], possibly resulting from impaired particle clearance [Warheit et al., 1997; Cullen et al., 2000]; lower doses cause lung inflammation in mice [Dankovic et al., 2007]. A single intratracheal dose of nanoTiO_2 can disrupt alveolar septa and induce emphysema-like changes in mouse lungs [Chen et al., 2006]. Particle accumulation in lungs and interstitial pneumonia associated with alveolar septal thickening results following intraperitoneal injection of nanoTiO_2 in mice [Chen et al., 2009]. A multispecies, subchronic, inhalation study demonstrated similar inflammatory responses, lung burdens, and pulmonary overload in both mice and rats exposed at high doses [Bermudez et al., 2004]. NanoTiO_2 is also cytotoxic and causes DNA damage and mutation in various animal and human cell lines [Rahman et al., 2002; Bermudez et al., 2004; Sayes et al., 2006; Warheit et al., 2006; Wang et al., 2007].

To increase its stability in paints, lotion, or creams, nanoTiO_2 in general are surface coated with silica, alumina, or other polymers. Surface-coated nanoTiO_2 in sunscreen lotions is expected to reduce its photoactivity. However, surface coatings and functionalization play a major role in nanoTiO_2-induced toxicity. For instance, mice challenged with nanoTiO_2 coated with silica exhibited significantly larger inflammatory responses than mice challenged with uncoated nanoTiO_2 or silica alone [Rossi et al., 2010]. Similarly, vanadium pentoxide-coated anatase nanoTiO_2 particles induced more cytotoxicity and genotoxicity than natural anatase [Bhattacharya et al., 2008]. Therefore, nanoTiO_2 toxicity is enhanced by surface modifications of the particles. However, it is unclear what changes arise at the molecular level that lead to differences in the toxicity induced by uncoated nanoTiO_2 relative to particles that are surface modified.

Genomic analyses provide a means to analyze the entire transcriptome of cells or tissues [Mei et al., 2010]. Early changes in critical pathways may be used to predict eventual health outcomes as well as derive the molecular mechanisms leading to toxicity [Hanahan and Weinberg, 2000; Bhattacharjee et al., 2001; Golpon et al., 2004; Ning et al., 2004; Spira et al., 2004; Granville and Dennis, 2005]. However, mRNA expression does not always correlate with related protein abundance and activity, as mRNA and proteins may be post-transcriptionally regulated. Small noncoding RNAs known as microRNAs (miRNAs) have recently been found to play a central role in the regulation of gene expression and protein translation. MiRNAs are typically 21–25 nucleotides in length and function in translational repression or mRNA degradation via the RNA interference pathway [Kim et al., 2009; Winter et al., 2009]. MiRNAs are also thought to play an important role in the maintenance of chromatin structure and are therefore critical mediators of gene expression and genome stability [Guil and Esteller, 2009]. MiRNAs are involved in numerous biological processes including apoptosis [Lynam-Lennon et al., 2009], cell cycle progression [Carleton et al., 2007; Bueno et al., 2008], development (reviewed in [Sun and Tsao, 2008]), and immune response (reviewed in [Tsitsiou and Lindsay, 2009]). Aberrant miRNA expression has been found in several human diseases (reviewed in [Wang et al., 2008]), and many cancers express unique miRNA signatures [Croce, 2009; Garzon et al., 2009]. As such, disruption of miRNA expression is now being widely investigated and various techniques have been developed for this purpose (reviewed in [Kong et al., 2009]).

We have recently investigated the developmental and neurological effects of surface-coated nanoTiO_2 particles [Hougaard et al., 2010]. We also reported pulmonary inflammation in nonpregnant adult female mice exposed by inhalation to a low, biologically relevant dose of rutile nanoTiO_2 particles that were surface modified and coated [Hougaard et al., 2010]. In this study, we apply DNA microarrays, pathway-specific real-time polymerase chain reaction (RT-PCR) arrays, focussed RT-PCR, and protein analysis to characterize the molecular changes associated with the observed pulmonary inflammation in the same

nonpregnant adult mice exposed to surface-coated nano-TiO_2 particles from the above study.

MATERIALS AND METHODS

Material, Animal Handling, and Tissue Collection

This study used UV-titan L181 with particle size of 20 nm (Kemira, Pori, Finland) enriched in rutile and modified with amounts of zirconium, silicon, aluminum, and coated with polyalcohol.

The study was conducted in parallel with a developmental toxicity study and therefore the particle dose and the time point were selected accordingly. Detailed information about animals, exposure, and exposure monitoring is described by Hougaard et al. [2010]. Briefly, 45 time-mated, nulliparous, adult female C57BL/6BomTac mice were treated as described by Hougaard et al. [2008] by whole-body inhalation to 42.4 ± 2.9 (SEM) mg nanoTiO_2/m^3 or to filtered air for 1 hr/day for 11 days. Seventeen (nine controls and eight exposed) of these animals did not conceive (lack of pups and interuterine implantation sites) and were used in this study as exposed adult females.

Animals were sacrificed by cardiac puncture 5 days after the last exposure. Left lung and a section of the liver were sliced randomly into portions and used for (a) total RNA extraction; (b) total RNA extraction enriched with small RNAs; and (c) total protein extracts. Samples were stored at −80°C until analysis. All procedures complied with EC Directive 86/609/EEC and Danish laws regulating experiments on animals (permit 2006/561-1123).

Material Characterization

Details of sample preparation and particle analysis are described in the Supporting Information provided by Hougaard et al. [2010]. In brief, physical particle size, morphology, and general state of agglomeration/aggregation were determined by transmission electron microscope (TEM, Tecnai G20, FEI Company, Hillsboro, OR). Crystalline phases and crystallite sizes were determined by powder X-ray diffraction (XRD) with a Bruker D8 Advance diffractometer equipped with a Lynxeye CCD detector (Bruker AXS, Madison, WI 53711-5373). Results were obtained by Rietveld refinement of the X-ray diffractograms using Bruker 10 TOPAS V4.1 software. Specific surface area was determined on a Quantachrome Autosorp-1 (Quantachrome GmbH & Co. KG, Odelzhausen, Germany) using multipoint Brunauer, Emmett, and Teller (BET) nitrogen adsorption method after 1 hr degassing at 300°C. Analysis was completed according to DIN ISO 9277 as a commercial service by Quantachrome GmbH & Co. KG. Elemental composition was analyzed by X-ray Fluorescence analysis on a Philips PW-2400 spectrometer as a commercial service by the Department of Earth Sciences, University of Aarhus, Denmark.

Exposure Monitoring

The fine particle exposure (<600 nm) was monitored using a GRIMM Sequential (Stepping) Mobility Particle Sizer (SMPS, Model No. 5.521; Serial No. 5LP 10209) connected to a GRIMM Condensation Particle Counter (Model 5.400). The SMPS data sampling and calculations were completed using the GRIMM software 5.477/02 v. 1.34 and operated in the fast scan mode. Data were corrected for both Classifier and CPC efficiency using the available software options. Particles were neutralized using a 3.7 MBq Am-241 source (Model No. 5.521). Coarse particle exposure (0.75 to > 15 μm) was measured using a GRIMM Dust Monitor (Model 1.105) at a resolution of 6 sec. The Dust Monitor particle sizes were subsequently recalculated to geometric means assuming an upper channel cut point at 20 μm.

Titanium in Tissue

Approximately 25–75 mg of lung tissue was weighed and digested in concentrated nitric acid (PlasmaPure, SCP Science, Quebec, Canada) in a microwave oven (Multiwave, Anton Paar, Graz, Austria), and titanium content was determined by quadrupole-based inductively coupled plasma mass spectrometer (ICPMS 7500ce, Agilent Technologies, Tokyo, Japan) equipped with a collision/reaction cell (CRC). The limit of detection (LOD) for titanium in tissues, based on three times the standard deviation of repeated blank measurements, was estimated to be 0.2–5 mg/kg depending on sample intake and dilution. Additional details are described by Hougaard et al. [2010].

Total RNA and MiRNA Extraction and Purification

Total RNA was isolated from the lung and the liver (n = 8/group) using TRIzol reagent (Invitrogen) and purified using RNeasy Mini Kit (Qiagen). The mirVana miRNA Isolation Kit (Ambion, Streetsville, ON, Canada) was used to prepare total RNA enriched with small RNA species from randomly selected left lung sections. RNA quality was confirmed by UV spectrophotometry and using an Agilent bioanalyzer (Agilent Technologies).

Microarray Hybridization

Individual total RNA (250 ng) samples from eight mice per treatment group (control or exposed) and universal reference total RNA (Stratagene) were used to synthesize double-stranded cDNA and cyanine labeled cRNA according to the manufacturer's instructions (Agilent Linear Amplification Kits, Agilent Technologies). Experimental samples were labeled with Cyanine 5-CTP and reference RNA with Cyanine 3-CTP (Perkin-Elmer Life Sciences). Cyanine-labeled cRNA targets were in vitro transcribed using T7 RNA polymerase, purified by RNeasy Mini Kit (Qiagen) and were hybridized to Agilent mouse 4 × 44 oligonucleotide microarrays (Agilent Technologies) at 60°C overnight. Arrays were washed and scanned on an Agilent G2505B scanner. Data were acquired using Agilent Feature Extraction software version 9.5.3.1.

MiRNA Expression Profiling

Freshly isolated individual total lung RNA samples from eight control and eight treated samples were labeled using the Agilent miRNA Complete Labeling and Hybridization Kit (Agilent Technologies). Briefly, 100 ng of total RNA was dephosphorylated by incubation with calf intestinal phosphatase at 37°C for 30 min, denatured using 100% DMSO at 100°C for 5 min, then labeled with pCp-Cy3 using T4 ligase by incubation at 16°C for 1 hr. The labeled RNA samples were hybridized to an individual array on 8 × 15K format Agilent mouse miRNA array slides. Hybridizations were performed in SureHyb chambers (Agilent) at 55°C for 20 hrs. Arrays were washed, scanned at a resolution of 5 μm using an Agilent G2505B scanner and data were acquired using Agilent Feature Extraction software version 9.5.3.1.

Statistical Analysis of Microarray Data

A reference design [Kerr, 2003; Kerr and Churchill, 2007] was used to analyze gene expression microarray data. Data were normalized using LOWESS in R [R-Development-Core-Team, 2004] and differential expression was determined using MAANOVA [Wu et al., 2003]. The Fs statistic [Cui et al., 2005] was used to test for treatment effects, and P-values were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach [Hochberg, 1995]. Fold change calculations were based on the least-square means. Significant genes were identified as having an adjusted P-value < 0.05 for any individual contrast.

Agilent MiRNA Microarray Analysis

Nonbackground subtracted raw data were quantile normalized [Bolstad et al., 2003]. Present calls were determined as signals that were >3 trimmed SDs above the trimmed mean of the (−)3 × SLv1 probes on the array. Probes with technical replicates for a miRNA were averaged using the median signal intensity. Boxplots and cluster analyses were used to identify potential outliers (poor quality chips). This quality control check resulted in the elimination of six arrays from the analysis. Identification of differentially expressed miRNA was carried out at the probe level as well as the miRNA level. The MAANOVA model included the sample identity as a random effect and the gene specific variance estimate (F1 Test) was used to test for differences between the control and treated samples. In this analysis, parametric *P*-values were obtained and were then FDR corrected.

Real-Time Polymerase Chain Reaction

Microarray

Primers were designed using Beacon design 2.0 (Premier BioSoft International). Approximately 2.5 μg of total RNA per sample was reverse transcribed and RT-PCR was performed in duplicate using an iCycler IQ real-time detection system (Bio-Rad). Threshold cycle values were averaged. Gene expression levels were normalized to the *GAPDH* and *Hprt* gene, which were stable on the DNA microarray. PCR efficiency was examined using the standard curve for each gene. Primer specificity was assured by the melting curve for each gene. A Students' t-test was used for statistical evaluation. A minimum of five samples/treatment group were used.

MicroRNA

The Qiagen miScript PCR system was used. For each sample ($n = 5$ per group), 1 μg of total RNA enriched with small RNA species was polyadenylated and then converted to cDNA using an oligodT primer with a universal tag and miScript Reverse Transcription mix. Real-time PCR was performed in duplicate for each sample, using a primer complementary to the universal tag and a miScript primer (Qiagen) specific for each miRNA. Amplified product was detected using SYBR Green and a CFX real-time detection system (Bio-Rad). Expression levels of miRNAs were normalized to expression levels of small nuclear RNAs RNU1A1 and RNU5A1. A Students' t-test was used to statistically evaluate the data.

Pathway-Specific PCR Arrays

Approximately 800 ng of total RNA per sample ($n = 6$/group) was reverse transcribed using a RT^2 first strand kit (SABiosciences™). Reverse transcription and real-time PCR reactions were carried out using RT^2 SYBR Green PCR Master Mix on 96-well PCR arrays designed for the evaluation of mouse inflammatory cytokines and receptors (No. PAMM-011D, SABiosciences™) using a CFX real-time Detection System (BioRad). Threshold cycle values were averaged. Relative gene expression was determined according to the comparative C_t method and normalized to the *Hprt* and β-*actin* housekeeping genes. Fold changes were calculated using online PCR array data analysis software (SABiosciences™). Statistical significance was calculated using the REST method [Pfaffl et al., 2002].

Pathway Analysis

Gene ontology was used to assign genes to functional categories in DAVID [Huang da et al., 2009]. KEGG pathways were used to identify specific biological pathways associated with the differentially expressed genes. In addition, we also conducted pathway analyses using two programs we developed to perform gene set enrichment analysis in R. KEGG gene sets were obtained using the mgug4121a.db R library. The first method is a rank-based test that takes into accounts the magnitude of the intensity value as well as the correlation between genes within a specific pathway. The advantage of this test is that no assumptions on distribution or independence are made. Genes in a pathway are ranked within each observation and the average rank is calculated for each treatment. The distance between the two treatments is calculated. Permutation analysis is used to obtain a *P*-value for each pathway (Table I in the manuscript). The second method (Design-based test) utilizes Euclidean distance between the estimated mean vector of two treatment groups as the test statistic. The null distribution of the test statistic distance is obtained using bootstrapping as described by Kerr and Churchill [2001]. The advantage of this method is that the experimental design used in the study is accounted for.

TABLE I. Gene Set Enrichment Analysis

Pathway	*P* value	Probe [a]Total	Probe [b]Changing
Rank-based test			
Complement and coagulation cascades	0.002	123	18
Hematopoietic cell lineage	0.002	253	13
Olfactory transduction	0.003	914	2
Pantothenate and CoA biosynthesis	0.008	26	1
Systemic lupus erythematosus	0.013	222	11
Cytokine-cytokine receptor interaction	0.018	652	33
gamma-Hexachlorocyclohexane degradation	0.021	27	3
Neuro active ligand-receptor interaction	0.041	421	3
Toll-like receptor signaling pathway	0.045	332	11
Design-based test			
Cytokine-cytokine receptor interaction	0.001	652	33
Pantothenate and CoA biosynthesis	0.001	26	1
Hematopoietic cell lineage	0.006	253	13
Complement and coagulation cascades	0.011	123	18
Systemic lupus erythematosus	0.017	222	11
ABC transporters	0.018	87	2
Natural killer cell mediated cytotoxicity	0.018	334	13
T cell receptor signaling pathway	0.019	352	11
gamma-Hexachlorocyclohexane degradation	0.026	27	3
Fc epsilon RI signaling pathway	0.029	243	13
Asthma	0.032	87	3
Jak-STAT signaling pathway	0.032	390	10
Linoleic acid metabolism	0.041	53	6

[a]Total indicates the total number of known probes in a given pathway.
[b]Changing indicates the number of probes changing in a given pathway following the exposure.

Preparation of Tissue Protein Extracts and Western Blotting

A random section of the frozen left lung lobe was homogenized in lysis buffer (5 M HEPES, pH 7.5, 5 M NaCl, 10% Glycerol, 1% Triton X-100, 2 M EGTA, 1 M $MgCl_2$, 0.5 M NaF, 0.2 M sodium pyrophosphate, protease inhibitor cocktail tablets (Roche Applied Science)) and centrifuged. The supernatant was quantified for protein content using a Bradford protein assay reagent kit (Bio-Rad).

Total lung protein extracts were immunoblotted on 14% SDS-PAGE gels and analyzed using antibodies against Saa3 (Santa Cruz Biotechnologies). Membranes were stripped and reprobed with anti-actin antibody for normalization. Band intensities were determined by averaging the densitometric readings from three biological replicates of control and treated samples from the same gel.

Total SAA Immunoassay

The Mouse SAA immunoassay (Invitrogen) was used to measure total Saa1 in lung tissue homogenates. The assay was conducted according to the manufacturer's instructions. Briefly, 100 µl of assay diluent and known quantities of controls (0–5 µg/ml mouse SAA supplied by the company) and individual samples (100 µg total tissue homogenates) in 50 µl of incubation buffer were loaded onto a microplate precoated with mouse Saa1 specific antibody followed by addition of biotinylated second monoclonal antibody. The plate was incubated at 37°C for 1 hr and unbound Saa1 was removed by washing. A streptavidin-HRP enzyme was then added to each well and incubated for 30 min at room temperature. Plates were washed to remove any unbound enzyme conjugate. One-hundred microliters of chromagen substrate solution was added to each well and incubated in the dark for 30 min at room temperature. The reaction was quenched by adding 100 µl of stop solution to individual wells. Optical Density for each well was determined at 450 nm using a microtiter plate spectrophotometer.

For analysis of Saa2, an Enzyme Immunoassay kit (Life Diagnostics) was used. One hundred micrograms of total protein from individual lung homogenates and controls (reference standard Saa2 0–500 ng/µl, supplied by the company) were loaded onto a plate precoated with anti-mouse Saa2 antibody along with horseradish peroxidase-conjugated polyclonal antibody. Plates were incubated for 1 hr at room temperature, wells were washed and 100 µl of chromagen was added to each well. Following incubation with chromagen for 20 min at room temperature, 100 µl of stop solution was added and the optical density was read at 450 nm in a microtiter plate reader. A minimum of 3 samples/treatment group were used.

RESULTS

Exposure and Particle Characterization

Mature female mice (n = 9/controls, 8/treated) were exposed for 1 hr per day for 11 consecutive days to 42.4 ± 2.9 (SEM) mg/m^3 nanoTiO_2 particles. Physicochemical characteristics of the nanoTiO_2 used in the study are presented in detail by Hougaard et al. [2010]. Briefly, the sample consisted of a rutile TiO_2-based material, which accounted for 70.8 wt% TiO_2. The other major constituents were modifiers of $SiO_2 > Al_2O_3 > ZrO_2$ and 5.2 wt% polyalcohol coating. Mass spectrometric analyses suggested that the polyalcohol consisted of a complex mixture with 4, 6, or 8 carbon chain length. BET measurements showed that the specific surface area was 107.7 m^2/g, which is slightly higher than what was reported by the manufacturer (70 m^2/g). Transmission electron microscopy showed that the powder particles consisted of aggregates and agglomerates of equidimensional to needle-shaped TiO_2 crystals with particle size ranging from less than 10 nm to more than 100 nm diameter. Rietveld analysis of powder X-ray diffractograms revealed that the average crystallite size of the TiO_2 was 20.6 ± 0.3 nm, but an elongation was also determined.

The mice were exposed to 42.4 ± 2.9 (SEM) mg nano-TiO_2/m^3 particles mass concentrations as indicated by periodic filter measurements. The number of particles in the exposure atmosphere was 1.70 ± 0.20 × 10^6/cm^3 with major particle sizes of ~100 nm and 4 µm. About 80% of the particles by number were between 40 and 200 nm with a maximum size of 12 µm. However, the mass–size distribution was dominated by micrometer-size particles. Sub-100 nm size particles made up less than 1% of the mass.

The concentration of TiO_2 particles in tissues was analyzed by ICPMS and showed 38 mg TiO_2/kg in the lungs of mice 5 days after the last exposure. The lungs of control mice did not contain TiO_2. No TiO_2 particles were detected in the liver tissues of the exposed or control mice [Hougaard et al., 2010].

Bronchoalveolar Lavage Fluid Analysis

To assess inflammatory response to nanoTiO_2 exposure in mouse lungs, inflammatory cell counts were performed on the bronchoalveolar lavage fluid (BALF) as described by Hougaard et al. [2010] and the response was reported in detail by Hougaard et al. [2010]. Briefly, the total number of cells in BALF from mice exposed to nanoTiO_2 increased by 21% compared with mice exposed to air only, a change that was not statistically significant. The percentage of neutrophils increased significantly from 2% in control (air exposed) mice to 43% in the nanoTiO_2-treated group (a 19-fold increase). However, the total number of macrophages decreased from 87% in controls to 49% in the treated group. There was a 6-fold increase in lymphocytes in the treated group and no significant changes in the total number of eosinophils and epithelial cells [Hougaard et al., 2010].

Gene Expression Analysis

MAANOVA analysis revealed 353 transcripts that were differentially expressed (FDR—P-values < 0.05) compared with matched controls. Of these, 53 had fold changes >1.5 for exposed versus control (50 upregulated and 3 downregulated) (full list in Additional File 1; File 1: Supporting Information Table S1). Complete microarray data are available through the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/), GSE 19487. A heatmap was generated using the Heatplus library [Ploner, 2008] in R [R-Development-Core-Team, 2009] using the log2 of the relative intensities. Any technical probe replicates were averaged using the mean. Outliers were removed based on the cluster analysis of the normalized log2 of the Cy3 and Cy5 channel. Hierarchical cluster analysis on differentially expressed genes revealed that samples within a treatment group were clustered together (Additional File 1; File 1: Supporting Information Fig. S1). Thus, a clear treatment effect was found as a result of exposure to nanoTiO_2. The genes with increased mRNA levels included the following: *serum amyloid A-3* (*Saa3*, 4.7-fold), *chemokine (C-X-C motif) ligand 5* (*cxcl5*, 4.4-fold), *lymphocyte antigen 6 complex, locus F* (*Ly6f*, 4.3-fold), *solute carrier family 26, member*

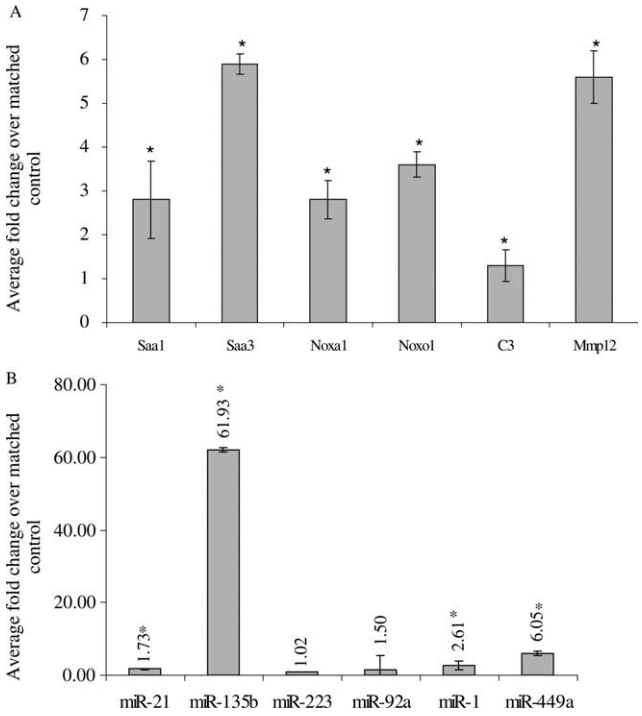

Fig. 1. Real time quantitative PCR validation of array results. **A**: Microarray results. Data (n = 5 mice/group, ±SEM) are presented as average fold changes over matched controls (air exposed). Gene expression values were normalized to internal reference gene *GAPDH* and *Hprt*. **B**: MicroRNA array results. Data are presented as average fold changes over matched controls (air exposed). (n = 5 mice/group, ±SEM). Expression values were normalized to small nuclear RNA RNU1A1. Numbers indicate fold changes. * indicates significant by Students' t-test.

4 (*Slc26a4*, 3.4-fold), *lipocalin 2* (*Lcn2*, 3.3-fold), and *NADPH oxidase organizer 1* (*Noxo1*, 3.0-fold). Among the genes with decreased mRNA levels, the largest decrease was observed for calcium-dependent phospholipid-binding protein, *Copine5* (*Cpne5*, 2.1-fold). Real time RT-PCR confirmed the altered mRNA levels of *serum amyloid A 1*(*Saa1*), *Saa3*, *Noxo1*, *NADPH oxidase activator 1* (*Noxa1*), *matrix metallopeptidase 12* (*Mmp12*), and *complement component 3* (*C3*) in the exposed mice (Fig. 1A).

Gene ontology analysis was used to assign genes to functional categories in DAVID [Huang da et al., 2009]. KEGG pathways were used to identify specific biological pathways associated with the differentially expressed genes. Pathway enrichment analysis applied two programs using the mgug4121a.db R library (Table I, rank-based test [Alvo et al., 2010], and a design-based test). The major pathways that were identified remained the same regardless of the method applied. These included cytokine–cytokine receptor interaction, metabolism, complement and coagulation cascade, hematopoeitic cell lineage, biosynthesis of steroids, and systemic lupus erythematosus.

TABLE II. List of all Acute Phase Response Genes Showing Fold Changes Higher Than 1.2 in exposed mice

Acute phase reactants	P value	Fold change[a]
Serum amyloid A1	**0.00**	**2.24**
Serum amyloid A3	**0.00**	**4.71**
Complement protein C3	**0.00**	**1.37**
Complement component 1, s (C1s)	**0.00**	**1.28**
Complement component 3a receptor 1 (C3ar1)	**0.00**	**1.15**
Complement component 1, q beta polypeptide (C1qb)	**0.00**	**1.30**
Complement component 1, r subcomponent (C1r)	**0.00**	**1.31**
Complement component C1RB (C1rb)	**0.00**	**1.21**
Fibrinogen	0.01	2.05
Coagulation factor II (F2)	0.01	1.72
Mannose binding protein	0.02	1.70
Albumin	0.01	1.79
apoA1	0.01	1.51
apoAII	0.03	1.61
alpha2-HS glycoprotein	0.00	1.85
S100A8 (calgranulin A)	0.01	−1.85
Serpina3n	0.00	1.37

Gene names in bold indicate FDR adjusted P value > 0.05.
[a]Average fold change compared with matched controls.

Induction of Acute Phase Response Reactants

NanoTiO_2 exposure altered the expression of several acute phase response genes (Table II) including *Saa1* and *3*. Previous work has demonstrated that acute phase response genes including *Saa* are induced in response to air pollution and inhaled particulates [Pope et al., 2004; Ruckerl et al., 2006]. Protein analysis was conducted to elucidate the role of *Saa* genes in nanoTiO_2-induced lung response. ELISA analysis of total lung tissue homogenates could not detect Saa2 in either treatment group (data not shown). A very modest decrease (0.86-fold) in total Saa1 protein was observed in the lung tissues of exposed mice (Additional File 1; File 1: Supporting Information Fig. S2A), in contrast to the gene expression results (Table II, 2.2-fold upregulated). This observation may have been due to outlying values from two control mice (expressing high amounts of Saa1 as indicated by the large error bars, Additional File 1; File 1: Supporting Information Fig. S2A.). Alternatively, *Saa1* may be targeted post-transcriptionally by a miRNA that is also induced in response to nanoTiO_2. Western blot analysis (no ELISA assay available) revealed a 2.2-fold increase in total Saa3 protein in the nanoTiO_2-treated group relative to matched controls (Additional File 1; File 1: Supporting Information Fig. S2B).

Induction of Inflammatory Cytokines and Receptors

NanoTiO_2 exposure resulted in increased mRNA levels of genes associated with cytokine–cytokine receptor sig-

TABLE III. Results of Pathway-Specific PCR Array (Mouse Inflammatory Cytokines and Receptors)

Gene name	*P* value	Fold change[a]
Chemokine (C-X-C motif) ligand 1 (Cxcl1)	0.000	7.00
Chemokine (C-C motif) ligand 2 (Ccl2)	0.000	4.20
Chemokine (C motif) receptor 1 (Xcr1)	0.000	1.80
Chemokine (C-X-C motif) ligand 5 (Cxcl5)	0.010	30.00
Secreted phosphoprotein 1 (Spp1)	0.010	2.00
Chemokine (C-C motif) ligand 6 (Ccl6)	0.021	1.90
Complement component 3 (C3)	0.021	1.70
Chemokine (C-C motif) ligand 22 (ccl22)	0.021	3.40
Chemokine (C-C motif) receptor 4 (Ccr4)	0.021	2.20
Chemokine (C-C motif) ligand 3 (ccl3)	0.024	2.20
Chemokine (C-C motif) ligand 12 (Ccl12)	0.026	2.00
Chemokine (C-C motif) ligand 9 (Ccl9)	0.028	1.70
Tumor necrosis factor (Tnf)	0.041	1.80
Chemokine (C-C motif) ligand 7 (Ccl7)	0.048	4.00

[a]Average fold change compared with matched controls.

naling and chemokine signaling pathways. These include chemokine (C-X-C motif and C-C motif) ligands (*cxcl5, cxcl1 and cxcl12, ccl7, ccl9, ccl2, ccl17, ccl12, and ccl19*). We confirmed the altered mRNA levels of these genes using pathway-specific PCR arrays (mouse inflammatory cytokines and receptors; SABiosciences™) containing 70 different cytokines and chemokines. Six individual samples from the control and treatment groups were analyzed. Twenty one genes were statistically significantly differentially expressed (1.5-fold) by t-tests (data not shown) and 14 genes by the REST method [Pfaffl et al., 2002] (Table III and Additional File 1; File 1: Supporting Information Table S2). The analysis showed significant increase in levels of *cxcl-5* (30.0-fold), *cxcl1* (7.0-fold), *ccl2* (4.2-fold), *ccl22* (3.4-fold), *ccl7* (3.8-fold), *ccr4* (2.1-fold), and cytokine *tumor necrosis factor* (*TNF*, 1.8-fold) in lung tissues of mice treated with nanoTiO$_2$. Expression of *interleukin-6*, *interleukin 1 beta*, and *IFN gamma* did not change. Overall, 14 transcripts that were significantly differentially expressed with FC $>$ 1.5 as measured by the microarray were validated using the PCR arrays. Eleven of them are in agreement with the results of the microarray.

Hepatic Gene Expression

We analyzed liver mRNA from the same mice and found differential expression of 11 genes (FDR *P*-value $<$ 0.05); however, these expression changes were modest; the largest change was a 1.6-fold downregulation of ''*similar to hepatocellular carcinoma-associated gene TD26*.'' The other 10 genes had fold changes below 1.2. The DNA microarray data are available through the NCBI (http://www.ncbi.nlm.nih.gov/geo/), GSE 19487. Overall ranking of all genes according to fold change demonstrated that gene expression was relatively unchanged in the livers.

TABLE IV. MiRNA Results

Probe	FDR *P* value	Fold change[a]
mmu-miR-449a	0.000	2.81
mmu-miR-1	0.000	1.92
mmu-miR-135b	0.000	1.83
mmu-miR-133b	0.004	1.59
mmu-miR-144	0.008	1.53
mmu-miR-21	0.000	1.52
mmu-miR-133a	0.018	1.39
mmu-miR-34b-5p	0.000	1.33
mmu-miR-193	0.048	1.29
mmu-miR-34c	0.000	1.28
mmu-miR-141	0.001	1.28
mmu-miR-33	0.003	1.26
mmu-miR-342-3p	0.000	−1.25
mmu-miR-92a	0.007	−1.26
mmu-miR-720	0.016	−1.28
mmu-miR-223	0.001	−1.39

[a]Average fold change compared with matched controls.

Global MiRNA Expression Changes

Agilent arrays containing 567 mouse probes were used to examine changes in miRNA following exposure to nanoTiO$_2$. The data are available through the NCBI (http://www.ncbi.nlm.nih.gov/geo/), GSE 19487. Fifty-five miRNAs were significantly altered by nanoTiO$_2$ exposure following FDR-adjustment (Table IV). The levels of six miRNAs were increased by more than 1.5-fold; the only downregulated miRNAs were miR-92a (1.3-fold) and miR-223 (1.4-fold)). Real time RT-PCR analysis confirmed the upregulation of miR-449a, miR-1, and miR-135b (Fig. 1B).

DISCUSSION

Short term or chronic exposure to nanoTiO$_2$ particles by inhalation or instillation has revealed that nanoTiO$_2$ particles induce a complex pulmonary response that potentially involves alterations in signaling cascades and associated expression of numerous genes and proteins [Lee et al., 1985; Bermudez et al., 2002; Bermudez et al., 2004; Warheit and Frame, 2006]. Changes in gene expression are presumed to occur in response to exposure and in the early stages of disease development, and thus lead to the downstream biological outcome. MiRNAs are proposed to be one of the mechanisms regulating mRNA and protein levels following toxicant exposure [Hudder and Novak, 2008; Taylor and Gant, 2008], and thus are potentially important mediators of toxic outcome. Concurrent analysis of mRNAs and miRNAs is a powerful approach to explore the direct response of the genome to toxicant exposure.

We have recently reported the developmental and neurological effects of surface-coated nanoTiO$_2$ particles [Hougaard et al., 2010]. Pregnant mice were exposed for 1 hr daily for 11 consecutive days to 42.4 $\pm$ 2.9 (SEM)

mg/m^3 suspended nanoTiO$_2$ particles via inhalation from gestational day 8–18. The mice that failed to conceive were sacrificed 5 days following the exposure. These mice (nonpregnant adult) exhibited evidence of pulmonary inflammation by analysis of cell counts in BALF. In this study, we characterize the global mRNA and miRNA response in the lungs of the nonpregnant adult mice, to provide insight into the molecular mechanisms contributing to this effect. We also analyze global hepatic mRNA response to the inhaled nanoTiO$_2$. We demonstrate alterations in pulmonary mRNA levels in pathways that are consistent with the observed and predicted effects of particle exposure and also in line with the observed phenotype. We also report perturbation of several miRNAs in the lungs of these mice. In contrast, gene expression was relatively unaffected in the livers of the same mice.

Pulmonary Inflammation

Analysis of different cell counts in BALF revealed an increase in the total number of neutrophils, supporting the presence of pulmonary inflammation, 5 days following nanoTiO$_2$ exposure in mature female mice from this study [Hougaard et al., 2010]. Long-term pulmonary inflammation following inhalation of nanoTiO$_2$ has previously been documented in both mouse and rat models [Bermudez et al., 2004; Ma-Hock et al., 2009; van Ravenzwaay et al., 2009]. For example, studies have demonstrated increased infiltration of neutrophils 2 weeks following inhalation of 100 mg/m^3 mixed anatase and rutile nanoTiO$_2$ for 6 hrs/day for 5 days [Ma-Hock et al., 2009; van Ravenzwaay et al., 2009]. Similarly, mice exposed to 10 mg/m^3 nanoTiO$_2$ for 6 hrs/day, 5 days/week for 13 weeks showed persistent increases in the number of neutrophils following a 52 day recovery period [Bermudez et al., 2004]. These studies, along with the data presented here, suggest that nanoTiO$_2$-induced pulmonary inflammation is characterized by neutrophil infiltration.

This study also supports previous work demonstrating that surface-treated nanoTiO$_2$ particles are inflammogenic. For example, intratracheal instillation of surface-treated (alumina or alumina and amorphous silica) nanoTiO$_2$ caused pulmonary inflammation in rats [Warheit et al., 2005]. High doses of silanized hydrophobic ultrafine TiO$_2$ instilled into rats resulted in increased lung toxicity [Pott et al., 1998]. Mild inflammatory changes were noted in rats instilled with low doses of silanized nanoTiO$_2$ [Rehn et al., 2003]. Similar inflammogenic responses were observed in several other studies using ultrafine nonsurface-modifed nanoTiO$_2$ both *in vitro* and *in vivo* [Baggs et al., 1997; Drumm et al., 1999; Bermudez et al., 2002; Bermudez et al., 2004; Renwick et al., 2004; de Haar et al., 2006]. The extent of inflammation observed in this study is in line with the overall calculated mass of nanoTiO$_2$ deposited in the pulmonary region (72.5 μg with a surface area of 77.6 cm^2). Differential cell count analysis of BALF revealed that 43% of the cells were neutrophils [Hougaard et al., 2010]. This finding is in close agreement with a previous experiment where mice exposed by instillation to nanoTiO$_2$ with a surface area of 50 cm^2 exhibited 45% neutrophils in BALF [Oberdorster et al., 2005b]. A linear correlation between the total number of neutrophils in BALF and particle surface area was noted in mice exposed for 24 hrs to five different types of nanoparticles, including the nanoTiO$_2$ studied in this study (Saber et al., personal communication). Similar relationships between surface area and total number of neutrophils has also been reported by Tran et al. [Tran et al., 2000]. Taken together, these studies demonstrate that nanoTiO$_2$ exposure promotes proinflammatory activities across a wide range of doses, necessitating an understanding of the molecular pathways involved in this response.

mRNA Expression Profiling

Global transcriptional profiling of lung samples from adult female mice exposed to nanoTiO$_2$ revealed significant differential expression of several inflammation modulators. Inhalation exposure to 42.4 ± 2.9 (SEM) mg/m^3 nanoTiO$_2$ for 1 hr every day for 11 consecutive days induced significant changes in the mRNA levels of several chemokine genes. These include *ccl2* (*MCP-1 alpha*), *cxcl1* (*keratinocyte cell-derived chemokine, KC*), *cxcl5* (*epithelial cell-derived neutrophil activating peptide ENA78*), *ccl22* (*macrophage-derived chemokine*), *ccl7* (*monocyte chemotactic protein 3, MCP3*), *ccl9*/*MIP1 gamma*, and *ccl3* (*monocyte inflammatory protein, MIP-1 alpha*). These genes participate in the modulation of chemotaxis, infiltration of neutrophils, and epithelial proliferation. Because it is difficult to interpret the biological relevance of small fold changes of individual genes, we emphasize changes in pathways and groups of genes (i.e., pathways, and Gene Ontology molecular functions and biological processes) in our discussion below. When many genes that interact together to carry out a biological function are perturbed, this is more likely to be biologically relevant than an individual gene within that family. Thus, the gene expression changes we observed collectively provide molecular insight into the findings on inflammation described earlier.

Neutrophil sequesteration is a critical event during pulmonary inflammation and involves multiple steps including the following: Increased production of chemokines and cell adhesion molecules, and enhanced interactions between cells. Cxcl1, macrophage inflammatory protein −2 (MIP2), and cxcl5 are the major chemokines for neutrophil recruitment and accumulation in mouse lungs. Cxcl1 and MIP2 are produced predominantly by the infiltrating myeloid cells (macrophages and neutrophils) and increases in their levels in response to a stimulant are rapid and transient. In contrast, cxcl5 is produced by resi-

dent cells, such as alveolar epithelial type II cells. Elevated levels of cxcl5 persist for days during lung inflammation. Preferential and persistent induction of cxcl5 in resident cells may be important in establishing cellular communication between myeloid cells and resident cells, which is required for initiation, maintenance and resolution of an inflammatory process [Jeyaseelan et al., 2005]. We observed high levels of *cxcl5* 5 days following the last exposure in this study. This finding indicates epithelial cell activation and may suggest continued lung injury and persistent pulmonary inflammation.

Increased expression of ccl3, cxcl5, and cxcl1 has been shown following nanoTiO$_2$ exposure in several *in vivo* mouse models; these findings were associated with the development of pulmonary inflammation, granulomas, emphysema, and pulmonary host defense [Driscoll et al., 1993; Park et al., 2010; Rossi et al., 2010]. Disregulation of inflammatory pathways following inhalation of nanoTiO$_2$ has also been shown by Chen et al. using a DNA microarray approach [Chen et al., 2006]. These authors used small (~6,000 clones) ''home-made'' cDNA microarrays to show that a single intratracheal instillation of 0.1 mg nanoTiO$_2$ (rutile, uncoated pure particles of 19–21 nm in size) caused severe pulmonary inflammation with alterations in genes involved in several pathways that overlap with this work (chemokines and coagulation complement cascade). Chemotaxis and immune response pathways, and Th1 and 2 type cytokines were also differentially regulated in the BALF of mice treated with a single intratracheal dose of 20 mg/kg nanoTiO$_2$ (P25, primary particle size 20 nm) 14 days after the last exposure [Park et al., 2009]. In many of the aforementioned studies, comparatively high doses of particles were directly deposited into the lungs of the animals by intratracheal instillation. In this study, mice were exposed using a biologically relevant exposure route to a relatively low dose of nanoTiO$_2$ (mimicking the occupational scenario in terms of the total mass administered) and similar changes in gene expression profiles were noted despite differences in doses and time points. In contrast to our data and the studies described earlier, Rehn et al. [2003] reported no signs of inflammation in rats exposed to a range of occupationally relevant low doses of surface-treated nanoTiO$_2$. Discrepancies between studies may be due to the targeted analysis of specific markers of inflammation and endpoints (e.g., total protein and BAL cells) applied by Rehn et al. [2003] compared with analysis of the entire transcriptome. Many nanotoxicological studies obtain data from a single endpoint representative of a particular mechanism of action, focusing on specific sets of genes or even a single pathway. The drawback of this approach is that the mechanism of action must be known beforehand; moreover, the expected phenotype or the physiological change may not be apparent in the very low dose range. Global gene expression profiles provide a snapshot of multiple cellular processes operating in a single experiment without prior knowledge of the mechanism of action. It is particularly suitable to identifying early molecular changes predictive of eventual pathological outcome. Thus, we demonstrate that global gene expression signatures support the observed phenotypes for exposure to nanoTiO$_2$ that response can be measured in the low-dose range, and the results obtained can be used to provide insight into the molecular mechanisms operating in response.

Pulmonary response to nanoTiO$_2$ also causes increase in levels of genes in the acute phase response pathway (Table II) including *Saa1, Saa3, C3*, complement components *1s* (*C1s*), *3ar1* (*C3ar1*), *1qb* (*C1qb*), *1r* (*C1r*), and *1rb* (*C1rb*). Moreover, several other acute phase proteins exhibited fold changes >1.5-fold with unadjusted *P*-values < 0.05. These include *C- reactive protein*, *fibrinogen*, *coagulation factor II*, *mannose binding protein*, *albumin*, *apoAI*, *apoAII*, and *a2-hs-glycoprotein* (regulator of SAA) (Table II). The complement cascade includes 30 proteins (some of which are enzymes), cofactors, inhibitors, and membrane-associated proteins. These molecules act in host defense by promoting phagocytosis and inflammation, which can activate the complement cascade resulting in chemotaxis and pulmonary inflammation. Acute-phase proteins have been used as biochemical markers of disease for numerous inflammatory processes. Induction of acute phase response has previously been observed in rodents following particle exposure. *Saa* is a generic name for a family of apolipoproteins [Meek and Benditt, 1986] synthesized in response to activated monocyte/macrophage-released cytokines such as IL-1 and IL-6 [Ganapathi et al., 1988; Jiang et al., 1995; Jensen and Whitehead, 1998]. Although the liver is the major site of synthesis of *Saa* [Morrow et al., 1981; Lowell et al., 1986], extrahepatic tissues including lung, spleen, adrenal gland, and others have been shown to express varying levels of individual members of *Saa* genes in response to different inflammatory stimuli [Meek and Benditt, 1986]. In this study, *Saa* genes (*1* and *3*) were strongly induced in the lungs of mice exposed to nanoTiO$_2$. The increase in *Saa3* mRNA levels was much greater (5.9-fold) than *Saa1* (2.8-fold). Corresponding protein levels of Saa3 were also changed in lung tissue homogenates of the treated group (Supporting Information Fig. S2B) but no change was observed for Saa1. Previous work has demonstrated that *Saa* genes are induced in mouse lung in response to dust and lipopolysaccharide (LPS) [Meek and Benditt, 1986; Andre et al., 2006; Park et al., 2010]. In keeping with this study, while injection of LPS resulted in increased mRNA levels of all three *Saa* genes in liver, *Saa3* was predominantly expressed in lungs. Similarly, the largest increase in *Saa3* mRNA levels was noted in mouse lungs after ultrafine particle exposure [Andre et al., 2006].

Select upregulation of Saa3 and associated proteins, together with increased gene expression of a suite of cyto-

kines, chemokines, and metalloproteases in our model suggests a critical role for Saa3 in nanoTiO_2-induced pulmonary inflammation/immune response. In premetastatic lungs, Saa3 induction by chemoattractants such as S100A8 plays a role in myeloid cell accumulation and acts as a positive feedback regulator for chemoattractant secretion [Hiratsuka et al., 2008]. In this study, *S100A8* mRNA levels were decreased compared with the controls (-1.9-fold; unadjusted P-value < 0.05) in the treatment group suggesting the involvement of a different regulatory mechanism for Saa3. Although the cytokines IL-1, IL-6, and TNF play a role in the expression of *Saa* genes, only *TNF* was marginally upregulated in our model. We also observed a marginal increase in TNF protein in BAL cells as well in lung tissue homogenates by protein arrays (data not shown), suggesting a possible role for TNF in *Saa3* expression in response to nanoTiO_2.

Several chemokines (*cxcl5, cxcl1*, and *ccl3*) and components of the acute phase pathway (*Saa3, c3*) measured in this study are rapidly induced in response to acute injury or stress. However, the increase in their expression levels is transient and falls rapidly once the stimulus is removed or the injury is repaired. Thus, high and persisting levels of these acute stressors 5 days after the last exposure may reflect the magnitude of the injury and indicate initiation of secondary pathological conditions.

In addition to confirming the response of these pathways, we also found a dramatic increase in *polymeric Ig receptor* (*PIgr*, 2.0-fold), *solute carrier family 26, member 4* (*Slc26a4*, 3.4-fold), and *lipocalin 2* (*Lcn2*, 3.3-fold). These genes are involved in mucosal immune defense. *NADPH oxidase organizer 1* (*Noxo1*), a gene associated with antimicrobial defense [Flo et al., 2004; Schneeman et al., 2005; Nakao et al., 2008], was also upregulated by 3-fold. We also found upregulation of the proinflammatory genes *Vanin-1* and *3* (*Vnn-1,−3*, 2.6- and 1.9-fold, respectively) in nanoTiO_2-exposed lungs [Nitto et al., 2008]. Thus, our results confirm strong activation of pulmonary immune and inflammation in response to nanoTiO_2 inhalation.

Despite significant upregulation of several genes associated with acute phase, inflammation and immune response in lung, the hepatic transcriptome of these mice was unaffected. These findings agree with our previous report showing few changes in hepatic transcription in mice exposed by inhalation to doses of carbon black and diesel exhaust that caused substantial pulmonary inflammation [Saber et al., 2009]. The results suggest that systemic effects in liver are secondary to pulmonary inflammation and may not require gene expression.

NanoTiO_2 Exposure

Based on the exposure conditions, each animal in this study was exposed to ~840 μg of nanoTiO_2 (11 days $\times$ 1 hr/day $\times$ 40 mg/m^3 $\times$ 0.0018 m^3 inhaled/hr = 0.840 mg/animal). It is predicted that the majority of nanosized particles deposit in the lung once inhaled. The deposition of particles in the lungs depends on the size of the particles. In this study, the mass of the airborne fraction contained predominantly micrometer-sized particles. On the basis of the deposition model described by Jacobsen et al. [2009], we expected a deposition of 72.5 μg in the pulmonary region, 48 μg in the tracheobronchial region, 356 μg in the gastrointestinal tract, and 267 μg of nanoTiO_2 in the skull. Hence, with an average lung weight of 274 mg, the estimated deposited pulmonary dose amounts to 265–439 mg/kg nanoTiO_2 corresponding to 112–159 mg nanotitanium (Ti)/kg in the lung, depending on whether pulmonary or bronchopulmonary regions are considered. Detection in lung tissue using ICPMS revealed 38 mg/kg Ti in the lungs [Hougaard et al., 2010], indicating that although significant clearing of the lungs had taken place during the 16 days after the first exposure, considerable amounts of nanoTiO_2 were still present 5 days postexposure. This corresponds to 24–34% of predicted nanoTiO_2 deposition in lungs. Our results are in agreement with Rossi et al. [2010], who exposed mice by inhalation to 10 mg/m^3 of surface-coated nanoTiO_2 (40 nm) for a total of 32 hrs. They showed ~60% of the deposited nanoTiO_2 was cleared over a 4-week period [Rossi et al., 2010]. This suggests that the small particle size leads to alveolar deposition with subsequent slow clearing [Oberdorster, 1995]. Depending on the particle size, some of the inhaled nanoTiO_2 is also expected to be deposited in the nasal region. Approximately 90% of 1 nm, 20–30% of 5–10 nm, and less than 10% of 20 nm particles deposited in the nasopharyngeal region. A small fraction of the total particle mass deposited in the nasal region has previously been shown to be efficiently translocated to olfactory bulb in brain through axonal transport via olfactory neurons [Oberdorster et al., 2002, 2004]. However, particles deposited in the nasal region of the respiratory system would be quickly cleared either by blowing the nose, sneezing, or by efficient mucociliary transport mechanism. As such, we have not assessed the deposition of particles in the upper respiratory tract and hence their uptake by the central nervous system. Since particles in the nasal region are rapidly cleared, and since we have measured the gene expression changes 5 days after the last exposure, we believe that the contribution of nasally deposited particles to overall outcome in the present model is likely to be minimal.

The dose administered in this study corresponds to the highest occupational exposure limit for dust (in terms of total mass administered, 5 mg/m^3 during an 8 hr working day) in Denmark. Thus, this study used a relatively low, occupationally relevant exposure regime in combination with robust and conservative statistical analysis of a sufficiently large sample size of animals. However, one could

argue that considering the slow clearance of the particles (as observed in this study), along with the dose used, lung overburden has occurred. Although the dose rate was high, the deposited dose is only a fraction of what would result in overload (i.e., slower motility of alveolar macrophages and impaired mucociliary clearance). Moreover, the particles in this study were surface coated with Si, Zr, and Al. It has been shown that surface-coated nanoTiO$_2$ are more toxic than benign pure particles of nanoTiO$_2$ [Rossi et al., 2010]. Although we did not test the possibility that surface coatings (Si, Al, and Zr) themselves contributed to the toxicity, it is documented in the literature that Si, Zr, and Al are less toxic when administered on their own [Rossi et al., 2010], suggesting that the slower clearance and retention of smaller size particles in the lung tissue could be the reason behind observed pulmonary response. Here, we confirm that nanoTiO$_2$ inhalation results in changes in proinflammatory, immune response and complement cascade-related genes that persist for relatively long periods of time after the final exposure. The observed fold changes for any particular gene in our study were much lower than the other reports, which may be due to the lower doses used, the differences in exposure set up, or the types of particles used. However, a large portion of these changes were validated with quantitative RT-PCR and RT-PCR arrays. Thus, we argue that the pulmonary response observed in our study is likely due to the highly toxic nature of the particles and not lung overburden.

Although the mice in this study were exposed to doses near the occupational exposure limit for dust in Denmark in terms of total mass administered, it might be argued that the dose is high if the hours of exposure are considered (i.e., delivered in 1 hr vs. exposure over an 8 hr work day). Genomic tools have not been extensively used to understand nanoassociated toxicity. Using animals that exhibit an inflammatory response following the exposure, this work was initiated to establish proof of principle that genomic tools can be applied to characterize the effects of nanoparticles at relatively low doses and reveal the molecular perturbations that persist at this time point that contribute to the observed phenotype. However, the observed overall response at the transcriptional level must be investigated in studies incorporating multiple doses, time points, and different types of nanoTiO$_2$, to truly dissect the mechanism of acute exposure and distinguish between immediate early effects and persistent effects. Thus, multiple time points and doses were not conducted in this study, but will be the subject of future work.

MiRNA Expression Profiling

Despite a dramatic increase in *Saa1* gene transcript levels, we did not see a correlated increase in its protein synthesis. Similarly, in contrast to the ~5-fold transcriptional increase of *Saa3* mRNA, Saa3 protein was increased by only 2-fold. One mechanism that controls translation of proteins involves targeted translational repression by miRNAs. Thus, to address the observed discrepancies between the transcription and protein expression profiles and to explore the potential role of miRNAs in mitigating mRNA and translation in response to particle exposure, we analyzed global changes in pulmonary miRNA expression using DNA microarrays. Total RNA from lung tissues of control and exposed mice was used. The analysis revealed 55 statistically significant changes; 16 were altered by more than 1.2-fold and six had fold changes >1.5 (Table IV). Because an individual miRNA may target hundreds of mRNAs, a small change in a miRNA may have significant repercussions on transcription and translation. As such, we explored changes both above and below the 1.5-fold threshold.

Some of the miRNAs that are altered in response to nanoTiO$_2$ have been implicated in inflammation and immune response. For example, upregulation of miR-21 and downregulation of miR-1 has recently been observed in IL-13 transgenic mice with allergic airway inflammation [Lu et al., 2009]. MiR-21 is expressed by inflammatory leukocytes in asthmatic lung [Jin et al., 2008], hematopoietic cells [Landgraf et al., 2007], and in macrophages and dendritic cells [Lu et al., 2009]. MiR-21 is induced in murine macrophages following LPS challenge [Lu et al., 2009], all suggesting a critical association for miR-21 in inflammation and immune response. MiR-1 levels increase during myocardial differentiation of mouse embryonic stem cells [Takaya et al., 2009]. Farraj et al. [2010] have shown that miR-1 and miR-21 are significantly downregulated in the myocardium of rats exposed to synthetic particulate matter. Other miRNAs affected by nanoTiO$_2$ exposure have been linked to a cancer phenotype. For example, differential expression of miR-135b is found in human colon cancer samples [Sarver et al., 2009] and miR-449a is associated with antitumor activity in prostate cancer cells [Noonan et al., 2009].

Analysis by qRT-PCR confirmed the higher levels of miR-449a (fold six), miR-1 (2.6-fold), and miR-135b (60-fold) in this study (Fig. 1B) but not the suppression of miR-223 and miR-92a. Analysis of curated targets in TarBase (http://diana.cslab.ece.ntua.gr/tarbase/) did not reveal any known targets of miR-449a or miR-135b. As such, we searched for mRNA targets of miR-135b and miR-449a using the target-prediction softwares TargetScan and Pictar. Predicted targets for miR-135b (TargetScan or Pictar) that were differentially expressed (downregulated) included the following: *checkpoint suppressor1*, *runt related transcription factor 2* (*runx2*), and *phosphodiesterase 8b*. Predicted targets of miR-449a that were differentially expressed included the following: *lymphoid enhancer-binding factor 1* (*lef1*) and *Kit ligand*. *Runx2* and *lef1* are the downstream targets of WNT signaling

suggesting that miR-135b and miR-449a may have a common function in regulating WNT signaling. However, it appears that the effect of the observed 6- and 60-fold induction of miR-449a and miR-135b on transcript levels of their predicted targets is negligible. There may be several reasons to explain the lack of changes in target mRNAs. First, the genes targeted by miR-449a and miR-135b in response to particle exposure may be different than the ones predicted *in silico*. Second, the majority of the genes changing in response to nanoTiO_2 in our model are acutely induced and therefore the magnitude of their induction is expected to be 10–100-fold higher immediately following the exposure. Therefore, even if some of these genes are directly targeted by the miRNAs at the transcriptional level, their suppression may not be below those of control levels. Third, it is also possible that the predominant mode of action for these miRNAs is through translational repression.

Currently, the causes and consequences of miRNA response following exposure to nanoTiO_2 are unknown. However, changes at the transcriptional level of several target genes are implicated in acute phase and inflammatory response. Thus, miR-1, miR-135b, and miR-449a may play a role in these biological processes. We have recently confirmed induction of miR-135b in other inflammatory models including particle-induced inflammation (Halappanavar et al., unpublished data). The induction of several inflammatory chemokines and acute phase components is well characterized in response to particle exposure, including nanoTiO_2 (as described in detail earlier). However, the possibility that these molecular pathways could be under the direct control of miRNAs that play a role in particle-induced response is yet to be demonstrated. We hypothesize that some of the genes, including the *Saa* family and chemokines, are the direct targets of miR-135b and that miR-135b could be acting to resolve the inflammation process. A similar hypothesis was also proposed by Jardim et al. [2009]. These authors suggest that disruption in miRNA expression in response to diesel exhaust particles (DEP) in human airway epithelial cells could be implicated in modulation of the inflammatory process induced by DEP. Further characterization of miR-135b responsive genes and their role in particle-induced pulmonary response is being carried out in our laboratory to determine the biological relevance of these changes.

The miRNA field is still developing in parallel with more powerful tools to study their expression and activity. At present, a number of published studies describe changes in the expression of several miRNAs in diseases (reviewed in [Sun and Tsao, 2008]) or in certain biological processes *in vitro* and *in vivo* [Bueno et al., 2008; Lynam-Lennon et al., 2009]. However, there are very few studies that have characterized changing mRNA and miRNA profiles in target tissues *in vivo* in response to toxic substances (reviewed in [Lema and Cunningham, 2010]). To our knowledge, this is the first report on particle-induced pulmonary miRNA changes. Integration of gene and miRNA expression profiles with reports examining multiple endpoints at the molecular, cellular, tissue, and physiological levels will be very useful in understanding the toxicity of particles in the context of the whole organism. Further studies incorporating acute, subchronic, and chronic doses with different types of nano TiO_2 particles and multiple time points are needed to validate our findings.

CONCLUSIONS

This work demonstrates the induction of acute phase reactants, chemoattractants, immune and host defense genes following exposure to occupationally relevant levels of surface-coated titanium dioxide nanoparticles via a biologically relevant exposure route. These findings are in keeping with the expected health outcome of chronic nanoparticle exposure, and the changes persist for up to 5 days following exposure. In parallel with perturbation of these genes, specific miRNAs are also induced in the lungs in response to nanoTiO_2. The role of miRNAs in response to particle-induced lung injury is now under investigation in our laboratory.

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PAPER III

Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan): Effects in prenatally exposed offspring on hepatic DNA damage and gene expression.

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Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan) in C57BL/6 mice: effects in prenatally exposed offspring on hepatic DNA damage and gene expression

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Abstract

We investigated effects of maternal pulmonary exposure to titanium dioxide (UV-Titan) on prenatally exposed offspring. Time-mated mice (C57BL/6BomTac) were inhalation exposed (1 h/day to 42 mg UV-Titan/m^3 aerosolised powder or filtered air) during gestation days (GDs) 8–18. We evaluated DNA strand breaks using the comet assay in bronchoalveolar lavage (BAL) cells and livers of the time-mated mice (5 and 26–27 days after inhalation exposure), and in livers of the offspring (post-natal days (PND) 2 and 22). We also analysed hepatic gene expression in newborns using DNA microarrays. UV-Titan exposure did not induce DNA strand breaks in time-mated mice or their offspring. Transcriptional profiling of newborn livers revealed changes in the gene expression related to the retinoic acid signalling pathway in the females, while gene expression in male offspring was unaffected. Changes may be a secondary response to maternal inflammation although no direct link was evident through gene expression analysis.

Keywords: : *In utero* exposure, lung inflammation, DNA strand breaks, retinoic acid pathway, acute-phase response

Introduction

Titanium dioxide (TiO_2) is an industrial product produced in large quantities worldwide. TiO_2 particles are used as a white pigment in cosmetics, food, plastics, paints and other products, and as a UV-filter in cosmetics. Fine TiO_2 particles are generally considered to be inert; however, when the particle size is reduced to the nanoscale, they become reactive (Sager et al. 2008). The activity of different crystalline forms of TiO_2 (rutile, anatase, brookite) generally varies. The anatase form appears to be more reactive compared with the rutile form and therefore may be potentially more toxic (Jiang et al. 2008; Warheit et al. 2007). Physico-chemical characteristics, such as particle aggregation, crystal phase and surface modifications, also contribute to particle-induced toxicity (Johnston et al. 2009). Thus, it is difficult to generalise the toxicity of TiO_2.

Pulmonary exposure to TiO_2 nanoparticles induces lung inflammation in mice and rats (Bermudez et al. 2004; Grassian et al. 2007; Ma-Hock et al. 2009; van Ravenzwaay et al. 2008; Hougaard et al. 2010). Of notable importance, nanosized TiO_2 particles have been detected in the lung tissues of rodents up to 4 weeks after inhalation exposure (Hougaard et al. 2010; Kreyling et al. 2010). Thus, acute as well as chronic effects of exposure must be considered. TiO_2 nanoparticles are suspected to be both genotoxic (Johnston et al. 2009) and carcinogenic (Mohr et al. 2006). Fine TiO_2 dust, when inhaled, has recently been classified by the International Agency for Research on Cancer as being possibly carcinogenic to humans (group 2B). However, currently, there is no evidence of TiO_2-related cancer in occupational settings (Boffetta et al. 2004; Ellis et al. 2010; Fryzek et al. 2003).

The work described above has focussed on the effects in adults. The effects of exposure to nanosized TiO_2 during prenatal and postnatal development are poorly understood. We previously reported that exposure of pregnant mice to surface-coated nanosized TiO_2 (UV-Titan designed for use in the paint and lacquer industry, 42 mg UV-Titan/m^3 for 1 h/day on gestation days 8–18), led to large neutrophil influx in bronchoalveolar lavage (BAL). The induced pulmonary inflammation was evident in the time-mated mice days to weeks after the last exposure (Hougaard et al. 2010).

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Moreover, gene expression analysis of exposed non-pregnant females 5 days after the exposure revealed increased expression levels of several genes implicated in inflammation, acute-phase and immune response (Halappanavar et al. 2011). Despite the observed effects, the exposure did not significantly affect traditional gestational parameters such as maternal weight gain during gestation and lactation; gestation length; offspring weight at birth, during lactation and maturation; litter size; gender ratio; number of implantations; and postnatal viability (Hougaard et al. 2010). However, exposed offspring exhibited minor behavioural changes at adulthood, despite a lack of detectable titanium content in newborn stomachs (containing mother's milk) and livers, and offspring livers at weaning (Hougaard et al. 2010).

Translocation of nanoparticles from the maternal lung to the circulation is considered to be slow. Only a fraction of a per cent of total deposited particles translocate beyond the lung cavity, regional lymph nodes or over the gastrointestinal mucosa. Therefore, after maternal pulmonary exposure, the direct translocation from maternal blood stream and across the placenta and into the foetus may also be negligible (Sadauskas et al. 2007). In agreement with this hypothesis, we were not able to detect TiO_2 in the offspring (Hougaard et al. 2010). However, nanoparticles may affect the foetus indirectly via maternal inflammation and genotoxicity. Maternal inflammation has been reported to potentially affect the immune and nervous system in developing offspring (Hodyl et al. 2007; Hodyl et al. 2008; Lasala and Zhou 2007; Surriga et al. 2009; Gilmore et al. 2004; Graciarena et al. 2010; Lasala and Zhou 2007; Marx et al. 2001), suggesting that events secondary to responses in maternal lungs could indirectly impact offspring development, such as observed behavioural changes (Hougaard et al. 2010).

In other studies, exposure of pregnant mice to carbon black nanoparticles by inhalation (42 mg Printex 90/m^3 for 1 h/day on gestation days 8–18) or by intratracheal instillation (total dose 11, 54 and 268 μg/animal on gestation days 7, 10, 15 and 18) resulted in no observable effects on traditional gestational parameters despite persistent pulmonary inflammation in the time-mated mice, measured by large neutrophil influx in BAL. Interestingly, increased levels of DNA strand breaks in the livers of time-mated mice exposed to Printex 90 via inhalation and offspring exposed *in utero* were found weeks after the end of exposure, while no changes in DNA strand breaks were found in the instilled mice or their offspring (Jackson et al. 2011a). We also analysed gene expression in the lungs of instilled dams and in the livers of their offspring (Jackson et al. 2011c). Exposed dams had dramatic changes in several genes and proteins related to lung inflammation. Newborn female offspring were more sensitive to maternal exposure than male offspring. Cellular signalling, inflammation, cell cycle and lipid metabolism were among the biological pathways affected in female offspring and were accompanied by minor behavioural changes (Jackson et al. 2011d). Male offspring, however, responded with subtle changes in metabolism-related genes. These data suggest that exposure to nanoparticles (1) induces persistent inflammation, immune and acute-phase signalling in exposed pregnant mice; (2) affects various biological pathways in prenatally exposed offspring; and (3) may be potentially genotoxic both to pregnant mice and to their developing offspring.

In the present study, we analysed genotoxicity and hepatic gene expression profiles in male and female offspring (PND 2 and 22) born to dams that exhibited profound pulmonary inflammation following exposure to nanosized TiO_2 (42 mg UV-Titan/m^3 × 1 h/day × 11 days) via inhalation during the gestational period. In parallel, DNA strand breaks were measured in the BAL and liver cells of time-mated mice 5 and 26–27 days after exposure. The aim of the study is to understand the potential health repercussions of exposure to UV-Titan nanoparticles during development and to provide mechanistic insight into the response.

Materials and methods

Animals and exposure

A detailed protocol was published previously (Hougaard et al. 2010). Briefly, nulliparous time-mated mice (C57BL/6BomTac, Taconic Europe, Ejby, Denmark) arrived on gestation day 3 (GD 3); day of mating was assigned as GD 0 and day of plug as GD 1. Housing was described in Hougaard et al. (2010). On GD 4, time-mated mice were weighed and assigned to groups of 22 and 23 animals each, with similar weight distributions. All the procedures complied with the EC Directive 86/609/EEC and the Danish law regulating experiments on animals (The Danish Ministry of Justice, Animal Experiments Inspectorate, Permit 2006/561-1123).

Time-mated mice were exposed to filtered clean air or ~42 mg/m^3 UV-Titan on GD 8–18 for 1 h/day by a whole-body exposure. Airflow was dynamic 20 l/min and a microfeeder aerosolised powder particles with a dispersion nozzle at a pressure of 5 bar. Particle and exposure characterisation for UV-Titan L181 (Kemira, Pori, Finland) were published previously (Hougaard et al. 2010) and results are summarised in Table I. Current scientific literature on prenatal exposure to nanoparticles is scarce and therefore recommended doses for developmental toxicity studies are not available. Although the 42 mg/m^3 UV-Titan on GD 8–18 for 1 h/day by a whole-body exposure dose chosen in the present study is relatively high, it is less than 1 day of occupational exposure at the 8-h time-weighted average occupational exposure limit by Danish Regulations (6.0 mg/m^3 TiO_2, The Danish Working Environment Authority 2007). Furthermore, exposure was timed after fertilisation, implantation and initiation of organ development. This exposure roughly corresponds to the first two trimesters of human pregnancy, where foetal organs are formed and developed. Because of slow particle clearance from the maternal lung, particles and particle effects were expected to remain throughout the entire lactational period. In agreement with this, substantial amounts of TiO_2 were found in lung tissue of dams at PND 26–27 (Hougaard et al. 2010). Exposure was terminated 2 days before expected delivery to avoid stressing the animals during birth preparations and potentially negative birth outcomes (premature birth and offspring loss). We have used similar exposure regimes

Table I. Key physico-chemical characteristics of titanium dioxide (UV-Titan L181).

Particle characteristics			
Declared particle size	17 nm		Kemira
Phase	Rutile		Kemira Hougaard et al. (2010)
Crystallite size	20.6 ± 0.3 nm (14.4–15.5 (100); 38.4 (001))		(Hougaard et al. 2010)
Surface area (BET)	70 m^2/g 107.7 m^2/g		Kemira (Hougaard et al. 2010)
Chemical composition	Na_2O	0.60 wt%	(Saber et al. 2011)
	SiO_2	12.01 wt%	
	Al_2O_3	4.58 wt%	
	ZrO_2	1.17 wt%	
	TiO_2*	70.81 wt%	
Loss of ignition	5.19 wt%		(Hougaard et al. 2010)
Thermogravimetric analysis (N_2 atmosphere, 40–800°C, 10°C/min)	6.1 ± 0.4 wt%		(Hougaard et al. 2010)
Inhalation exposure			
Particle size distribution in number	Aggregates and agglomerates were equidimensional to needle-shaped TiO_2 crystallites with diameters from less than 10 nm to more than 100 nm along the shortest and longest axis, 50% <97 nm		(Hougaard et al. 2010)
Particle number concentration	1.70 ± 0.20 × $10^6/cm^3$		(Hougaard et al. 2010)
Geometric mean size	97 nm		(Hougaard et al. 2010)
Mass-size distribution	75% were >1600 nm and <1% were <100 nm		(Hougaard et al. 2010)
Total inhaled dose	840 μg/animal		(Hougaard et al. 2010)
Estimated deposition	73 μg/animal in pulmonary region 315 μg/animal in extra-pulmonary region 365 μg/animal in gastro-intestinal tract		(Hougaard et al. 2010)

*UV-Titan is coated with polyalcohol adding to the remaining wt%.

previously (Hougaard et al. 2008; Hougaard et al. 2010; Jackson et al. 2011a) and found that maternal stress induced by the exposure regime does not affect gestation or lactation (Jackson et al. 2011b).

After exposure termination on GD 18, females were singly housed. Delivery was expected on GD 20 and designated postnatal day (PND) 0. Offspring were counted and sexed on PND 1. Dams and individual offspring were weighed on PND 1, 8, 11, 16, 19 and 22. On PND 2 (2 days after birth to avoid disturbing the dams and ensuring survival of the remaining offspring), one female or male offspring (newborn) per litter was killed by decapitation. Exposed and control newborn groups for analysis were composed of 5–6 females or males. Organs were dissected, weighed, placed in NUNC cryotubes, snap frozen in liquid N_2 and stored at −80°C until analysis. At least five living offspring remained in litters. On PND 23–24 (at weaning), one male and one female per litter were killed. Organs were treated as described for newborns.

Exposed time-mated mice that failed to conceive (non-pregnant females) were killed on PND 3 (5 days after the last inhalation exposure) and "dams" on PND 24–25 (26–27 days after the last inhalation exposure). Mice were anaesthetised with Hypnorm and Dormicum and killed by withdrawal of heart blood (stabilised in 0.17 mol/l K_2EDTA). BAL was collected, put immediately on ice and treated as described in Jackson et al. (2011a). The number of uterine implantation sites was determined and organs were dissected, weighed, placed in NUNC cryotubes, snap frozen in liquid N_2 and stored at −80°C until analysis.

Measurement of endotoxin in particle suspensions

An amount of 4.05 mg of TiO_2 was suspended in 1.0 ml pyrogen-free water with 0.05% Tween 20, sonicated twice for a total of 8 min (pulse 10 sec on and 10 sec off), centrifuged at 20,000 rpm for 6 min and the supernatant was used for the endotoxin assay. The endotoxin contents were analysed in duplicate using the kinetic Limulus Amebocyte Lysate test (Kinetic-QCL endotoxin kit, Lonza, Walkersville, MD, USA). A standard curve (ranging from 0.05 to 50 EU/ml) obtained from an *Escherichia coli* O55:B5 reference endotoxin was used to determine the concentrations in terms of endotoxin units (EU) (15.0 EU = 1.0 ng). An inhibition/enhancement control was prepared by spiking the supernatant with 10 μl of a 0.5 EU/ml solution of endotoxin.

Detection of DNA strand breaks

The levels of DNA strand breaks in frozen BAL and liver cells were determined using the alkaline comet assay as previously described (Jackson et al. 2011a), based on a protocol by McNamee et al. (2000). BAL cell suspensions in freezing medium with 10% DMSO were thawed quickly at 37°C. For liver, deep-frozen samples (~40 mg) were pressed through a metal stapler (diameter 0.5 cm, mesh size 0.4 mm) into Merchant's media (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) for inhibition of endogenous DNA cleaving enzymes (Brunborg et al. 1996). Cell suspensions were embedded in agarose on GelBond film (85 mm × 100 mm, Cambrex Bioscience, ME; 130 μl per sample; eight samples per film)

and subjected to alkaline electrophoresis (four films per electrophoresis). The slides were fixed, stained with SYBR Gold fluorescent dye (Molecular probes, Denmark) and 50 randomly selected cells were scored by fluorescent microscopy. DNA damage was quantified as % DNA in the tail and as tail length. Frozen standard A549 cells untreated or treated with H_2O_2 were used as internal controls. The % DNA was calculated to estimate the number of lesions per million base pairs (Forchhammer et al. 2010).

Global gene expression

Isolation of total RNA for DNA microarrays and RT-PCR

Random sections of the liver tissue were used to isolate total RNA (same livers as in comet analysis). RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) and purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). RNA samples showing A260/280 ratios between 2.0 and 2.1 and RNA integrity (analysed using Agilent 2100 Bioanalyzer, Agilent Technologies, Mississauga, ON, Canada) numbers higher than 8.0 were used for analysis.

Gene expression profiling using Agilent 4 × 44K mouse arrays

Global mRNA profiling was conducted on liver tissues of five male and five female newborn PND 2 offspring each from the control and exposed (UV-Titan) groups. In total, 20 samples were analysed. Individual total RNA (200 ng) samples and universal reference total RNA (Stratagene, Agilent Technologies, Mississauga, ON, Canada) were used to synthesise double-stranded cDNA and cyanine-labelled cRNA according to the manufacturer's instructions (Agilent Linear Amplification Kits, Agilent Technologies, Mississauga, ON, Canada). Experimental samples were labelled with Cyanine 5-CTP, and reference RNA with Cyanine 3-CTP (Perkin-Elmer Life Sciences, Woodbridge, ON, Canada). Cyanine-labelled cRNA targets were *in vitro* transcribed using T7 RNA polymerase and purified by RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). Labelled cRNA was hybridised to Agilent mouse 4 × 44 oligonucleotide microarrays (Agilent Technologies, Mississauga, ON, Canada) at 60°C overnight. Arrays were washed and scanned on an Agilent G2505B scanner at 5 µm resolution. Data were acquired using Agilent Feature Extraction software version 9.5.3.1.

Raw and normalised microarray data were deposited in a MIAME-compliant database (Gene Expression Omnibus, GEO) according to the detailed instructions provided by the MGED Society website (http://www.mged.org/Workgroups/MIAME/miame.html).

Gene expression microarray analysis

Since the Agilent slides contain four arrays per slide, the design was blocked on the slide. A reference design (Kerr and Churchill 2007; Kerr 2003) was used to analyse gene expression microarray data. The background fluorescence was measured using the (-)3xSLv1 probes; probes with median signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of the (-)3xSLv1 probe were flagged as absent (within the background signal). Data were normalised using LOWESS in R Development Team (2004). Ratio intensity plots and heat maps for the raw and normalised data were constructed to identify outliers. One male control sample was removed from the analysis based on clustering. Genes that were differentially expressed as a result of treatment were determined using the MAANOVA library in R (Wu et al. 2003). The main effect in the model was treatment. This model was applied to the log2 of the absolute intensities. The Fs statistics (Cui et al. 2005) was used to test for treatment effects. The *p*-values for all statistical tests were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach (Hochberg 1995). The fold change calculations were based on the least-square means. Significant genes were identified as having an adjusted p-value < 0.05 for any individual contrast.

Quantification of hepatic mRNA levels in offspring

To evaluate the fate of significantly differentially expressed genes detected by microarrays in the newborn offspring liver, RT-PCR was conducted on liver samples from both newborns and weaned offspring. Samples from newborns were newly purified to be able to compare values in newborns and offspring at weaning. For logistical reasons, the analysis was performed in a different laboratory from the microarray experiment, and therefore there were small differences in the RNA purification methodologies. The genes transthyretin (*Ttr*); UDP glycosyltransferases 3 family, polypeptide A2 (*Ugt3a2*); and cytochrome P450, family 26, subfamily b, polypeptide 1 (*Cyp26b1*) were analysed using commercial probes (*Ttr* = Mm00443267_m1, *Ugt3a2* = Mm01304077_m1, *Cyp26b1* = Mm00558507_m1; Applied Biosystems, Nærum, Denmark). RNA isolation and cDNA synthesis were performed as previously described in Bornholdt et al. (2007) and Saber et al. (2009). Briefly, total RNA was isolated from tissue lysates in a QIAGEN tissue lyser (QIAamp®, Copenhagen, Denmark) as recommended by the manufacturer. The RNA was treated with QIAGEN RNase-Free DNase kit (Copenhagen, Denmark), cDNA was synthesised using Taq-Man reverse transcription reagents (Applied Biosystems, Nærum, Denmark) and RT-PCR was performed using Universal Mastermix (Applied Biosystems, Nærum, Denmark). PCR was performed on an ABI PRISM® 7500 sequence detector (PE Biosystems, Foster City, CA, USA) as described (Saber et al. 2009). 18S (4310893E, Applied Biosystems, Nærum, Denmark) was used as the reference gene. Expression for each gene was quantified in separate wells. Samples were quantified in triplicates; standard deviation was below 20%. Run-to-run variation was controlled by quantifying the mRNA levels for the same control sample. The standard deviation in separate runs was lower than 25%. No template and RT controls were included in all runs.

Statistical analyses

Analyses were performed in SYSTAT Software Package version 9. The accepted level of significance was 0.05. Offspring results are presented either as a litter average or as litter average of female and male offspring. Data were analysed by

analyses of variance (three- or two-way ANOVA), with repeated measures (treatment × time of sampling × pregnancy – for time-mated females, and sex – for offspring). Significant results were followed by group analyses (one-way ANOVA). Pregnancy was included as cofactor in maternal data analysis. Data that were not normally distributed were log10 transformed.

Results

Measurement of endotoxin in particle suspensions

The supernatant of the UV-Titan suspension did not inhibit or enhance the measured endotoxin concentration and the concentration was 0.05 EU/mg UV-Titan or 3.0 pg endotoxin/mg UV-Titan. The time-mated females inhaled a total dose of 0.84 mg UV-Titan/animal, thus the total inhaled daily dose of endotoxin was 0.16 EU/kg BW or 10.5 pg/kg BW to a total of 1.74 EU/kg BW or 116 pg/kg BW over 11 days.

The UV-Titan particle suspension inhibited the assay, and it was not possible to measure the endotoxin concentration of the particle suspension. Similar findings were previously reported by Schulze (2010).

Detection of DNA strand breaks

Inhalation of UV-Titan did not affect the levels of DNA strand breaks in BAL or liver cells in the non-pregnant females and dams compared with their controls sampled 5 or 26–27 days after last exposure, respectively (two-way ANOVA BAL cells p-value = 0.20; liver cells p-value = 0.87; Figure 1A and Figure 1B).

Prenatal exposure to UV-titan did not affect the levels of DNA strand breaks in the livers of newborn or weaned offspring compared with their controls (two-way ANOVA p-value = 0.71; see Figure 1C). The newborn offspring, irrespective of nanoparticle exposure, had higher levels of DNA strand breaks compared with the offspring at weaning (p-value = 0.001), similar to previously published results (Jackson et al. 2011a). The samples from weaning were run and analysed twice with identical results.

Global gene expression

Prenatal exposure to UV-Titan caused subtle but significant changes in hepatic gene expression in both male and female offspring. Thirty-five genes in female offspring and seventeen genes in male offspring were differentially expressed with

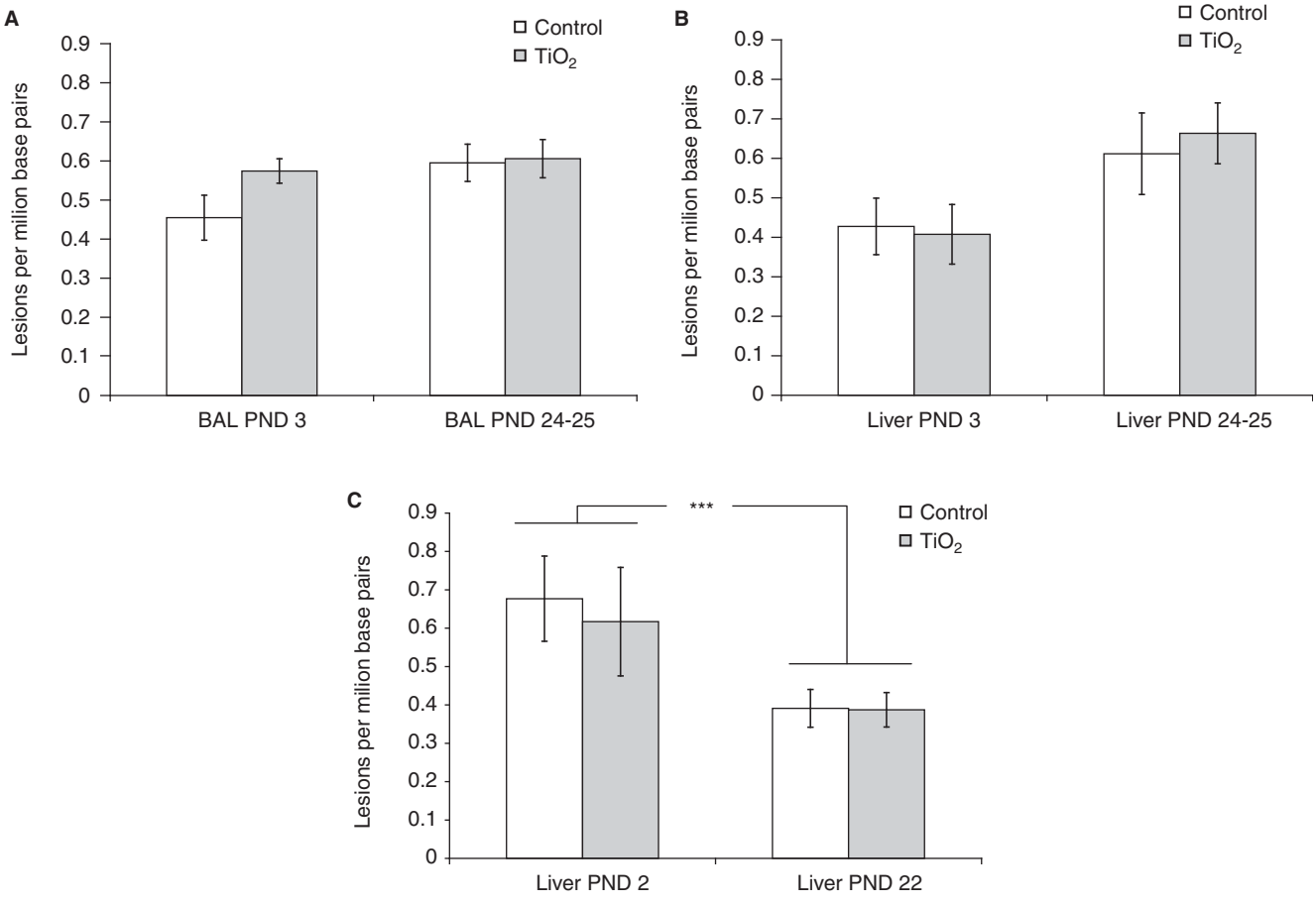

Figure 1. Levels of DNA strand breaks analysed by comet assay for females, dams and offspring exposed by whole-body inhalation exposure to 42 mg/m^3 titanium dioxide (UV-Titan) for 1 h/day on gestation days 8–18 and control mice exposed to filtered air.
(A) Time-mated mice (females and dams) – bronchoalveolar lavage (BAL) cells (two-way ANOVA exposure and post-natal days (PND), p-value = 0.20); females (PND 3, 5 days after exposure end, n = 8–9); dams (PND 24–25, 26–27 days after exposure end, n = 10–14). (B) Time-mated mice – liver cells (two-way ANOVA exposure and PND, p-value = 0.87); females (PND 3, 5 days after exposure end, n = 8–9); dams (PND 24–25, 26–27 days after exposure end, n = 10–14). (C) Offspring – liver cells (two-way ANOVA exposure and PND, p-value = 0.71; with PND interaction p-value = 0.001); offspring, average of one male and female offspring from each litter, where possible (PND 2, 2 days after birth, n = 11; PND 22, at weaning, n = 13–14).
Data are presented as mean lesions per 106 base pairs ± SEM (calculated from %DNA in tail results).

FDR adjusted p-values ≤ 0.05. In female offspring, 28 of the 35 genes showed fold changes above 1.3, with 21 genes upregulated and 7 genes downregulated in response to the treatment (Table II). Cytochrome P450, family 26, subfamily b, polypeptide 1 (*Cyp26b1*), aldolase 2, B isoform (*Aldob*) and albumin 1 (*Alb1*) showed the highest fold changes of 1.8. In males, however, only four genes showed fold changes higher than 1.3 (two upregulated and two downregulated), suggesting that male offspring were less responsive to prenatal UV-Titan exposure than females (see Table III). The complete microarray dataset is available through the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE29067.

Gene ontology analysis was used to assign genes to functional categories in DAVID (Huang et al. 2009). Specific biological pathways associated with the differentially expressed genes were explored using the Kyoto Encyclopaedia for Genes and Genomes (http://www.genome.jp/kegg/) pathways. Ingenuity Pathway Analysis (IPA) was used to perform comparative analysis of biological networks associated with the altered genes in female offspring. Although the observed changes in gene expression profiles in female offspring were subtle, a large portion of them were functionally associated with four major biological networks. These networks included (1) cardiovascular system and haematological development and function (13 molecules/genes); (2) cell signalling and cell–cell interaction, vitamin and mineral metabolism (six molecules/genes); (3) embryonic development (one molecule/gene); and (4) carbohydrate and nucleic acid metabolism (one molecule/gene). Since networks 3 and 4 consisted of only one molecule/gene, they were excluded from further processing. Networks 1 and 2 were merged to better understand the biological significance of these networks. The results indicate that many of the molecules in the network are associated with retinoic acid (RA) receptor signalling (Supplementary Figure 1). The male offspring were less responsive compared with females. Three of the four statistically significantly and differentially expressed genes belonged to phase-2 xenobiotic metabolism, gluconeogenesis and amino acid metabolism,

Table II. Transcripts exhibiting statistically significant changes (FDR p-value < 0.05) in the livers of newborn female offspring on PND 2 in response to maternal whole-body inhalation exposure to 42 mg/m^3 UV-Titan for 1 h/day on gestation days 8–18.

Gene Description	*FC
Cardiovascular system and haematological development and function, cell signalling and interaction	
Aldolase 2, B isoform (Aldob; NM_144903.2)	1.81
Albumin 1 (Alb1; NM_009654.3)	1.81
Integrin alpha 2b (Itga2b, NM_010575.2)	1.50
Integrin beta 3 (Itgb3; NM_016780.2)	1.41
Coagulation factor XIII, A1 subunit (F13a1; NM_028784.3)	1.41
Glycoprotein 5 (platelet) (Gp5; NM_008148.3)	1.41
Glycoprotein 9 (platelet) (Gp9; NM_018762.1)	1.34
Glycoprotein Ib, beta polypeptide (Gp1bb; NM_001001999.1)	1.40
Insulin-like growth factor binding protein 5 (Igfbp5; NM_010518.2)	1.40
Leukotriene B4 receptor 1 (Ltb4r1; NM_008519.2)	1.40
Von Willebrand factor homolog (Vwf; NM_011708.3)	1.38
Pleckstrin (Plek; NM_019549.2)	1.33
Cytochrome P450, family 4, subfamily a, polypeptide 12 (Cyp4a12; NM_177406.3)	-1.52
Chemokine (C-X-C motif) ligand 10 (Cxcl10; NM_021274.1)	-1.49
Cell signalling, molecular transport, vitamin and mineral metabolism	
Cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1; NM_175475.3)	1.82
Transthyretin (Ttr; NM_013697.5)	1.48
Proteoglycan 4 (megakaryocyte-stimulating factor, articular superficial zone protein) (Prg4; NM_021400.3)	-1.36
Embryonic development, cellular development, cellular growth and proliferation	
C-type lectin domain family 1, member b (Clec1b; NM_019985.3)	1.40
Ovarian cancer marker	
WAP four-disulphide core domain 2 (Wfdc2; NM_026323.2)	1.47
Calcium signalling	
Triggering receptor expressed on myeloid cells-like 1 (Treml1; NM_027763.1)	1.37
Lung function	
Thrombospondin type I, domain containing 4 (Thsd4; NM_001040426.3)	1.31
Oncogene	
Myeloblastosis oncogene (myb; NM_001198914.1)	-1.40
Unknown	
cDNA sequence BC089597 (BC089597; NM_145424.2)	1.45
Gene model 92, (NCBI) (Gm92, NM_unknown)	1.44
7 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:A730054G07 (AK043081.1)	1.39
RIKEN cDNA 2900041A09 gene (2900041A09Rik; CM000221.2)	-1.38
Adult male bone cDNA, RIKEN full-length enriched library, clone:9830125P17 (AK036518.1)	-1.40
Clone IMAGE:3983821, mRNA, partial cds. (BC021831.1)	-1.71

*FC, fold change, values are over matched control.

Table III. Transcripts exhibiting statistically significant changes (FDR p-value < 0.05) in the livers of newborn male offspring on PND 2 in response to maternal whole-body inhalation exposure to 42 mg/m^3 UV-Titan for 1 h/day on gestation days 8–18.

Gene Description	*FC
Gluconeogenesis and amino acid metabolism	
Glutamic pyruvate transaminase (alanine aminotransferase) 2 (Gpt2; NM_173866.3)	-1.39
Phase II xenobiotic metabolism	
UDP glycosyltransferases 3 family, polypeptide A2 (Ugt3a2; NM_144845.3)	1.67
Sulphotransferase family 5A, member 1 (Sult5a1; NM_020564.3)	1.38
Oncogene	
Myelocytomatosis oncogene (Myc; Tnfsf3c; NM_033622.1)	-1.34

*FC, fold change, values are over matched control.

suggesting that the responses in male and female are different.

To further understand the link between maternal exposure and offspring response, the relationship between gene expression profiles in the time-mated non-pregnant females and the female offspring was examined in detail. To do this, statistically significant and differentially expressed genes in the time-mated females measured 5 days after the last inhalation exposure (PND 3) (previously published (Halappanavar et al. 2011)) and significant genes found in the newborn female offspring from PND 2 were analysed using IPA for their implications in diseases and disorders. The results revealed several biological networks associated with inflammation response, cellular movement, cellular growth and proliferation, haematological system development and function and immune cell trafficking in exposed time-mated non-pregnant females 5 days after inhalation exposure. By contrast, the major biological networks in the newborn prenatally exposed female offspring were mainly related to development and function of cardiovascular and haematological system, tissue development and vitamin, and mineral and lipid metabolism. Merging of the networks derived from the time-mated females and the newborn female offspring resulted in a complex biological network (data not shown) without a clear connection between pathways. A few indirect associations between the altered inflammatory modulators in the time-mated females and affected genes in the newborn female offspring were observed such as Von Willebrand factor and glycoproteins.

Quantification of hepatic mRNA levels in offspring

The most differentially expressed genes in the microarray experiment (*Cyp26b1, Ttr,* and *Ugt3a2)* were analysed by independent RT-PCR analysis of liver tissue from newborns and weaned offspring. Table IV presents a comparison of microarray and RT-PCR data. While gene expression in the microarray analysis was highly significant, the gene expression analysed by RT-PCR was less significant. However, the magnitude and direction of fold changes were similar between microarray and RT-PCR for *Ttr* and *Ugt3a2*. A discrepancy was noted for *Cyp26b1*. We chose to analyse significantly expressed genes by independent analysis in newly isolated RNA, which may account for some of differences in gene expression between the two technologies.

Discussion

We evaluated the effects of gestational exposure by maternal inhalation of surface-coated nanosized titanium dioxide (TiO_2, UV-Titan designed for use in the paint and lacquer industry) on DNA damage and global gene expression. Although, the chosen exposure dose was relatively high, the daily dose corresponded to ~1 day of exposure that Danish workers would experience at the current 8-h time-weighed average occupational exposure limit 6 mg TiO2/m3, The Danish Working Environment Authority. To characterise genotoxicity from the exposure, we quantified BAL and liver DNA damage in the time-mated mice and liver DNA damage in the offspring using the comet assay. In parallel, we analysed changes in gene expression in the liver of offspring using DNA microarrays to shed light on other potential effects of exposure *in utero* to UV-Titan and to explore mechanisms of action. We demonstrate that levels of DNA strand breaks were similar in time-mated mice 5 and 26–27 days after inhalation exposure to UV-Titan or filtered air. In addition, there were no statistically significant changes in the number of DNA strand breaks in offspring exposed prenatally to UV-Titan. However, hepatic transcriptional profiling of newborns revealed changes in the expression of genes related to the RA signalling pathway in the female offspring, while

Table IV. Microarray validation data by real-time polymerase chain reaction in newborn offspring on PND 2 and offspring at weaning on PND 22.

		Microarray			RT-PCR				
		PND† 2			PND† 2	PND† 22	Overall	PND† 2	PND† 22
		FC‡	p-Value	p-Value FDR§	FC‡	FC‡	p-Value#	p-Value$	
Cyp26b1	Male	-	-	-	1.29	-1.28	0.86	0.59	0.74
	Female	1.82	< 0.001	< 0.001	1.05	1.35	0.92	0.92	0.81
TTR	Male	-	-	-	2.03	2.99	0.29	0.21	0.79
	Female	1.48	< 0.001	0.04	1.60	1.00	0.95	0.65	0.53
Ugt3a2	Male	1.67	< 0.001	< 0.001	3.06	1.43	0.01	0.05	0.76
	Female	-	-	-	2.83	1.22	0.35	0.70	0.26

Cyp26b1, Cytochrome P450, family 26, subfamily b, polypeptide 1; TTR, Transthyretin; Ugt3a2, UDP glycosyltransferases 3 family, polypeptide A2; †PND, Post-natal day (days after birth); ‡FC, Fold change relative to control; §FDR, False discovery rate; #Two-way ANOVA (exposure, PND); $One-way ANOVA (exposure).

gene expression in male offspring was relatively unaffected by exposure.

Nanosized TiO_2 is a well-established genotoxin *in vitro* (reviewed by (Johnston et al. 2009)), but results *in vivo* are conflicting. Trouiller et al. (Trouiller et al. 2009) demonstrated that exposure to a large dose of nanosized TiO_2 in drinking water was genotoxic in peripheral blood, bone marrow and liver of mice. However, the effects of pulmonary exposure to nanosized TiO_2 are less clear (Driscoll et al. 1997; Rehn et al. 2003; Saber et al. 2011). Driscoll et al. demonstrated persistent genotoxic effects in rats, up to 15 months following two consecutive intratracheal instillations of TiO_2 to a final dose 100 mg/kg in alveolar type II cells (Driscoll et al. 1997). The authors suggest that the increased mutation frequency was associated with neutrophilic inflammation.

Work from our laboratory has not supported a similar relationship between inflammatory response and genotoxicity in cells isolated from BAL (Bornholdt et al. 2002; Madsen et al. 2008; Saber et al. 2005; Saber et al. 2009). In one study, mice were exposed to four intratracheal instillations of 54 μg/animal of highly inflammogenic biofuel dust. No DNA strand breaks were observed in BAL cells 1 h after the last instillation (Madsen et al. 2008). In another study, mice were exposed by intratracheal instillation to 54 μg/animal (2.8 mg/kg) of different nanoparticles including UV-Titan and Printex 90. Printex 90 exposure caused a threefold higher neutrophil influx than UV-Titan; however, DNA strand breaks in BAL cells 24 h after instillation were only elevated for UV-Titan (i.e., there was no significant increase in DNA strand breaks in Printex 90-exposed mice relative to controls) (Saber et al. 2011). Saber et al. observed that nose-only inhalation exposure to 20 mg/m^3 for 90 min/day on four consecutive days induced DNA strand breaks in BAL cells 1 h after the last exposure, while inflammation was evident as cytokine expression in the lung without neutrophil influx (Saber et al. 2005). Similarly, it was observed that DNA strand break levels in BAL cells induced by ozone exposure peaked and were repaired before induction of proinflammatory cytokines (Bornholdt et al. 2002). Thus, DNA strand breaks detected by the comet assay do not correlate strongly with lung inflammation. Moreover, DNA strand breaks are very transient, probably because the DNA damage is rapidly repaired by DNA repair enzymes (Bornholdt et al. 2002). In the present study, we did not observe DNA strand breaks in BAL cells 5 and 26–27 days after inhalation exposure to UV-Titan (42 mg/m^3 1 h/day for 11 days, ~840-μg inhaled dose, 72.5-μg estimated pulmonary dose = 3.3 mg/kg). We cannot rule out that induced DNA damage was repaired by 5 or 26–27 days after inhalation exposure. Thus, given the increased inflammation observed in these mice (Hougaard et al. 2010) and the lack of observed genotoxicity, our results do not support a direct relationship between inflammation and genotoxicity.

Contrary to the observations of the present study, we previously reported that inhalation exposure to Printex 90 using a similar set-up (42 mg/m^3, 1 h/day for 11 days, ~826-μg inhaled dose, 287-μg estimated pulmonary dose equal to 13.1 mg/kg) resulted in increased DNA strand breaks in the livers of exposed mothers and their offspring. However, similar to the current study, DNA strand breaks were not increased in liver cells of intratracheally instilled mice with a comparable pulmonary deposition dose (four instillations with a final dose 268 μg/animal equal to 12.2 mg/kg) (Jackson et al. 2011a). In addition, inhaled Printex 90 induced less inflammation than instilled Printex 90. These data also demonstrate that inflammation is likely not directly connected to genotoxicity, corroborating our results above and previous findings (Bornholdt et al. 2002; Bornholdt et al. 2007; Saber et al. 2011). We hypothesised that deposition patterns of the inhaled versus instilled doses determine the different genotoxicities of Printex 90. While the total pulmonary dose was the same by instillation and inhalation, the estimated intragastric deposition of Printex 90 was much larger by inhalation than instillation. We speculated that differences in the amount of ingested Printex 90 could explain the observed difference in liver DNA damage. Thus, the DNA strand breaks in the liver cells of mice exposed to Printex 90 were likely not related to pulmonary inflammatory responses, but rather were induced by the intragastric deposition of particles transported from the upper airways.

Comparing the Printex 90 study (Jackson et al. 2011a) with the present UV-Titan study (Hougaard et al. 2010), the total inhaled masses were similar (826 and 840 μg/animal, respectively). However, the mean particle size distribution of aerosolised Printex 90 was considerably smaller than that of aerosolised UV-Titan. The mean particle size (in number) of the aerosolised Printex 90 was 45 nm, while the mean particle size (in number) of aerosolised UV-Titan was 97 nm. Based on the mass size distribution of Printex 90, 5% was below 100 nm and 75% above 200 nm; for UV-Titan, less than 1% was below 100 nm and 75% was over 1600 nm. Therefore, a higher estimated pulmonary deposition was likely achieved with Printex 90 compared with UV-Titan (287 and 73 μg/animal, respectively, for Printex 90 and UV-Titan). Conversely, the estimated intragastric deposition of Printex 90 was therefore less than that of UV-Titan (137 and 365 μg/animal, respectively, for Printex 90 and UV-Titan). Thus, it is unlikely that differences in intragastric deposition explain why inhalation of Printex 90 leads to hepatic DNA damage while inhalation of UV-Titan does not. However, Printex 90 is an efficient generator of reactive oxygen species (Jacobsen et al. 2008) and induces more oxidative stress than UV-Titan (Saber et al. 2011). Reactive oxygen species production and oxidative stress are linked to DNA damage (Borm et al. 2006; Jacobsen et al. 2010; Møller et al. 2010) and therefore may play a causal role in the observed differences between these exposures. It is also possible that there is less uptake of UV-Titan agglomerates in the digestive system compared with Printex 90 (Carr et al. 1996; Kreyling et al. 2002; Kreyling et al. 2009). In line with this, it has previously been reported that intragastric dosing of carbon-based nanoparticles causes an increase in oxidative DNA damage in liver (Danielsen et al. 2008; Folkmann et al. 2009).

Based on the particle size of the inhaled UV-Titan, we expect that only a small fraction of nanoparticles was translocated outside the lung cavity or the gastrointestinal mucosa

(Kreyling et al. 2010; Kreyling et al. 2009; Sadauskas et al. 2009; van Ravenzwaay et al. 2008), and thus few particles would reach the placental barrier and the foetus (Myllynen et al. 2008; Sadauskas et al. 2007; Takahashi and Matsuoka 1981; Wick et al. 2010). Analysis of UV-Titan in maternal lung, maternal liver and offspring liver confirmed our expectation; significant amounts of UV-Titan were restricted to the maternal lung (Hougaard et al. 2010). However, small amounts of nanoparticles would not be detected due to a high analytical detection limit. The UV-Titan was coated with Zr, Si, Al, Na as well as complex polyalcohols, which could leach and cause toxicity. Thus, it should also be noted that it is possible that impurities leached from the particle coating reached the blood stream and crossed the placenta. To evaluate the possible effect of particle impurities on observed inflammation, we analysed levels of endotoxin using the limulus amebocyte lysate test in the particle extracts. The daily inhalation exposure was calculated to contain 0.16 EU/kg BW or 10.5 pg/kg BW. The level of endotoxin was below the endotoxin tolerance limit for human and veterinary drugs (5 EU/kg BW/h or 330 pg/kg BW/h) (USP 30 NF 25 2007). For comparison, the positive LPS control included in the study by Saber et al. (Saber et al. 2009) received 12.5 mg LPS/kg BW.

We hypothesise that the inflammatory cytokines and acute-phase proteins induced in the lungs of the UV-Titan-exposed time-mated mice (Halappanavar et al. 2011; Hougaard et al. 2010) may cross the placenta and induce effects in the offspring. The inflammatory cytokines interleukin-1beta (*IL-1*β), interleukin-6 (*IL-6*) and tumour necrosis factor alpha (*TNF-*α) are important for immune changes associated with pregnancy. IL-1β regulates embryogenesis and foetal development, and inflammatory cytokines increase as a signal for the onset of labour (similarly to "transplant rejection") (Protonotariou et al. 1999). However, excessive inflammation during pregnancy negatively affects offspring development. Maternal inflammation during pregnancy, induced by exposure to LPS, resulted in reduced activity of the hypothalamic–pituitary–adrenal axis and altered immune response in offspring later in life (Hodyl et al. 2007; Hodyl et al. 2008; Lasala and Zhou 2007; Surriga et al. 2009). Proinflammatory insult during gestation may also affect neuronal development and memory (Gilmore et al. 2004; Graciarena et al. 2010; Lasala and Zhou 2007; Marx et al. 2001). While the dams in the above studies received doses with several hundreds of micrograms of LPS per kilogram, the UV-Titan-exposed dams in the present study received levels of endotoxin below the regulatory limits, and therefore the observed maternal inflammation was likely due to nanoparticle exposure. Based on these results, we speculate that nanoparticle-induced inflammatory signals from the mother could induce changes in hepatic gene expression in the offspring.

To further evaluate the effects of prenatal exposure to UV-Titan, global gene expression using microarrays was evaluated in the newborn offspring. This analysis was performed on liver tissue, because foetal liver receives maternal blood directly after placenta passage and consequently may be a site of primary toxicity following maternal exposures. Nanoparticles have been shown to accumulate in foetal liver after intrafoetal administration (Challier et al. 1973). Liver plays an important role in various processes including detoxification, regulation of metabolic homeostasis and endocrine function. These functions may be compromised by prenatal toxicant exposure. Balanced synthesis and secretion of hormones and growth factors are essential for the promotion of postnatal growth. Prenatal exposure to toxicants may affect growth, development and developmental programming, leading to disease susceptibility.

We have reported recently (Jackson et al. 2011c) that hepatic gene expression is significantly altered in newborn female offspring prenatally exposed to the carbon black nanoparticle Printex 90 via maternal pulmonary exposure by intratracheal instillation (total dose 268 μg/animal on gestation days 7, 10, 15 and 18). By contrast, male offspring are relatively unaffected by maternal Printex 90 instillation exposure. Stronger responses are also observed in female offspring compared with male offspring following prenatal exposure to Printex 90 by maternal inhalation exposure (42 mg/m^3 1 h/day for 11 days, ~840 μg inhaled dose, 72.5 μg estimated pulmonary dose = 3.3 mg/kg) (Jackson et al., unpublished results).

In the current study, hepatic gene expression profiling of prenatally exposed offspring revealed differential expression of 35 genes in females and 17 in males (p-value < 0.05) relative to control animals. RT-PCR analysis confirmed the upregulation of *Cyp26b1*, *Ttr* and *Ugt3a2*, but only *Ugt3a2* in male offspring reached statistical significance. The differentially expressed genes in female offspring are implicated in the basic biological pathway for RA receptor signalling (Supplementary Figure 1), while male response is related to metabolic processes. Gender-specific differences in RA signalling pathways have been documented in adult animals. Deficiency of retinoid x receptor α in livers of 6-month-old mice has larger effects on cancer-related gene expression in males compared with females (Guo et al. 2008). It has also been shown that males are more sensitive to vitamin A deficiency and that the metabolic rate of RA is different between genders (Marchetti et al. 1997; Klein-Szanto et al. 1980). However, the present study suggests that prenatally exposed females exhibit larger transcriptional responses than male offspring. We have observed a similar pattern in gender-specific gene expression in offspring prenatally exposed to carbon black (Printex 90) (Jackson et al. 2011c). This suggests that prenatal exposure to nanoparticles has a stronger effect in the prenatally exposed females than in males.

As described above, the altered transcripts in the female offspring are implicated in RA receptor signalling, which plays an important role in embryonic growth, foetal development, neurogenesis, limb growth, organogenesis and regulation of fertility (Mark et al. 2009; Niederreither and Dolle 2008). RA receptors themselves were not differentially expressed in the prenatally exposed mice. However, activation of transcription by nuclear receptors occurs through ligand binding and heterodimerisation and does not require

transcription of the receptors (de Lera et al. 2007). It has been reported that LPS-induced inflammation in the rat can potentially disrupt the balance of RA metabolism and maintenance of RA homeostasis by regulating genes involved in RA degradation, which may then affect the expression of other RA-regulated genes (Zolfaghari et al. 2007). It is possible that the prenatal exposure to UV-Titan and accompanying pulmonary inflammation and acute-phase response induce a similar transcriptional response to the one induced by LPS.

Proinflammatory cytokines are also reported to activate RA signalling in vascular smooth muscle, which in turn may modulate inflammatory response in the vascular wall (Gidlof et al. 2001). As a possible feedback mechanism, Il-1β downregulates RA-binding proteins and receptors in mice lung tissue (Bry and Lappalainen 2006). RA directly reduces synthesis of Il-1β and response to inflammatory stimuli in human monocytes (Gross et al. 1993). Similar processes may be possible in the foetal tissue, where maternal inflammatory mediators could increase signalling through the RA pathway in the foetus, to attenuate the inflammatory response in the offspring. RA signalling also plays an important role in hepatic differentiation and likely during hepatic regeneration (Huang et al. 2009), as well as in carcinogenesis (Fields et al. 2007). Thus, changes in RA signalling may be a response to liver injury. While disturbance in RA signalling could trigger a variety of effects in developing offspring, our results lack supporting evidence for this model. Hence, further studies are necessary to fully explore the consequences of such perturbations *in utero* on adult health. Large fold changes were also observed for *Aldob* and *Alb1* by microarray analysis. *Aldob* is associated with carbohydrate metabolism and *Alb1* is implicated in acute stress response. We do not know the potential implications of these genes on the development of offspring exposed *in utero* to nanoparticles. Further work is needed to explore the effects of these transcriptional changes on health.

In conclusion, maternal inhalation exposure to 42 mg UV-Titan/m^3 1 h/day for 11 days did not induce genotoxicity in BAL or liver cells of exposed mothers, or the livers of their offspring. However, exposure induced persistent inflammation and acute-phase response in mothers, and affected gene expression in the liver of offspring, with increased response in female offspring. The observed changes in gene expression in the newborn offspring 2 days after birth suggest that anti-inflammatory processes were activated in the female offspring related to RA signalling.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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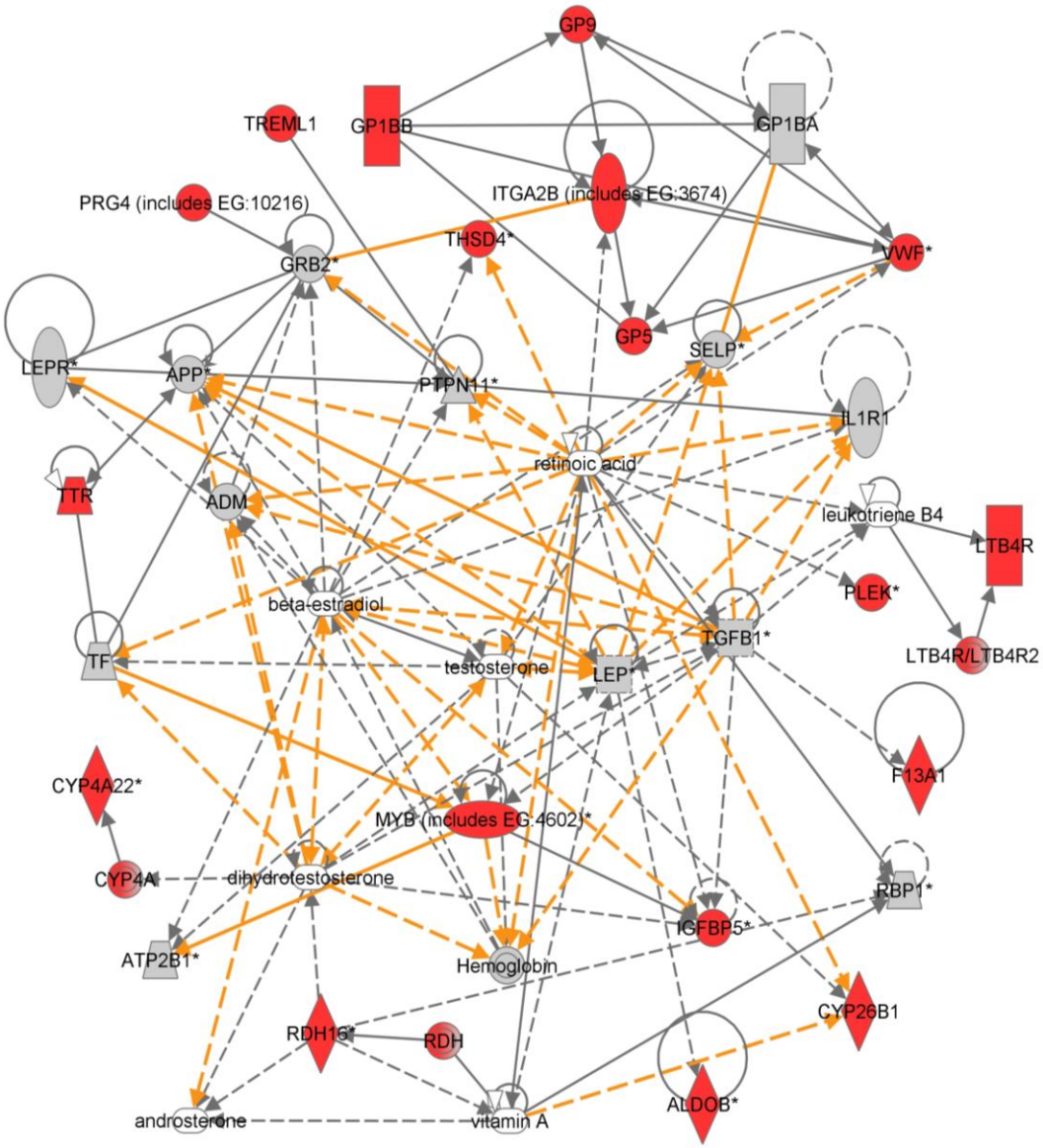
Supplementary material available online

Supplementary Figure 1.

Supplementary Figure 1: Gene network for female offspring exposed *in utero* to titanium dioxide (UV-Titan) by maternal whole-body inhalation exposure to 42 mg/m^3 for 1h/day on gestation day 8-18. The image was derived by merging two biological networks associated with the altered genes in female offspring; cardiovascular system and hematological development and function (grey lines), cell signaling and cell-cell interaction (orange lines). In Red are the genes significantly altered in response to the exposure. In Grey are the unaltered genes.



PAPER IV

An Experimental Protocol for Maternal Pulmonary Exposure in Developmental Toxicology.

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An Experimental Protocol for Maternal Pulmonary Exposure in Developmental Toxicology

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Abstract: To establish a protocol for studying effects of pulmonary exposure in developmental toxicity studies, the effects of intratracheal sham instillation under short-term isoflurane anaesthesia were evaluated with a protocol including multiple instillations during gestation. Twelve time-mated mice (C57BL/6BomTac) were anaesthetized with isoflurane and intratracheally instilled with saline containing 10% bronchoalveolar lavage (BAL) on gestation days 8, 11, 15 and 18. In addition, the early effects of the procedure were assessed in naive female mice. Control animals were not handled. Dams were followed until weaning, and the offspring were observed from birth to sexual maturation. The cell composition of BAL was examined in the females early after treatment (3 days) and in the dams at weaning (25 days). DNA damage in BAL and liver cells was determined by the comet assay. The procedure did not affect gestation or viability, growth and sexual maturation of the offspring. Lung markers of inflammation and DNA damage were comparable in control and treated dams. Livers of the anaesthetized and instilled females, dams and their offspring displayed no induction of DNA damage. Intratracheal instillation under isoflurane anaesthesia did not induce observable effects in pregnant mice or their offspring. We suggest that this procedure can be used as a means of exposure through the airways in studies of developmental toxicity.

Occupational exposures to hazardous chemical substances occur primarily in the airways [1]. Inhalation toxicology is therefore important for the evaluation of occupational exposures, and this requires special facilities and procedures. These complex experimental procedures may have an effect on the tested animals, which may devaluate the significance of the overall validity of the results. This is specifically problematic when exposing animals during gestation. The dam and the offspring may be especially sensitive to experimental interference [2–4]. Therefore, exposure during gestation may hamper the ability to detect adverse effects of the tested substances, e.g. if stress caused by experimental procedures intensify chemically induced effects in the pregnant animal or in the offspring [5]. It is exceptionally difficult to separate maternal chemical toxicity from maternal general stress, if at all possible [3]. This problem is normally solved by inclusion of a control group, but if there is an interaction between the chemical exposure and the experimental procedures, an elaborated study design is necessary, or the applied methods should be tested beforehand in the absence of test material. Therefore, this article addresses pulmonary exposure during gestation for testing of chemical substances.

Exposure through the airways may take place by whole-body inhalation exposure, nose-only inhalation exposure or intratracheal instillation exposure. For exposures by inhalation, whole-body inhalation exposure is less invasive to the animals because movement of the animal is not as restricted. However, this method may involve the deposition of chemicals in the fur of the animals, thus complicating exposure evaluation, because additional exposure might occur during the oral route via fur grooming and from cutaneous absorption. In case of exposure to highly toxic, carcinogenic or radioactive compounds, the exposure may also be associated with a potential risk for the personnel handling of the substances or the animals. During nose-only inhalation exposure, the chemical exposure is restricted to the airways but involves the restraining of the animals. Restraint has been shown to cause stress [6]. When applied at specific times during gestation, stress may induce e.g. pre- and post-implantation loss, reduce maternal weight gain and offspring foetal and post-natal growth, cause malformations, increase plasma corticosterone in maternal as well as foetal plasma and alter sexual differentiation [6].

Intratracheal instillation represents an alternative method for airway delivery [7,8]. The substance to be tested is suspended in a liquid solution injected into the lumen of the trachea of the animal, which is under general anaesthesia. Thus, intratracheal instillation only requires a small amount of test chemical, which is beneficial when the supply of material is limited or very valuable. Compared to whole-body and nose-only exposure, a precise dose can be introduced into the lung, and dose–response studies are easily achieved. Instillation does not imply substantial deposition in the nasal

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cavity or the fur of the experimental animals. It may, however, interfere with gestation and affect the experimental outcome: (i) the chosen anaesthesia may be toxic to the dams and/or the foetuses; (ii) the dams may be stressed during handling and anaesthesia; and (iii) the instillation procedure may affect the airways directly by inducing lung inflammation or because of mechanical damage.

The purpose of this study was to evaluate a protocol with repeated intratracheal instillations under isoflurane anaesthesia during gestation in the absence of test material, for use in developmental toxicity testing. Primary end-points were classical gestational, lactational and developmental parameters. Injecting fluid into the lung may be an invasive process, which may cause inflammation and inflammation-related DNA damage. Furthermore, isoflurane anaesthesia has, in some human studies, been associated with DNA damage [9]. Therefore, maternal lung inflammation (cell composition in the lung epithelial lining) and DNA damage and repair (comet assay in both mother and offspring) were assessed. Both newborn and weaned offspring were analysed, and non-pregnant females were included to be able to assess the effects on the females early after exposure termination.

Materials and Methods

Animals. Twenty-four time-mated, nulliparous, young adult mice (C57BL/6BomTac; Taconic Europe, Ejby, Denmark) were received on gestation day 3 (day of plug gestation day 1). Ten naive female mice (later termed 'females') of same origin and age arrived the same day. The animals were assigned to four experimental groups [time-mated control group (12), anaesthetized and instilled time-mated group (12), female control group (5) and anaesthetized and instilled female group (5)]. The mice were housed as described earlier in [10,11]. The mice were weighed on gestation days 4, 7, 10, 13, 16 and 18.

The animal welfare committee, appointed by the Danish Ministry of Justice, granted ethical permission for the study. All procedures complied with the EC Directive 86/609/EEC and Danish law regulating experiments on animals (permission 2006/561–1123).

Exposure. The mice were anaesthetized and instilled on days corresponding to gestation days 8, 11, 15 and 18 to simulate an inhalation exposure protocol of repeated inhalation of particles for 1 hr on gestation days 8–18 [11]. Treatment took place between 8 and 11 a.m. During this time, the cages with treated groups were placed in a Scantainer (Scanbur A/S, Karlslunde, Denmark) located in the laboratory. The mice were treated by groups, one cage at a time, females first and dams afterwards. Control females and dams were not handled or moved on treatment days.

The mice were instilled as described in [12], with minor modifications. The anaesthetized mice were placed on their backs on a platform with 40-degree slope. A diode light was placed touching the larynx. The tongue was pressed towards the lower jaw by a small spatula. The trachea was intubated using a 24-gauge BD Insyte catheter (Ref: 381212, Becton Dickinson, Brøndby, Denmark) with a shortened needle. The correct location of each intubation was tested by a small but highly sensitive pressure transducer developed by our laboratory in collaboration with John Frederiksen (FFE/P, Copenhagen, Denmark). When the catheter was correctly placed, this was indicated by a clicking sound triggered by the pressure variation as air was inhaled and exhaled. The mice were instilled with 40 µl solution followed by 160 µl air in a 1000-µl sterile disposable plastic syringe (300013, BD Plastipak™, Franklin Lakes, NJ, USA). The instillation solution consisted of sterile and pyrogen-free 0.9% NaCl solution, mixed 10:1 with bronchoalveolar lavage (BAL) fluid from C57BL/6BomTac mice. BAL fluid was prepared from 10 female mice, 12–14 weeks old, by flushing the lungs twice with 0.6-ml sterile and pyrogen-free 0.9% sodium chloride solution. BAL cells were isolated by centrifugation at $400 \times g$ for 10 min. at 4°C. The supernatants were pooled, diluted and frozen at −80°C. Before instillation, BAL fluid was thawed at room temperature and sonicated in Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (Model number: 101-147-037) for 2×8 min., with alternating 10-sec. pulses and 10-sec. pauses (total 4 min. on +4 min. off). The amplitude was set at 10% and maximum temperature at 60°C. Samples were continuously cooled on ice during the sonication procedure. Sonication was introduced into this protocol to assess all methodological steps used when instilling particles.

During the induction of anaesthesia, the mice were placed in a whole-body inhalation chamber (Art. No. 8329002; AgnTho's AB, Lidingö, Sweden) attached to a Sigma Delta anaesthetic vaporizer (Art. No. 8329001, AgnTho's AB). The chamber was a clear acrylic box (inner dimensions $18 \times 10 \times 10$ cm) attached to a scavenging system. The anaesthetic vaporizer delivered 3% isoflurane mixed with sterile filtered air under constant flow. Each mouse was anaesthetized for a few minutes until fully unconscious. The level of unconsciousness was judged by a pattern of deep breathing and absence of pain reflex. The mouse was immediately taken out of the chamber and instilled with the test solution. Mice remained fully unconscious for approximately 30–45 sec. Mice that regained consciousness before instillation could be accomplished were anaesthetized one more time and then instilled.

Isoflurane (Isoba® vet, Schering-Plough A/S, Ballerup, Denmark) was stored refrigerated until use. The concentration recommended by the manufacturer for isoflurane induction of anaesthesia for small mammals is 2–3%.

Parturition and lactation. After the last exposure on gestation day 18, the dams were housed alone and monitored for birth. Gestation day 20 was the expected day of delivery and was assigned as post-natal day 0 for the offspring.

On post-natal day 1, all dams and females were weighed. Offspring were counted, weighed, and sex was determined. The dams that had not given birth and the naive females were anaesthetized with a mixture of hypnorm-dormicum diluted in sterile water 1:2 (hypnorm: fentanyl citrate 0.75 mg/kg and fluanisone 23.8 mg/kg, VetaPharma Ltd., Leeds, UK; dormicum: midazolam 11.9 mg/kg, Roche a/s, Hvidovre, Denmark). BAL was collected. Liver and lungs of all mice were dissected and snap frozen in cryotubes (NUNC) in liquid N_2. The number of uterine implantation sites was determined in time-mated females. One time-mated instilled female died as a result of tissue growth in the abdominal cavity and was excluded from the study.

On post-natal day 2, up to one male and one female offspring were removed from litters, leaving at least three and preferably five offspring with the dam. Offspring were weighed, decapitated, and liver and lungs were dissected and snap frozen in cryotubes in liquid N_2. Remaining offspring were weighed on post-natal days 8, 13, 18 and at weaning on post-natal day 22.

At weaning on post-natal day 22, anogenital distance was measured with a slide gauge in all offspring. Relative anogenital distance was calculated (AGD/cube root of body-weight) [13]. One male and one female, in litters were possible, were kept for the observation of onset of puberty. Remaining offspring were euthanized by cervical dislocation. Liver and lungs from one male and one female offspring from each litter were isolated and snap frozen in cryotubes in liquid N_2. The dams were handled on post-natal day 23, as described for females on post-natal day 1.

Sexual maturity, vaginal opening in females and preputial separation in males were recorded on post-natal days 26, 29, 31, 33, 36, 38, 40 and 43 for females and post-natal days 26, 33, 36, 38, 40 and 43 for males. Females were euthanized on post-natal day 43 by cervical dislocation. Males were euthanized by cervical dislocation on

post-natal day 85; their testicles were dissected, weighed and snap frozen in cryotubes in liquid N_2.

Bronchoalveolar lavage preparation and analyses. BAL was performed in hypnorm-dormicum anaesthesia by washing lungs four times with 0.8 ml 0.9% sterile saline through the trachea. BAL fluid was immediately cooled on ice and preserved as described [14]. The total number of cells and of dead cells in BAL samples was determined by a NucleoCounter (NucleoCassetteTM–941-0002, Chemometec, Denmark), following the standard kit procedure.

The numbers of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were determined in the total number of cells in the BAL fluid by counting 200 cells collected in 40 µl cell suspension centrifuged at $55 \times g$ for 4 min. in a Cytofuge 2 (StatSpin, Bie and Berntsen, Herlev, Denmark). All slides were fixed with 96% ethanol, stained with May-Grünwald-Giemsa stain, randomized and blinded before counting. Forty microlitres of cell suspension was mixed with 160 µl freezing medium, HAM F-12 supplemented with 10% FBS and 10% DMSO (1.02950.0500, Merck, VWR international, Herlev, Denmark) and stored at −80°C for comet analysis.

Detection of DNA strand breaks by the comet assay. The lung is the initial recipient of the test compound, but the compound may of course transfer to other organs. For many chemicals, metabolism takes place in the liver. Therefore, DNA strand breaks were determined by comet assay [15] with minor modifications [16] in BAL [17] and liver cells. Briefly, BAL cells or liver cell suspensions were embedded in agarose on GelBond film (85 × 100 mm, Cambrex Bioscience, Rockland, ME, USA) and subjected to alkaline electrophoresis. The slides were fixed and stained by SYBR Gold fluorescent dye (Molecular Probes, Invitrogen, Taastrup, Denmark), and 50 randomly selected cells were scored by fluorescent microscopy. DNA damage was quantified as % DNA in tail and as tail length. Frozen standard A549 cells untreated or treated with H_2O_2 were internal controls. The results were recalculated from % DNA results to estimate the number of lesions per million base pair [18].

Statistical analyses. The accepted level of statistical significance was 0.05. Litter was considered the statistical unit. Gestational parameters were analysed by Kruskal–Wallis one-way analysis of variance. Weight data were analysed by analyses of variance (ANOVA), with treatment as factor and time of sampling (post-natal day) as repeated measure. Litter size was used as co-variable for gestational weight. Remaining data were analysed by analyses of variance (ANOVA) with the following factors: treatment, time of sampling (post-natal day) and sex (where relevant). Numbers of litters were analysed by Fisher's exact test. Sexual maturation data were analysed by PROC LIFETEST. Analyses were performed on SYSTAT Software Package version 9 (Systat Software Inc., Chicago, IL, USA) and SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± SEM.

Results

Early effects of instillation.
The weight gain of females (including three mated females that did not give birth) that were saline/BAL instilled under isoflurane anaesthesia was comparable to weight gain of control females (including three mated females that did not give birth); (treated 0.3 ± 0.21 g, control 0.5 ± 0.22 g, p = 0.73). Liver and lung weights were unaffected by treatment (table 1).

Maternal and offspring parameters.
Gestational and developmental parameters were similar in treated and control groups (weight increase during gestation and lactation, length of gestation, number and loss of implantations, litter size and viability, sex ratio, AGD at weaning and sexual maturation, cf. table 2). Organ weights of livers and lungs from dams and offspring were unaffected by treatment (table 1). The weight of the testicles at post-natal day 85 was slightly reduced in male offspring of treated dams (treated 0.69 ± 0.02 g, control 0.74 ± 0.02 g, p = 0.09).

Lung inflammation.
There were no statistically significant differences in BAL fluid cell composition between treated and control females and dams (table 3), indicating that the employed exposure protocol did not induce inflammation that could be detected 3 days after the last exposure.

DNA strand breaks.
The amount of DNA damage in BAL cells as analysed by comet assay was similar in treated and control females, 3 days after treatment (p = 0.28) (table 4). DNA damage was not determined in dam BAL cells at weaning, 25 days after last anaesthetization and instillation, because there was

Table 1.
Relative lung and liver weight of isoflurane-anaesthetized and saline/BAL-instilled females or dams and offspring, and control mice.

Relative lung weight			Relative liver weight		
Control	Treated	p-value	Control	Treated	p-value
Females 3 days after anaesthetization and instillation (n = 8 and 8)					
1.26 ± 0.02	1.24 ± 0.06	0.66[1]	5.27 ± 0.18	4.96 ± 0.13	0.31[1]
Dams 25 days after anaesthetization and instillation (n = 9 and 8)					
0.96 ± 0.03	0.95 ± 0.02	0.66[1]	5.93 ± 0.10	5.97 ± 0.08	0.31[1]
Offspring PND 2 (n^2 = 9 and 7)					
2.98 ± 0.09	2.78 ± 0.14	0.36[3]	3.21 ± 0.11	3.24 ± 0.07	0.33[3]
Offspring at weaning PND 22 (n^2 = 9 and 8)					
1.17 ± 0.02	1.18 ± 0.03	0.36[3]	3.96 ± 0.14	4.21 ± 0.17	0.33[3]

PND, post-natal day (days after birth); BAL, bronchoalveolar lavage.
Data are presented as mean relative weight (%) ± SEM.
[1]Overall p-value adult females.
[2]Litter average.
[3]Overall p-value offspring.

Table 2.
Gestation, lactation and developmental parameters of isoflurane-anaesthetized and saline/BAL-instilled dams and control dams.

End-point	Control	Treated	p-value
Time mated	12	11	–
Weight gain, GD 4–18 (g)	13.8 ± 0.62	12.9 ± 1.03	0.47
Weight gain[1], GD 7–18 (g)	12.8 ± 0.63	11.6 ± 1.02	0.21
Weight gain, PND 1–23 (g)	2.5 ± 0.50	4.0 ± 0.40	0.69
Gestation length (days)	20	20	1.00
Number of litters	11	8	0.71
Implantations	8.5 ± 0.39	8.1 ± 0.68	0.97
Implantation loss (%)	23.8 ± 7.29	33.7 ± 9.80	0.30
Live offspring per litter, PND 1	6.5 ± 0.71	5.7 ± 0.99	0.59
Dead offspring per dam during lactation	0	0.4 ± 0.34	0.11
Birth weight female (g)	1.4 ± 0.05	1.4 ± 0.07	0.35
Birth weight male (g)	1.4 ± 0.03	1.5 ± 0.06	0.41
Weight gain female, PND 1–22 (g)	7.4 ± 0.25	7.1 ± 0.33	0.56
Weight gain male, PND 1–22 (g)	7.8 ± 0.32	8.2 ± 0.63	0.58
Sex ratio[2]	0.55 ± 0.07	0.38 ± 0.06	0.13
AGD at weaning females (mm) (n^3 = 9 and 7)	5.25 ± 0.17	5.21 ± 0.22	0.90
AGD at weaning males (mm) (n^3 = 8 and 9)	8.37 ± 0.25	8.90 ± 0.41	0.32
Relative AGD at weaning females	2.55 ± 0.08	2.54 ± 0.09	0.94
Relative AGD at weaning males	4.01 ± 0.10	4.12 ± 0.13	0.51
Vaginal opening (n = 9 and 5)	Majority at PND 36	Majority at PND 36	0.74
Preputial separation (n = 7 and 8)	Majority at PND 33	Majority at PND 33	0.35

AGD, anogenital distance; GD, gestation day (pregnancy day); PND, post-natal day (days after birth).
Dams were allowed to deliver their pups on gestation day (GD) 20, equal to post-natal day (PND) 0. Weights of dams and individual pups were recorded on PND 1, and pups were counted and sex determined. Time-mated mice were examined for the number of implantation sites, allowing for calculation of implantation loss. Females that did not give birth were killed on PND 1 (day after expected birth) and the dams on PND 23 (after weaning). Offspring were observed for puberty start after weaning. Data are expressed as mean ± SEM.
[1]Weight before instillation.
[2]Per cent of females.
[3]Litter average.

no increase in the level of DNA damage at the earlier time of collection.

DNA damage was quantified in liver (table 4). Levels of DNA damage in treated females 3 days after treatment and dams at weaning were similar to control levels ($p = 0.96$). No increase in DNA damage was found in offspring of isoflurane-anaesthetized and saline/BAL-instilled dams at post-natal day 2 and post-natal day 22 ($p = 0.62$).

Discussion

The purpose of the present study was to evaluate a procedure for pulmonary treatment of pregnant animals to assess whether this procedure is associated with adversity in dams or offspring. The tested procedure included repeated intratracheal instillation under isoflurane anaesthesia, in the absence of test material for use in pregnant animals. The present study diverges from an ordinary toxicological study, because preferably the effects of the treatments should be negligible. We did not observe any effects of the procedure on the assessed end-points of gestation or lactation, and the offspring of treated dams survived and developed similarly to the offspring of control dams. Maternal lung inflammation and maternal and offspring DNA damage were comparable in the sham exposed and control animals.

Experimental conditions and procedures may interfere with gestation and foetal development by inducing stress in the dams and their foetuses, thus hampering the ability to detect outcome of toxicological and pharmaceutical evaluations of substances administered during gestation [5]. Repeated blood collection in pregnant rats is one example of a procedure that may affect the course of gestation. Collection of blood four times during gestation was thus associated with decreased body-weight gain and food consumption in the treated dams, while gestational parameters were unaffected [2]. A few studies have examined the protocols of inhalation exposure for potential effects on the course of gestation, in the absence of a test chemical. Tyl *et al.* [3] compared whole-body and nose-only inhalation exposure for 6 hr on gestation days 6–15 in pregnant mice, without the inclusion of an unhandled control group. Also in this study, no effects on gestation and foetal parameters were observed. Some maternal effects, demonstrated by reduced body-weight gain and liver weight, were observed in females subjected to nose-only inhalation exposure [3]. Exposure to filtered air for 1, 2 or 6 hr on gestation days 6–17 by nose-only inhalation did not induce major changes in rat reproduction and gestational parameters (uterine weight, foetal viability and weight, resorptions and malformations). Some maternal weight loss was observed on the first day of exposure, but the dams had recovered by last day of exposure [4].

In the current study, the mice were intratracheally instilled with saline containing 10% bronchoalveolar lavage four times during gestation. The time-points of instillation were

Table 3.
BAL cell composition of isoflurane-anaesthetized and saline/BAL-instilled females or dams and control mice.

	Total cells	Dead cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils	Epithelia
Females 3 days after anaesthetization and instillation							
Control (n = 8)	119,250 ± 13,000	13,500 ± 1350	106,688 ± 11,313	1058 ± 324	1028 ± 398	289 ± 200	10,189 ± 1778
Treated (n = 8)	161,250 ± 19,654	1050 ± 327	137,355 ± 18,975	2411 ± 595	3874 ± 1069	7478 ± 2719	10,133 ± 1418
Dams 25 days after anaesthetization and instillation							
Control (n = 9)	150,667 ± 24,051	14,444 ± 1819	130,280 ± 23,085	2397 ± 884	4287 ± 1616	4647 ± 2931	9057 ± 890
Treated (n = 8)	172,500 ± 11,438	13,750 ± 2374	145,380 ± 7859	2940 ± 972	8246 ± 3622	7658 ± 7061	8276 ± 1817
Overall p-value	0.09	0.28	0.19	0.22	0.11	0.21	0.78

BAL, bronchoalveolar lavage.
Data are presented as mean number of cells in BAL ± SEM.

Table 4.
DNA damage in BAL cells and liver cells of isoflurane-anaesthetized and saline/BAL-instilled females or dams and offspring, and control mice.

End-point	Control	Treated	p-values
Females 3 days after anaesthetization and instillation (n = 8 and 8)			
BAL cells	1.52 ± 0.11	2.20 ± 0.50	0.28
Liver cells	2.79 ± 0.76	3.11 ± 0.52	0.96[1]
Dams 25 days after anaesthetization and instillation (n = 9 and 8)			
Liver cells	1.86 ± 0.32	1.60 ± 0.22	0.96[1]
Offspring PND 2 (n^2 = 11 and 7)			
Liver cells	2.15 ± 0.18	2.79 ± 0.20	0.62[3]
Offspring at weaning PND 22 (n^2 = 9 and 8)			
Liver cells	3.69 ± 0.44	3.42 ± 0.48	0.62[3]

BAL, bronchoalveolar lavage; PND, post-natal day (days after birth).
Data are presented as mean lesions/10 × 6 base par calculated from %DNA results ± SEM.
[1]Overall p-value adult females.
[2]Litter average.
[3]Overall p-value offspring.

chosen to cover the major part of the embryonal and foetal periods and to ensure continued exposure to the test substance, even if the substance is cleared away from the lungs. A limited number of instillations are probably most suited for testing particle exposure where slow clearing of the lungs is to be expected. The repeated instillations also serve to diminish inter-animal variation in deposition of the dosed substance. The treatment period was chosen to avoid stressing the dams during sensitive periods, thereby interfering with implantation and delivery. Instillation was performed under general anaesthesia with isoflurane to enable the introduction of catheter into the upper airways during the instillation procedure. The alternative would be anaesthetization with e.g. hypnorm-dormicum mixture, which involves administration by injection and a long-lasting period of unconsciousness [19]. Isoflurane is a commonly used agent for anaesthetizing experimental animals [20]. Because of its low blood/gas partition coefficient, isoflurane anaesthesia takes affect fast and is short lasting. In our study, anaesthesia and recovery lasted less than 5 min. in total. This short duration limits isoflurane exposure of the foetus, even though isoflurane readily passes the placenta. Importantly, maternal isoflurane anaesthesia does not seem to interfere with foetal development, even at anaesthetic dose levels (reviewed in [9]). Isoflurane exposure, *per se*, was therefore not expected to affect dams or foetuses adversely and seem preferable to injection anaesthesia.

The applied experimental procedure must be expected to induce some degree of stress in the animals, however. This may be enhanced further by isoflurane that increases the plasma level of the stress hormone corticosterone in blood in rodents [21–23]. Still, the investigated gestational and developmental parameters were comparable in treated and non-handled dams.

Instilling a small volume of fluid into the lung may induce physical injury or cause lung inflammation. However, in the present study, cell composition in the BAL was comparable in treated and control females. This indicates that there was no significant lung inflammation in the treated females after 3 days, suggesting that the lungs were not overtly affected by the instillation procedure. Similarly, the levels of DNA damage were similar in the lungs and livers of exposed females and dams and in the offspring liver. In some human studies, isoflurane has been associated with DNA damage [9]. It cannot be excluded that short-lasting DNA damage and inflammation may have occurred, because it may have been repaired at the time of analysis.

In conclusion, repeated intratracheal instillation under isoflurane anaesthesia did not seem to interfere with gestation or prenatal and post-natal development. Overall, the intratracheal procedure presents some obvious benefits for airway exposure of pregnant animals compared with the whole-body and nose-only inhalation exposure. Furthermore, precise dosing directly to the lung allows better control of the exposure level and requires smaller amount of test material. The procedure therefore seems applicable in studies requiring airway exposure during the course of gestation and when little material is available for testing.

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Conflict of interests

The authors report no conflict of interests.

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Corrigendum

The following error appeared in the article entitled ''An Experimental Protocol for Maternal Pulmonary Exposure in Developmental Toxicology'', published in *Basic & Clinical Pharmacology & Toxicology* 2011;**108:**202-207.

An incorrect conversion factor was used in the article. The correct conversion factor for converting %DNA in tail to number of lesions per million base pairs is 0.0544. The corrected data is found in the table below. The correction has no effect on data validity or statistical results.

On behalf of the authors, we apologize for this error.

Table 4.

DNA damage in BAL cells and liver cells of isoflurane anaesthetized and saline/BAL instilled females or dams and offspring, and control mice.

End point	Control	Treated	*p*-values
Females 3 days after anaesthetization and instillation (n = 8 and 8)			
BAL cells	0.29 ± 0.02	0.41 ± 0.09	0.28
Liver cells	0.53 ± 0.14	0.59 ± 0.10	0.96[1]
Dams 25 days after anaesthetization and instillation (n = 9 and 8)			
Liver cells	0.35 ± 0.06	0.30 ± 0.04	0.96[1]
Offspring PND 2 (n^2 = 11 and 7)			
Liver cells	0.40 ± 0.03	0.53 ± 0.07	0.62[3]
Offspring at weaning PND 22 (n^2 = 9 and 8)			
Liver cells	0.70 ± 0.08	0.64 ± 0.09	0.62[3]

BAL, Bronchioalveolar lavage; PND, Postnatal day (days after birth)
Data are presented as mean lesions/10*6 base pairs calculated from %DNA results ± SEM.
[1]Overall *p*-value adult females.
[2]Litter average.
[3]Overall *p*-value offspring.

PAPER V

Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: Effects on liver DNA strand breaks in dams and offspring.

Jackson P, Hougaard KS, Boisen AMZ, Jacobsen NR, Jensen KA, Møller P, Brunborg G, Gutzkow KB, Andersen O, Loft S, Vogel U and Wallin H.

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Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: Effects on liver DNA strand breaks in dams and offspring

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Abstract

Effects of maternal pulmonary exposure to carbon black (Printex 90) on gestation, lactation and DNA strand breaks were evaluated. Time-mated C57BL/6BomTac mice were exposed by inhalation to 42 mg/m^3 Printex 90 for 1 h/day on gestation days (GD) 8–18, or by four intratracheal instillations on GD 7, 10, 15 and 18, with total doses of 11, 54 and 268 μg/animal. Dams were monitored until weaning and some offspring until adolescence. Inflammation was assessed in maternal bronchoalveolar lavage (BAL) 3–5 days after exposure, and at weaning. Levels of DNA strand breaks were assessed in maternal BAL cells and liver, and in offspring liver. Persistent lung inflammation was observed in exposed mothers. Inhalation exposure induced more DNA strand breaks in the liver of mothers and their offspring, whereas intratracheal instillation did not. Neither inhalation nor instillation affected gestation and lactation. Maternal inhalation exposure to Printex 90-induced liver DNA damage in the mothers and the *in utero* exposed offspring.

Keywords: *Carbon black, nanoparticles, genotoxicity, inflammation, pulmonary exposure, in utero exposure, gestation and lactation*

Introduction

The need for risk assessment and an understanding of the toxicity of particles in ambient air and engineered nanoparticles is becoming more evident. It is concerning that some nanoparticles have the ability to induce DNA damage (Borm et al. 2004; Knaapen et al. 2004; Brauner et al. 2007; Schins and Knaapen 2007; Jacobsen et al. 2009; Møller et al. 2010). The primary genotoxicity of nanoparticles is related to their ability to induce reactive oxygen species (ROS) (Jacobsen et al. 2008b). It is less likely that insoluble nanomaterials such as Printex 90 cause DNA damage, because they only contain minute amounts of organic compounds and transition metals (Jacobsen et al. 2008a). Particles can however induce inflammation and thereby mediate secondary genotoxicity (Knaapen et al. 2004). Human exposure to ultrafine particles in the ambient air has been associated with increased risk of lung cancer, allergy, pulmonary and cardiovascular disease (Delfino et al. 2005; Pope and Dockery 2006; Weichenthal et al. 2007; Brunekreef et al. 2009; Krewski et al. 2009). Organisms under development may display increased sensitivity to nanoparticle toxicity. During development, frequent cell divisions allow only a short time for repair of DNA damage and the immune system is not fully functional. Early-life exposure might therefore predispose to cancer and other diseases later in life (Barton et al. 2005).

Little is known of potential health effects of nanoparticle exposure during fetal life and postnatal development. Epidemiological evidence indicates that environmental air pollutants, including fine particles,

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are associated with adverse pregnancy outcomes, such as premature birth, reduced birth weight, stillbirth, and postnatal respiratory deaths (Dejmek et al. 1999; Lacasana et al. 2005; Šrám et al. 2005; Sláma et al. 2007; Brauer et al. 2008; Pope et al. 2010). Maternal exposure to air pollution during pregnancy has been associated with increased levels of bulky DNA adducts and micronuclei in umbilical blood of newborns (Pedersen et al. 2009). Adverse effects of diesel exhaust particles and titania-based nanoparticles in mothers and their offspring have been reported in a few animal studies (Reliene et al. 2005; Hougaard et al. 2008, 2010). It has been suggested that the fetus could be affected either: (1) Directly by particle translocation through the placenta; (2) by altered placental function; or (3) indirectly by circulating cytokines or other secondary messengers from an inflammatory process in the mother (Hougaard et al. 2011).

Soot from most combustion sources, such as diesel exhaust soot, partly consists of a carbonaceous core and inorganic and organic compounds, e.g., polycyclic aromatic hydrocarbons (Utsunomiya et al. 2004). Printex 90 is a well characterized carbonaceous core particle that has been used extensively as a benchmark and as a model for diesel emission particles without adhered chemicals and metals. Some chemical and physical features are similar to other engineered carbon-based nanoparticles, e.g., single and multi-wall carbon nanotubes and C_{60} fullerenes that are handled in workplaces and occur in consumer products. Printex 90 consists of carbon with less than 1% organic and inorganic impurities (Brown et al. 2000; Wilson et al. 2002; Borm et al. 2005; Jacobsen et al. 2007). Health effects reported after exposure to carbon black are therefore assumed to be caused by the insoluble particle core rather than by associated compounds. Carbon black nanoparticles possess an intrinsic potential to generate reactive oxygen species (Wilson et al. 2002; Jacobsen et al. 2007, 2010; Folkmann et al. 2009; Yang et al. 2009). It is well known that pulmonary exposure to carbon black by instillation or inhalation induces an inflammatory response *in vivo* in rats (Driscoll et al. 1997; Brown et al. 2000; Wilson et al. 2002; Gallagher et al. 2003; Renwick et al. 2004; Sager and Castranova 2009) as well as mice (Saber et al. 2005; Jacobsen et al. 2009; Totsuka et al. 2009; Hougaard et al. 2010). Carbon black is also reported to be mutagenic (Driscoll et al. 1996; Jacobsen et al. 2007, 2010; Totsuka et al. 2009) and it induces lung tumors in rats (Mohr et al. 2006). It is uncertain whether occupational exposure to carbon black is related to cancer risk (Puntoni et al. 2001; Morfeld and McCunney 2007; Sorahan and Harrington 2007; Ramanakumar et al. 2008), but carbon black has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Baan et al. 2006).

The purpose of the present study was to assess the effect of maternal gestational exposure to pure carbon black nanoparticles on the development of the offspring exposed *in utero*. Pulmonary exposure to carbon black nanoparticles causes pulmonary inflammation and genotoxicity. Therefore, we examined the effect of maternal pulmonary exposure to Printex 90 on DNA damage in the exposed offspring, along with traditional gestational and litter parameters.

Methods

The study was comprised of two parts: An inhalation study and an instillation dose-effect study, with the highest dose being similar to the inhaled dose estimated to be deposited in the pulmonary region. The end points studied are classical gestational and lactational parameters. This was related to lung inflammation and DNA damage (see Figure 1 for details of the design and sampling point terminology).

Animals

Time-mated, nulliparous adult female mice (C57BL/6BomTac, Taconic Europe, Ejby, DK) were received on gestation day three (GD 3). The mice were immediately distributed in cages of five or six. Housing conditions have been described previously (Hougaard et al. 2008, 2010). On GD 4, mice were weighed and assigned to experimental groups. Body weight was also recorded before exposure on GD 7, and GD 10, 13, 15 (or 16) and 18.

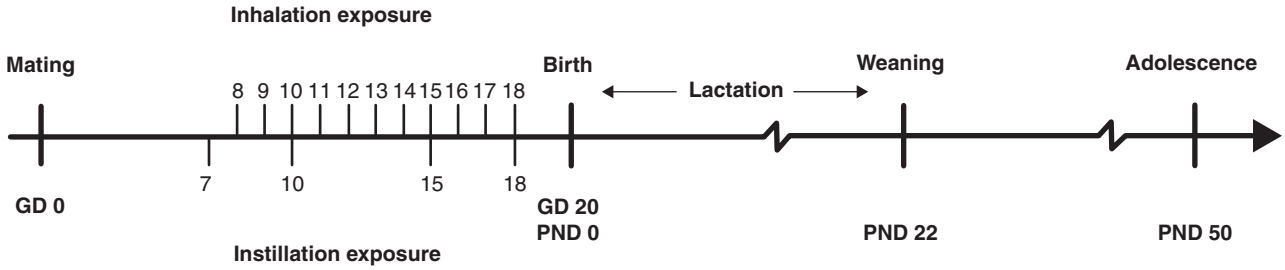
All the procedures complied with the EC Directive 86/609/EEC and the Danish law regulating experiments on animals (The Danish Ministry of Justice, protection of experimental animals (Dyreforsøgstilsynet) with Animal Experiments Inspectorate Permission 2006/561-1123).

Printex 90

The Printex 90 was a gift from Degussa-Hüls, Frankfurt, Germany (see Table I for previously published particle characterization).

Particle exposure

Inhalation. A total of 44 time-mated mice were exposed by whole-body inhalation exposure as described previously (Hougaard et al. 2008, 2010). Time-mated mice were placed in perforated steel-cage in a steel-framed pyrex glass exposure chamber.



Samples collected:	Females	Newborns	Weaned offspring	Dams	Adolescents
Inhalation study:	PND 3 (DAE 5)	PND 2	PND 22-23	PND 22-23 (DAE 24-25)	PND 50
Instillation study:	PND 1-2 (DAE 3-4)	PND 2	PND 23	PND 24-25 (DAE 26-27)	PND 47

Figure 1. Experimental design. GD, gestation day (pregnancy day); PND, post natal day (days after birth); DAE, days after exposure. 'Time-mated mice' term was used for all exposed mice during gestation and when referring to results of 'females' and 'dams' together. Time-mated mice that had not given birth or had only few offspring were termed 'females'.Time-mated females that gave birth were termed 'dams'. PND 1 and PND 2 offspring were termed 'newborns'. Offspring on PND 22–23 was termed 'offspring at weaning'. Offspring on PND 50 (47) termed 'adolescents' had not reached sexual maturity. Time-mated mice were exposed by inhalation and intratracheal instillation to Printex 90. Time-mated mice inhaled 42 mg/m^3 Printex 90 or filtered air for 1 hour/day for 11 consecutive days on GD 8–18. The daily dose would correspond to 12 hours at the Danish Occupational Exposure Limit of 3.5 mg/m^{3}for carbon black. The total instilled doses were 0, 11, 54 and 268 μg/animal were distributed over four instillations on GD 7, 10, 15 and 18. The highest dose was chosen as to be similar to the estimated deposited dose in the pulmonary region from the inhalation study.

The animals were exposed whole-body to HEPA-filtered air or 42 mg/m^3 aerosolized Printex 90 for 1 h per day from GD 8–18. Maximally 12 mice could be exposed at a time. Four groups of mice were exposed on each exposure day and the mice order was changed each time.

Printex 90 was fed into a small airstream by a rotating perforated disc micro-feeder (Fraunhofer-Institut für Toxikologie und Experimentelle Medizin, Hannover, Germany) and it was dispersed into the nozzle with pressurized air (20 L/min; 5 bars). The mice were exposed at a slightly negative pressure in the exposure chamber between 07:30 and 14:30 h. The high dose-rate and short exposure time were chosen to avoid unnecessary stressing of the dams during gestation. We chose a relatively high dose

Table I. Key physico-chemical characteristics of Printex 90.

Declared particle size	14 nm	Degussa-Hüls
Geometric mean size	65 nm (carbon spheres)	(Saber et al. 2005)
Morphology	Individual carbon black spheres mainly occurred in open-structured long chain aggregates and fewer large dense aggregates	(Saber et al. 2011)
Particle size distribution	The aggregates cover a wide size-range from <100 nm to 20–30 μm; the typical aggregate size is approximately 200 nm	(Saber et al. 2011)
Surface area	295–338 m^2/g	(Saber et al. 2005) (Jacobsen et al. 2008b)
Pycnometric particle density	2.1 g/cm^3	(Saber et al. 2005)
Chemical composition	99% C, 0.8% N and 0.01% H_2	(Jacobsen et al. 2008b)
The total PAH content (Carbon black extract – Soxhlet)	0.0742 μg/g	(Jacobsen et al. 2007)
The total PAH content (DEP extract – NIST SRM 1650)	216 μg/g	(Jacobsen et al. 2008a)

PAH, polycyclic aromatic hydrocarbon (data included for comparison); DEP, diesel extract particles.

because there are virtually no data on the developmental toxicity of nanoparticles. Still, the dose used (1 h exposure to 42 mg Printex 90/m^3) corresponds to only one-and-a-half day exposure that Danish workers might experience at the time-weighted average occupational exposure limit (3.5 mg/m^3 for carbon black) (The Danish Working Environment Authority 2007).

Instillation. The particle preparation and instillation procedures were described previously (Jackson et al. 2011). Printex 90 was sonicated for 8 min (10 s pulses and 10 s pauses, total sonication time 4 min) at a concentration of 1.675 mg/mL (67 μg/instillation) in 0.2 μm filtered, γ-irradiated Nanopure Diamond UV water (Pyrogens: < 0.001 EU/ml, Total Organic Carbon: < 3.0 ppb), using a 400 W Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) mounted with a disruptor horn and operated at 10% amplitude. This dispersion was used for the high dose and diluted 1:5 for the medium dose (13.4 μg/instillation) and diluted futher 1:5 for the low dose (2.7 μg/instillation). Eighty time-mated mice were anesthetized with 3% Isoflurane and instilled with a vehicle or one of the three concentrations of Printex 90 dispersions (40 μL solution followed by 160 μL air) on GD 7, 10, 15 and 18. We chose to instill Printex 90 at times that would cover the major part of the fetal development. We tried to distribute the dose over that period assuming that a fraction of the particles would have been cleared rapidly, but that much of the dose would remain in the lungs for several weeks. Exposure took place between 08:30 and 14:30 h. Time-mated mice were instilled in different order each day, to reduce any variation that might be related to the time of exposure. The total instilled doses were 11, 54 and 268 μg/animal.

Exposure control and characterization

Inhalation. Total aerosolized Printex 90 was sampled periodically from the exposure chamber using Millipore cassettes mounted with Millipore Fluoropore Filters (diameter 2.5 cm, pore size 0.45 μm). Filters were weighed immediately on a Sartorius Microscale (Type M3P). If needed, the airborne mass concentration was adjusted after the control measurement to the target concentration of 40 mg/m^3.

The particle concentration-size-distribution was monitored on-line using a GRIMM Sequential (Stepping) Mobility Particle Sizer (Model No. 5.521) connected to a Condensation Particle Counter system (SMPS+C) and a GRIMM Dust Monitor (Model 1.105) for small (9.8–492.2 nm) and coarse particles (0.75–1.00 to >15 μm), respectively. The SMPS+C system was operated in fast scan mode (*ca*. 3 min and 40 s per spectrum) using correction for particle density and Stokes settling. The Dust Monitor collected data at a resolution of 6 s. The SMPS data were quality controlled omitting spectra collected during larger rapid concentration changes, which occurred during adjustments of exposure concentrations and results in false size distribution spectra.

Instillation. The particle size distribution in the Printex 90 dispersions was determined with a 633 nm He-Ne Dynamic Laser Scatter (DLS) Zetasizer nano ZS (Malvern Inc., UK). Data were analyzed using the Dispersion Technology Software (DTS) vs. 5.0 (Malvern Instruments Ltd). Samples were measured at 25°C in 1 mL disposable polystyrene cuvettes. For calculations of hydrodynamic size, we used the refractive (R_i) and absorption indices (R_s) of 2.020 and 2.00, respectively, for Printex 90 and standard properties for H_2O.

The dispersion of Printex 90 instillation fluid was also analyzed by Scanning Electron Microscopy (QUANTA 200 FEG MKII with EDX). Samples were prepared by placing one drop of the dispersions onto holey carbon-coated TEM Cu-grids (200 mesh) placed on filter paper in a Petri dish for quick absorption of liquid. The prepared Cu-filters were allowed to dry under a tilted lid in a HEPA-filtered LAF-bench (Microflow Advanced Biosafety Cabinet (ABS) Class II; now Bioquell Ltd, Hampshire, UK). Samples were transferred to polymer sample vials for storage as individual samples until analysis.

Parturition and lactation

For terminology used see Figure 1. After the last exposure on GD 18, the time-mated mice were housed alone and monitored for birth. The expected day of delivery, GD 20, was assigned as post-natal day zero (PND 0) for the offspring. On PND 1, the offspring were counted and sex determined. Dams and newborns were weighed on PND 2 (inhalation study) and on PND 1 (instillation study). The remaining dams and offspring were also weighed on PND 8 (9), 12, 17 and at weaning on PND 22.

Time-mated mice. On PND 3 (5 days after the last inhalation exposure) and on PND 1–2 (3–4 days after the last instillation), the females were anesthetized with a mixture of Hypnorm-Dormicum and killed by withdrawal of heart blood. Bronchoalveolar lavage (BAL) fluid from each female was collected. The number of uterine implantation sites was determined; organs were dissected, placed in NUNC cryotubes,

snap frozen in liquid N_2 and stored at −80°C until analysis. After weaning, at PND 22–23 (24–25 days after the last inhalation exposure) and PND 24–25 (26–27 days after the last instillation) the dams were killed and treated as described above for females.

Offspring. On PND 2, in the inhalation study, all except two male and two female offspring in each litter were removed and killed by decapitation. In the instillation study, one male and one female in each litter were removed and killed, leaving 3–5 offspring for further investigations. From the newborns, liver and lungs were dissected, placed in NUNC cryotubes, snap frozen in liquid N_2 and stored at −80°C until analysis.

On PND 22, male and female offspring were randomly distributed into balanced experimental groups: A group for collection of organs at weaning PND 22–23, an adolescent group for maturation data and organs at PND 50 (47), and a group for behavioural testing and mating for a 2nd generation (to be published elsewhere). In the inhalation study all dissected organs were weighed. This included lungs and liver from the newborns; and lungs, liver, kidneys, spleen, heart and brain from the females, dams, offspring at weaning and adolescents. In the installation study only thymus of newborns and offspring at weaning was weighed, other organs were rapidly frozen to preserve the tissue quality. Relative organ weight was calculated as (organ weight/bodyweight)*100.

No time-mated mice or offspring died as a result of particle exposure. However, some time-mated mice were lost during the instillation study apparently due to other causes: One time-mated mouse assigned to the control group died before the start of the exposure; four time-mated mice from the low dose group and two time-mated mice from the medium dose group died during instillation. Data from these mice were excluded from the study. One control, one low dose, three medium dose, and five high dose dams were also lost due to spontaneous acute intestinal pseudo-obstruction, commonly observed in lactating C57B1/6 mice (described in Percy and Barthold 2001). Offspring of these dams were also killed immediately. Since the cause of this disease was related to lactation only, the gestation data and newborns data on PND 2 were included in the study.

BAL preparation and analyses

BAL was collected under hypnorm-dormicum anaesthesia by washing lungs four times with 0.8 mL 0.9% sterile saline through the trachea. The BAL was immediately put on ice until BAL fluid and BAL cells were separated by centrifugation at 4°C and 400 *g* for 10 min. The BAL cells were re-suspended in 100 µL medium (HAM F-12 with 10% fetal bovine serum and 1% penicillin-streptomycin). The number of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were determined in 40 µL re-suspension by counting 200 cells prepared and analyzed as described (Jackson et al. 2011). Counts are presented relative to the total cell number in the BAL fluid. The total number of living and dead cells in BAL samples was determined in further diluted suspension (20 µL cells in 180 µL HAM F12 medium with FBS and PS) by counting in a hemocytometer with trypan blue dye (inhalation study samples) or in a NucleoCounter (instillation study samples), following the standard kit procedure (Chemometec, Denmark).

The remaining re-suspension (40 µL) was mixed with 160 µL freezing medium (HAM F-12, 10% FBS, 1% PS, containing 10% DMSO) and stored at −80°C for later comet assay analysis.

Detection of DNA strand breaks

The level of DNA strand breaks in frozen BAL and liver cells was determined by the alkaline comet assay as described in (Dybdahl et al. 2004; Bornholdt et al. 2007) based on a protocol by (McNamee et al. 2000). The strand breaks measured by the assay represent a mixture of direct strand breaks, alkaline labile sites and transient breaks in the DNA due to repair processes (Collins 2009). BAL cell suspensions in freezing medium with 10% DMSO were thawed quickly at 37°C. For liver, deep frozen samples (*ca*. 40 mg) were pressed through a metal stapler (diameter 0.5 cm, mesh size 0.4 mm) into Merchant's media (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM NaEDTA, pH 7.4) for inhibition of endogenous DNA cleaving enzymes (Brunborg et al. 1996). Samples were rapidly embedded in agarose moulded onto a hydrophilic polyester film, a GelBond® film (Lonza Rockland Inc. ME, USA), which was then quickly immersed into lysing solution at 4°C. Samples were alkaline treated and subjected to alkaline electrophoresis (pH > 13) at 25 V and current of 292–296 mA for 20 min in circulating electrophoresis solution. The gels were fixed, and later stained with SYBR Gold fluorescent dye (Molecular probes, Denmark; 1:10,000) and 50 randomly selected comets were scored by fluorescent microscopy using Kinetics® image analysing system (version 3.9). DNA damage was quantified as %DNA intensity in the comet tail. Electrophoresis efficiency was validated by including identical H_2O_2 exposed A549 cells as positive controls. In independent experiments the

absolute frequency of lesions (per million base pairs) was calibrated using ionizing radiation (Møller et al. 2004). The primary comet assay endpoints were recalculated to the number of lesions per million base pairs, assuming that one unit increase in the %DNA in the tail corresponds to 0.0554 lesions/10^6bp.

Two different comet methods were used to process the inhalation and instillation samples. Inhalation samples were cast in polyethylene moulds with eight wells (well diameter 19.5 mm with 130 μL per sample). Four films with eight samples each were processed per electrophoresis. The instillation samples were analyzed using a high throughput protocol allowing 48 samples per GelBond® film, developed at Norwegian Institute of Public Health (Gunnar Brunborg and Kristine Bjerve Gutzkow) within the COMICS EU Project. Cell/agarose suspension was dripped with a multichannel pipette onto a GelBond(R) film (7 μL per sample). Eight films were processed per electrophoresis, in two parallel electrophoresis tanks. Due to preparation time, the lysing procedure varied between 1–2 h for samples in the present study (up to 3.5 h). The high volume protocol allowed processing of all related samples on one film and reduced the variation caused by increased processing time and different electrophoreses.

The level of oxidatively damaged DNA in the liver from offspring of dams exposed to Printex 90 by inhalation was also assessed as formamidopyrimidine DNA glycosylase (FPG) (kindly donated by Andrew Collins, Oslo, Norway) enzyme sensitive sites (Folkmann et al. 2007). FPG sites were recalculated to lesions/10^6bp by factor 0.0261.

To prepare liver samples for comet analysis, the inhalation liver samples were cut on dry ice. This procedure was later modifyed such that the samples were crushed in liquid N_2, a method that gives results with smaller variation and reduced background. Instillation liver samples were cut from fresh livers; samples were immediately frozen and not handled until analysis. Strand breaks were reported as lesions/10^6 bp for all experiments. However as the comet analyses were performed in different experiemetnal set-ups and because H_2O_2 exposed controls were used that do not allow direct estimation of the number of induced strand breaks, the levels of strand breaks may not be directly comparable between experiments. Consequently emphasis was put on the comparison within experiments.

Data analyses

The accepted level of statistical significance was 0.05. Litter was considered the statistical unit. Gestational parameters were analyzed by Kruskal-Wallis One-Way Analysis of Variance. Weight data were analyzed by analyses of variance (ANOVA), with treatment as factor, and day of weighing (GD, PND) as repeated measure. Litter size was used as co-variable for weight data during gestation. The number of litters was compared by Fisher's exact test. Remaining data (BAL results, comet assay results, organ weights) were analyzed by analyses of variance (ANOVA), with treatment, day of sampling (PND) and sex (where relevant) as factors. Significant results from overall analyses were analyzed by pair wise comparisons. Data were analyzed separately for each day of sampling and instillation study results were further analyzed by dose in Fisher's Least-Significant-Difference Test. Females sampled 3–5 days after exposure and dams sampled after weaning were compared, even though the groups differed by timing of sampling and also by pregnancy status. The "female" group consisted of pregnant mice with small litters and non-pregnant mice. Pregnancy is reported to alter the level of inflammatory response (Fedulov et al. 2008; Lamoureux et al. 2010), thus different background levels had to be accepted. Analyses were performed on SYSTAT Software Package version 9 and Statistical Tables for PC users.

Results

Particle exposure

Inhalation. Time-mated mice inhaled 42 mg/m^3 (the variation of the dose between groups was 41.73 ± 0.01 mg/m^3) Printex 90 or filtered air for 1 h/day for 11 consecutive days. This would correspond to 12 h at the Danish Occupational Exposure Limit of 3.5 mg/m^3 for carbon black. The particle number concentration in the exposure atmosphere was 4.09 ± 0.03 × 10(6)/cm^3. The average particle size-distribution was multimodal and highly dominated by sub-100 nm particles. The most abundant size number was in the order of 41 nm, which was also the average size (see Figures 2A and 2B). The average size by mass was 310 nm, and the mass size distribution was bimodal with one mode around 290 nm and a coarser mode at *ca.* 1.5 μm (see Figure 2B). Only 5% of the mass was below 100 nm, 83% of the particles were in this ultrafine size range by number.

Based on a deposition model revised from (Jacobsen et al. 2009), 34.8% of the particle mass was expected to deposit in the pulmonary region and 20.1% of particles were expected to deposit in the extra-pulmonary region (11.9% bronchial region, 0.9% trachea, 0.6% larynx, and 6.7% scull). An

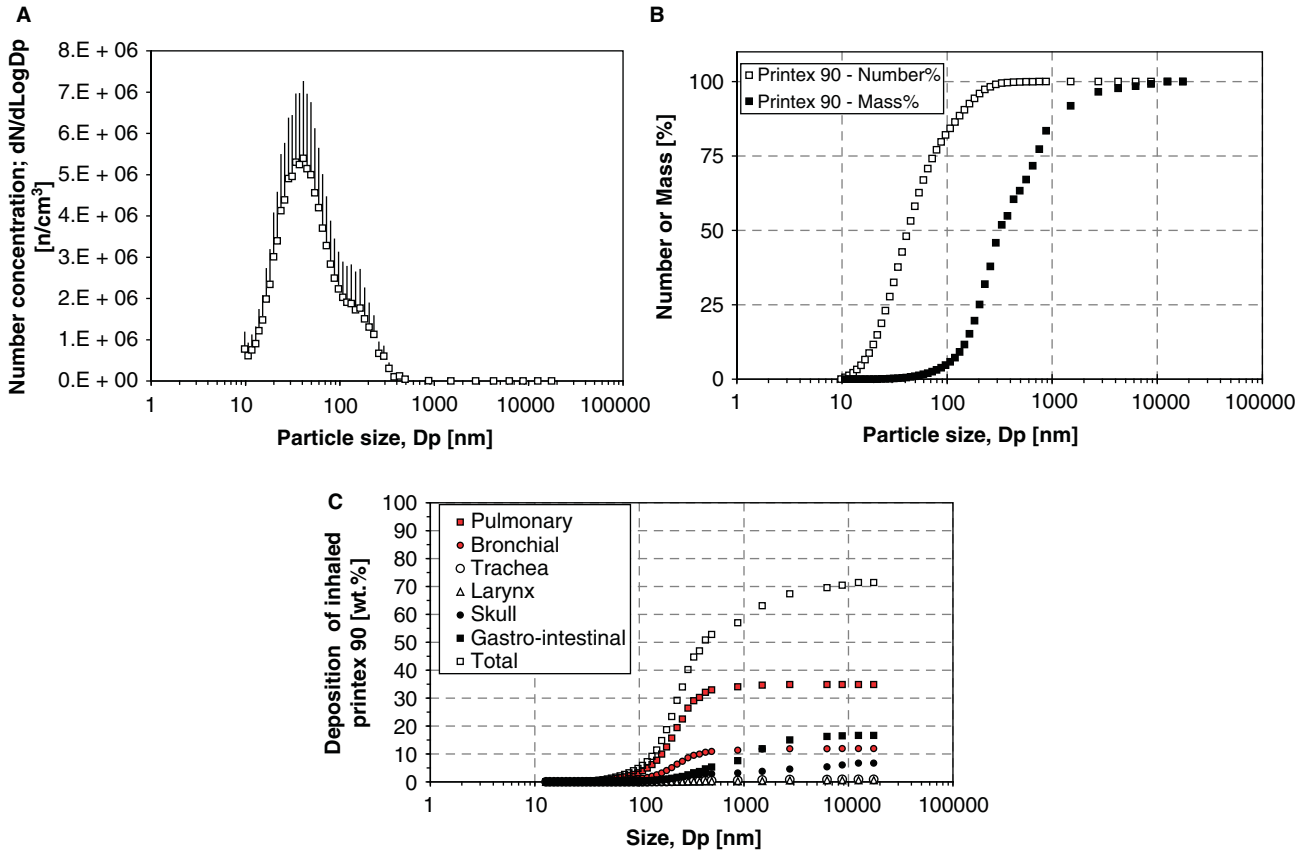


Figure 2. Size distribution data for the inhalation exposure and calculated deposition in mice. (A) Average number size distribution of Printex 90 dust in the inhalation exposure chamber. Error bars are the standard deviation of the different individual exposure runs. (B) Accumulated average number and mass distribution of particles in the exposure chamber. (C) The calculated accumulated particle deposition in the mice using a modified version of the model applied in Jacobsen et al. (2009).

additional 16.6% was expected to deposit in the gastrointestinal tract. The calculated accumulated deposition pattern is presented in Figure 2C. The total inhaled dose was 826 μg Printex 90 (1 h/day × 11 days × 41.7 μg/dm^3 × 1.8 dm^3/h). Based on deposition estimates, the pulmonary dose would be 287 μg/animal; or 13.1 mg/kg, based on an average body weight of 22 g on GD 4 (287 μg/22 g). The corresponding inhaled particle surface area was at least 0.085 m^2/animal (295 m^2/g × 0.000287g/animal); or 3.9 m^2/kg (0.085 m^2/0.022 kg) equal to 243 m^2/kg lung (0.085 m^2/0.00035 kg lung).

In addition to the particles that directly enter the gastrointestinal region, a contribution from the extra-pulmonary region was expected, because particles are removed from the lungs by the mucociliary escalator and are ultimately swallowed. Therefore, up to 36.7% (303 μg/animal) of Printex 90 nanoparticles was expected to enter the animal via the gastrointestinal tract, increasing the final particle mass to 590 μg/animal; or 26.8 mg/kg. In the whole-body exposure used in the present study, particles were deposited on the fur and grooming can therefore be expected to increase the total dose even further.

Instillation. Time-mated mice were instilled four times during gestation on GD 7, 10, 15 and 18 with Printex 90 or vehicle. The instilled dose in the highest dose group 268 μg/animal, 12.2 mg/kg; 0.080 m^2/animal; or 3.6 m^2/kg. The dose was similar to the estimated dose deposited in the pulmonary region, calculated from the estimates in (Jacobsen et al. 2009). The final instillation doses in the lower dose groups were 54 and 11 μg/animal, respectively; 2.5 and 0.5 mg/kg; 0.016 and 0.003 m^2/animal respectively; or 0.72 m^2/kg and 0.15 m^2/kg, respectively. The particle size distribution was similar in the three instilled dispersions with concentrations of 1675, 335 and 67 μg/mL (see Figure 3), and was stable for more than 1 h. The average zeta-size was approximately 140 nm and the hydrodynamic number size-distributions had a peak size between 50 and 60 nm (see Figure 3). When converted to volume-distributions, minor amounts of μm-size particles and two smaller size modes with peak sizes around

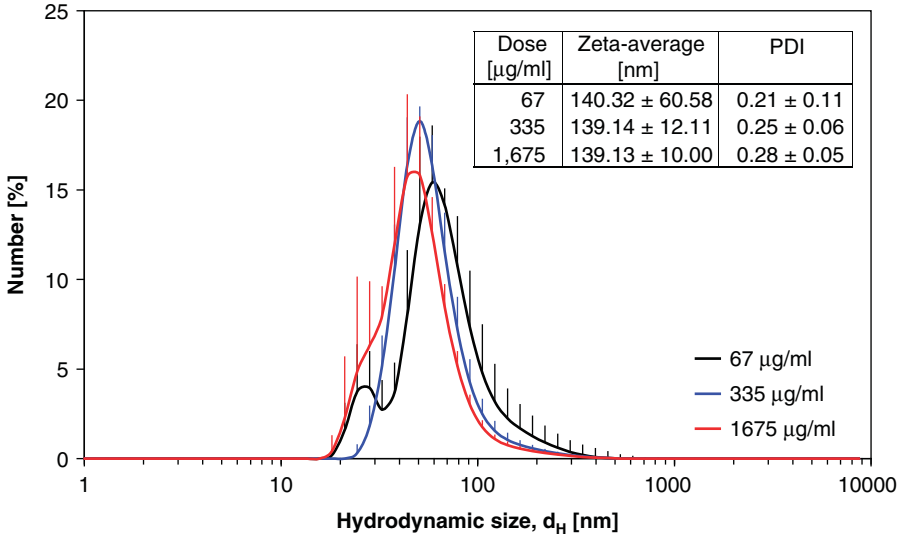


Figure 3. Hydrodynamic size distribution of intratracheally instilled Printex 90 dispersions. Particle size distribution at the three instillation concentrations for the intratracheal instillation exposure measured by Dynamic Light Scattering. Error bars show the standard deviation of six measurements. Inserted table shows the average intensity size and polydispersivity index.

50–60 nm and 200–400 nm were identified. The observed DLS sizes were confirmed by TEM and SEM, with a wide size distribution of nm- to µm-size free and agglomerated particles. The agglomerates consisted of spherical to sub-spherical carbonaceous particles as well as minor amounts of free single primary spheres (see Figures 4A and 4B).

Lung inflammation in the time-mated mice

Analysis of BAL fluid cell composition by differential cell count indicated the presence of inflammation in the lungs of time-mated mice exposed to Printex 90 both by inhalation and instillation (see Table II).

Inhalation. Time-mated mice exposed to Printex 90 by inhalation had more neutrophils in BAL fluid compared to their controls 5 and 24 days after exposure (5 days: 11.4-fold increase, $p = 0.008$; 24 days: 11.6-fold increase, $p < 0.001$). Females exposed to Printex 90 by inhalation had more lymphocytes in BAL fluid 5 days after exposure (3.4-fold increase, $p = 0.020$) and total cell counts were higher at both time-points (5 days: 1.5-fold increase, $p = 0.032$; 24 days: 1.2-fold increase, $p = 0.057$).

Instillation. Change in the counting method caused a sustained increase in all types of cells in BAL, except epithelial cells, when comparing the instillation and inhalation control groups. It is possible that some

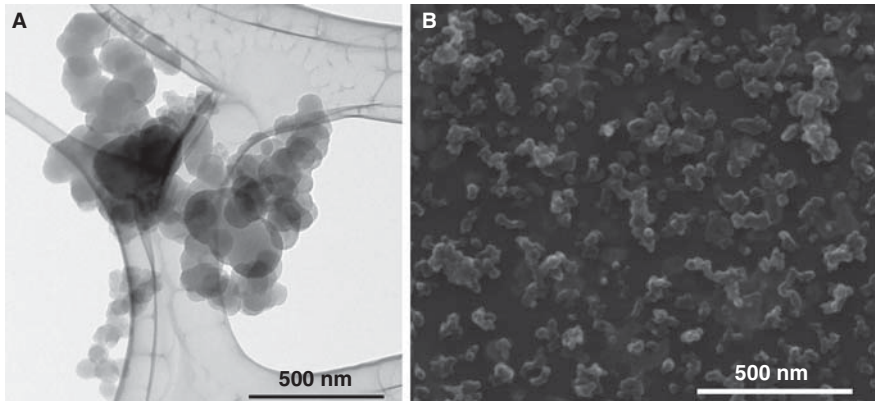


Figure 4. Printex 90 intratracheal instillation exposure characterization by TEM and SEM. (A) Transmission Electron Micrograph of agglomerated small- and medium size Printex 90 aggregates (67 µg/mL). (B) Scanning Electron Micrograph illustrating the overall size and morphologies of Printex 90 aggregates and agglomerates in the dispersions (1.675 µg/mL).

Table II. BAL cell composition of females or dams exposed to Printex 90 by inhalation or intratracheal instillation and control mice.

	Inhalation		Instillation			
	Control	Printex 90 41.7 mg/m^3 ~ 287 µg/animal	Control	Printex 90 11 µg/animal	Printex 90 54 µg/animal	Printex 90 268 µg/animal
Females 3–5 days after exposure						
Total count	66.07 ± 6.03	96.88 ± 10.72*	168.00 ± 45.03	175.50 ± 15.56	200.40 ± 19.22	292.00 ± 54.15
Dead cells	1.08 ± 0.36	0.79 ± 0.32	14.00 ± 7.21	12.50 ± 4.27	11.20 ± 5.24	18.67 ± 5.93
Macrophages	56.35 ± 4.76	69.21 ± 6.74	139.74 ± 42.31	128.79 ± 12.01	154.45 ± 20.45	115.51 ± 22.57
Neutrophils	1.01 ± 0.61	11.47 ± 3.06**	3.69 ± 2.08	5.50 ± 1.68	9.32 ± 4.75	105.75 ± 26.19***
Lymphocytes	0.76 ± 0.18	2.59 ± 0.63*	3.50 ± 0.95	7.18 ± 3.12	7.22 ± 1.52	27.03 ± 9.68**
Eosinophils	0.46 ± 0.31	3.97 ± 2.58	5.92 ± 3.66	22.24 ± 10.04	11.37 ± 6.37	33.04 ± 9.07
Epithelial cells	7.50 ± 1.28	9.63 ± 1.61	15.15 ± 4.22	11.80 ± 2.73	18.04 ± 3.43	10.67 ± 3.77
Dams at weaning 24–27 days after exposure						
Total count	44.08 ± 3.37	54.81 ± 4.26(*)	137.25 ± 5.64	93.43 ± 8.27	154.50 ± 10.80	361.29 ± 43.40***
Dead cells	0.68 ± 0.10	0.45 ± 0.11	6.88 ± 1.94	3.43 ± 2.26	4.50 ± 2.23	19.43 ± 2.75***
Macrophages	31.90 ± 3.25	37.73 ± 3.20	113.68 ± 4.91	82.98 ± 7.50	128.78 ± 9.50	131.79 ± 16.44
Neutrophils	0.41 ± 0.07	4.82 ± 1.07***	2.85 ± 0.90	0.90 ± 0.16	5.23 ± 1.65	173.26 ± 22.66***
Lymphocytes	1.35 ± 0.34	2.02 ± 0.50	7.71 ± 2.14	3.38 ± 0.67	6.94 ± 1.10	34.12 ± 8.38***
Eosinophils	0.37 ± 0.29	0.67 ± 0.47	2.26 ± 0.97	0.33 ± 0.16	2.84 ± 2.28	1.29 ± 0.42
Epithelial cells	10.03 ± 0.85	9.56 ± 0.80	10.76 ± 1.77	5.83 ± 1.18	10.71 ± 1.09	20.72 ± 4.53

BAL, bronchoalveolar lavage. Data presented as mean cell number × 10^3 in BAL ± SEM. (*)$p \sim 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

increase is also due to the instillation procedure, the contribution is however minor (Jackson et al. 2011). The time-mated mice exposed to Printex 90 by instillation also had more neutrophils in the BAL 3 and 26 days after exposure compared to the instilled control animals. This was statistically significant in the high dose group only (3 days: 28.7-fold increase, $p < 0.001$; 26 days: 60.9-fold increase, $p < 0.001$). Also, more lymphocytes were observed 5 and 26 days after exposure in the high dose group (3 days: 7.7-fold increase, $p = 0.005$; 26 days: 4.4-fold increase, $p < 0.001$). Printex 90 instilled dams had more total cell counts and more dead cells in BAL fluid 26 days after exposure in the high dose group (total cell count increased 2.6-fold, $p < 0.001$; dead cells increased 2.8-fold, $p < 0.001$).

DNA strand breaks

DNA strand breaks were evaluated by the Comet assay in time-mated mice BAL cells and liver cells, and in liver cells of offspring (see Table III).

Inhalation

Time-mated mice. Inhalation of Printex 90 did not affect the level of DNA strand breaks in BAL fluid cells 5 and 24 days after exposure in the exposed time-mated mice compared to their controls ($p = 0.20$). Exposure induced higher levels of DNA strand breaks in the liver 5 and 24 days after exposure in the time-mated mice compared to their controls (5 days: 1.3-fold increase, $p = 0.04$; 24 days: 1.6-fold increase, $p < 0.001$).

Offspring. In the offspring exposed to Printex 90 by maternal inhalation exposure, the level of DNA strand breaks was higher in offspring liver at weaning and in adolescents, compared to their controls (weaning: 1.4-fold increase, $p = 0.001$; adolescents: 1.5-fold increase, $p = 0.011$). Overall, newborns displayed higher levels of DNA strand breaks in liver tissues compared to tissues from the older offspring at weaning and from adolescents, both in the Printex 90 and the control group ($p < 0.001$). Each data point represents an average value of two separate comet assay runs.

The level of oxidatively generated DNA damage in the liver of offspring from the inhalation study was also determined by the level of formamidopyrimidine DNA glycosylase (FPG) enzyme sensitive sites. There was no consistent increase in oxidatively generated DNA damage in the offspring liver cells in newborns, at weaning or in adolescents (newborn exposed 0.91 ± 0.27 vs. control 0.71 ± 0.20; weaning exposed 1.05 ± 0.12 vs. control 1.28 ± 0.13; adolescents exposed 0.87 ± 0.10 vs. control 1.20 ± 0.11;

Table III. Level of DNA strand breaks for females, dams or offspring exposed to Printex 90 by inhalation or intratracheal instillation and control mice.

	Inhalation		Instillation			
	Control	Printex 90 41.7 mg/m^3 ~ 287 μg/animal	Control	Printex 90 11 μg/animal	Printex 90 54 μg/animal	Printex 90 268 μg/animal
Females 3–5 days after exposure						
BAL	0.55 ± 0.06	0.70 ± 0.10	0.90 ± 0.04	0.91 ± 0.09	0.78 ± 0.07	0.72 ± 0.07
Liver	2.57 ± 0.19	3.25 ± 0.21*	0.66 ± 0.06	0.55 ± 0.07	0.57 ± 0.07	0.52 ± 0.07
Dams at weaning 24–27 days after exposure						
BAL	0.58 ± 0.05	0.60 ± 0.04	0.62 ± 0.02	0.69 ± 0.06	0.58 ± 0.01	0.50 ± 0.03**
Liver	1.24 ± 0.07	1.94 ± 0.11***	0.52 ± 0.05	0.55 ± 0.09	0.48 ± 0.02	0.58 ± 0.04
Offspring						
Liver newborns PND 2	3.50 ± 0.28	3.64 ± 0.37	0.66 ± 0.02	0.65 ± 0.03	0.73 ± 0.02	0.64 ± 0.03
Liver offspring at weaning PND 22–23	1.13 ± 0.09	1.56 ± 0.08***	0.39 ± 0.03	0.41 ± 0.04	0.42 ± 0.04	0.42 ± 0.03
Liver adolescents PND 50 (47)	1.10 ± 0.16	1.69 ± 0.14**	0.54 ± 0.04	0.48 ± 0.04	0.47 ± 0.04	0.52 ± 0.03

BAL, bronchoalveolar lavage; PND, post natal day (days after birth). Data are presented as mean number of lesions per 10^6 base pairs ± SEM (calculated from %DNA results). Offspring data are calculated as litter average, when sibling liver tissues were analyzed at the same collection point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

two-way ANOVA $p = 0.60$; all data are presented as lesions per 10^6 base pairs) (data not graphically shown).

Instillation

Time-mated mice. Intratracheal instillation of Printex 90 did not affect the level of DNA strand breaks in BAL cells in the females ($p = 0.30$), while the dams exposed by instillation had significantly less DNA strand breaks in BAL cells in the high dose group 26 days after exposure compared to control dams (20% reduction, $p = 0.007$). No increase in the level of DNA strand breaks was observed in the liver of time-mated mice exposed to Printex 90 by instillation compared to their controls (two-way ANOVA $p = 0.85$). Each data point represents an average of duplicate scored in two separate rounds.

Offspring. In the offspring exposed to Printex 90 by maternal intratracheal instillation, the level of DNA strand breaks in liver cells was comparable to their controls ($p = 0.8$). Interestingly, as we also observed in the inhalation study, the level of DNA strand breaks was generally higher in liver cells from newborns, compared to tissues from older siblings at later time points ($p < 0.001$).

Maternal and litter parameters

Gestational and litter parameters in exposed dams and their offspring were similar to controls both after inhalation or instillation of Printex 90 (weight gain during gestation and lactation, gestation length, offspring weight at birth, during lactation and maturation, litter size, gender ratio, number of implantations, and postnatal viability; see Table IV).

Organ weights

Inhalation. A higher relative brain weight was found in time-mated mice 5 days after exposure to Printex 90 by inhalation, compared to their controls (exposed 2.09 ± 0.04% vs. control 1.86 ± 0.06%, $p = 0.005$). At weaning, relative lung weight was higher in exposed compared to control dams (exposed 1.20 ± 0.03% vs. control 1.10 ± 0.02%, $p = 0.005$). Other organs did not differ.

Organ weights in offspring of dams exposed to Printex 90 were similar to their controls, except that the exposed female offspring had smaller relative heart weight at weaning (exposed 0.68 ± 0.01% vs. control 0.71 ± 0.01%, $p = 0.052$). Furthermore, exposed adolescent males had a higher relative weight of testes (exposed 2.10 ± 0.05% vs. controls 1.58 ± 0.20%, $p = 0.024$).

Instillation. The thymus weight of exposed newborn and weaned offspring was similar to their controls. Other organs were not weighed.

Discussion

Printex 90 carbon black is one of the best studied materials in particle toxicology. On one hand, it is

Table IV. Gestation, lactation and developmental parameters of dams and offspring exposed to Printex 90 by inhalation or intratracheal instillation and control mice.

	Inhalation		Instillation			
	Control	Printex 90 41.7 mg/m^3 ~ 287 µg/animal	Control	Printex 90 11 µg/animal	Printex 90 54 µg/animal	Printex 90 268 µg/animal
Time mated/exposure groups	22	22	24	17	17	22
Dam arrival weight, GD 4 (g)	22.21 ± 1.47	21.82 ± 1.27	23.01 ± 1.33	22.37 ± 1.20	22.59 ± 1.03	22.87 ± 0.89
Number of litters PND 1	18	17	20 (-1[†])	10 (-4[†])	11 (-2[†])	20
Dam weight gain, GD 7–18 (g)[‡]	11.25 ± 0.58	10.64 ± 0.71	11.94 ± 0.33	12.00 ± 0.73	11.00 ± 0.61	10.79 ± 0.36
Dam lactation weight gain PND 1(2)–17 (g)	4.31 ± 0.27	4.34 ± 0.41	4.40 ± 0.43	4.38 ± 0.81	4.85 ± 0.45	4.93 ± 0.46
Gestation length (days)	19.89 ± 0.07	20.06 ± 0.06	20.00 ± 0.00	20.00 ± 0.00	20.09 ± 0.09	20.05 ± 0.05
Implantations	7.39 ± 0.56	7.59 ± 0.54	9.25 ± 0.21	9.27 ± 0.57	8.08 ± 0.62	8.32 ± 0.46
Implantation loss (%)	14.59 ± 3.88	17.01 ± 4.57	21.52 ± 2.52	30.74 ± 8.42	25.61 ± 7.75	24.16 ± 3.70
Live pups per litter PND 1	5.09 ± 0.91	4.95 ± 0.74	7.30 ± 0.34	7.00 ± 0.67	6.82 ± 0.52	6.20 ± 0.34
Offspring dead during lactation (%)	4.10 ± 1.98	1.72 ± 1.19	5.49 ± 2.06	10.00 ± 10.00	1.30 ± 1.30	3.26 ± 1.94
Birth weight females (g)	1.40 ± 0.04	1.41 ± 0.05	1.33 ± 0.03	1.30 ± 0.03	1.31 ± 0.04	1.30 ± 0.03
Birth weight males (g)	1.43 ± 0.03	1.42 ± 0.05	1.35 ± 0.02	1.38 ± 0.04	1.36 ± 0.04	1.35 ± 0.04
Weight gain females PND 1(2)–22 (g)	8.02 ± 0.23	7.66 ± 0.32	6.37 ± 0.29	7.34 ± 0.47	6.96 ± 0.45	7.50 ± 0.29
Weight gain males PND 1(2)–22 (g)	8.28 ± 0.18	7.89 ± 0.38	7.12 ± 0.32	7.33 ± 0.62	7.86 ± 0.60	7.86 ± 0.24
Sex ratio[§]	0.42 ± 0.06	0.51 ± 0.06	0.46 ± 0.04	0.66 ± 0.06	0.56 ± 0.06	0.45 ± 0.04

GD, gestation day (pregnancy day); PND, post natal day (days after birth). Dams were allowed to deliver their offspring on gestation day (GD) 20, equal to post natal day (PND) 0. Weights of dams and individual offspring were recorded on PND 2 (1), and offspring were counted and sex determined. Time mated mice were examined for the number of implantation sites, allowing for calculation of implantation loss. Females that did not give birth or had small litters were killed on PND 3 (1–2) and the dams on PND 22–23 (24–25). Data are expressed as mean ± SEM, offspring data are calculated as litter average. [†]Died during instillation. [‡]Weight before exposure. [§]Females in litter (%).

considered to be a low-toxicity insoluble material, but on the other hand, it is a potent generator of reactive oxygen species (Jacobsen et al. 2008b), it induces DNA strand breaks and oxidatively generated DNA damage (Jacobsen et al. 2008b, 2009), and it is mutagenic (Jacobsen et al. 2007, 2010). Moreover, carbon black induces tumors in rats (Mohr et al. 2006) and is possibly carcinogenic to humans (Baan et al. 2006). Physically and chemically Printex 90 resembles carbonaceous cores of diesel engine combustion particles. Because it is engineered to have nanosize (i.e., the primary particles are smaller than 100 nm), Printex 90 is therefore a representative of an engineered carbonaceous nanoparticles. Although developmental effects of particulate air pollution have been reported in the offspring of human subjects, very few experimental mechanistic studies are available.

We found that maternal inhalation exposure to Printex 90 induced DNA strand breaks in the liver of time-mated mice and in the offspring even weeks after the end of exposure. There were no changes in the levels of DNA strand breaks in mice intratracheally instilled with a similar pulmonary dose. Despite this, we did not observe any gestational or developmental toxicity in the offspring.

Effects in time-mated mice

The mother is the route of exposure for the offspring exposed to xenobiotics *in utero*. The effects of maternal pulmonary exposure to Printex 90 were assessed at two time points in the time-mated mice. Females with few or no offspring were used to evaluate the early effects of exposure, while dams were examined at the end of lactation, at weaning. To make inhalation and instillation comparable, we estimated the dose deposited in the pulmonary region by inhalation, and instilled a similar dose to the highest of three instilled doses (268 µg/animal). As expected, we observed a massive influx of neutrophils in the BAL fluid of exposed females and dams, which persisted for 24–27 days after the end of exposure. After inhalation of Printex 90, the pulmonary inflammation (by polymorphonuclear neutrophil infiltration) in time-mated mice was of similar magnitude as in the mice instilled with the medium dose. We and others have previously found that instilled particles induce

stronger inflammatory responses in the lung compared to inhaled particles (Osier and Oberdörster 1997; Driscoll et al. 2000; Jacobsen et al. 2009). This may be because a greater fraction of the instilled particles is deposited deeper into the lung and, consequently, is cleared more slowly.

Generally, translocation of nanoparticles from the lung into circulation is considered to be slow, and it has been reported that only a fraction of a percent gets beyond the lung cavity and regional lymph nodes (van Ravenzwaay et al. 2008; Kreyling et al. 2009; Sadauskas et al. 2009b). In addition, insoluble nanoparticles do not seem to readily pass over the gastrointestinal mucosa in rodents (Carr et al. 1996; Kreyling et al. 2002, 2009). Once in circulation, the distribution to the fetus also seems to be very small (Takahashi and Matsuoka 1981; Myllynen et al. 2008; Wick et al. 2010). However, it is likely that this differs much depending on size, surface and other properties.

We observed DNA strand breaks in liver cells of the exposed time-mated mice and the offspring after inhalation exposure. The exposure procedure is a key determinant for particle size-distribution and consequently for deposition and uptake (Landsiedel et al. 2008). Most Printex 90 particles that would reach the circulation are expected to accumulate in the liver with possible ROS-induced primary genotoxicity. Nanoparticles may persist in the Kupffer cells of the liver for months (Oberdörster et al. 2002; Sadauskas et al. 2007, 2009a). Consequently, only a few liver cells would be directly exposed to ROS generated from Printex 90. Pulmonary exposure to Printex 90 resulted in pulmonary production of cytokines (Saber et al. 2005), but no liver inflammation or acute phase response was found in liver after four consecutive nose-only inhalation exposures to Printex 90 or diesel exhaust particles (NIST) (Saber et al. 2009). Thus, it is unlikely that the observed DNA strand breaks are caused by liver inflammation induced by pulmonary exposure. It is also unlikely that the DNA strand breaks were caused by circulating cytokines, because we observed the strongest pulmonary inflammation in instillation exposed mice. Inhalation exposure results in a larger immediate exposure to the gastrointestinal tract, because inhaled nanoparticles deposited in the extra-pulmonary region are transported up by mucociliary transport and swallowed. The time-mated mice were exposed by whole-body inhalation exposure and therefore it can be expected that they received even a greater dose in the gastrointestinal tract due to fur grooming. Intra-gastric exposure to 0.64 mg/kg Printex 90 induced DNA damage in the liver of rats 24 h after exposure, whereas the same dose administered by intratracheal instillation caused no DNA damage in the liver or lung (Danielsen et al. 2010). Similarly, intra-gastric administration of other carbonaceous nanoparticles (such as single-wall carbon nanotubes, C_{60} fullerenes and diesel exhaust particles) at the same or even lower doses, caused DNA base oxidation damage in the liver and lung of rats (Danielsen et al. 2008; Folkmann et al. 2009). Therefore, the observed DNA damage in the liver may be a result of the inhalation-associated gastrointestinal exposure rather than from exposure in the lungs.

Effects in the offspring

The background level of DNA strand breaks was higher in newborns compared to older siblings. These DNA strand breaks might be related to a high proliferation rate during tissue maturation and/or the naturally occurring high level of oxidative stress at birth (Randerath et al. 1996; Cindrova-Davies et al. 2007; McArt et al. 2010). This may have reduced the sensitivity of the comet assay to detect differences between the exposure groups.

A few molecular genotoxins have been demonstrated to pass from the mother to the fetus and generate DNA damage in fetal tissues (Brunborg et al. 1996; Tripathi et al. 2008). However, we expect that only a small fraction of Printex 90 particles can translocate from the lungs of the mothers to the fetuses because the particles would have to pass two compartmental barriers, i.e., in the lung and placenta. The observed effects of *in utero* exposure are therefore more likely due to changes in signalling cascades. It is possible that inflammatory molecules are transferred from the maternal to the fetal compartment (Jonakait 2007) and affect the fetus. Thus, the increased levels of DNA strand breaks in liver tissue of the offspring may be caused by maternally induced inflammatory mediators after Printex 90 inhalation exposure.

DNA strand breaks in offspring liver of the inhalation exposed dams were still evident in 50-day old offspring. At this time, the offspring were independently fed and had no contact with the dams. Therefore, it is unlikely that secondary genotoxicity caused by inflammatory signalling from the dams caused the observed DNA strand breaks in the older offspring.

Neither inhalation nor instillation of Printex 90 affected gestational or lactational parameters, and offspring of exposed dams survived and developed similarly to control offspring. This is in agreement with findings in two other published studies; an instillation study of carbon nanoparticles (200 μg/mouse on gestational days 7 and 14) (Yoshida et al. 2010) and a study of TiO_2 from our laboratory using a

set-up similar to the present inhalation exposure (Hougaard et al. 2010), suggesting that these inhaled nanosize particles are not toxic during development.

Human exposure to air pollution has been associated with adverse effects *in utero* exhibited by reduced growth, increased mortality and increased risk of perinatal diseases (Dejmek et al. 1999; Lacasana et al. 2005; Šrám et al. 2005; Sláma et al. 2007; Brauer et al. 2008; Pedersen et al. 2009; Pope et al. 2010). Children born and raised in areas with high air pollution have systemic inflammation and increased levels of urinary 8-oxodeoxyguanosine, a marker of oxidative damage to DNA (Calderon-Garciduenas et al. 2008; Švecová et al. 2009). Our data indicate that inhalation exposure to carbon black Printex 90 may have long-lasting genotoxic effects on the exposed organism.

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PAPER VI

Prenatal exposure to carbon black (Printex 90):
Effects on sexual development and neurofunction.

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Prenatal Exposure to Carbon Black (Printex 90): Effects on Sexual Development and Neurofunction

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Abstract: Maternal pulmonary exposure to ultrafine particles during pregnancy may affect the health of the child. Developmental toxicity of carbon black (Printex 90) nanoparticles was evaluated in a mouse model. Time-mated mice were intratracheally instilled with Printex 90 dispersed in Millipore water on gestation days (GD) 7, 10, 15 and 18, with total doses of 11, 54 and 268 μg Printex 90/animal. The female offspring prenatally exposed to 268 μg Printex 90/animal displayed altered habituation pattern during the Open field test.

Maternal exposure to particulate matter in the air is associated with negative birth outcomes (reviewed in [1]), and several underlying mechanisms including oxidative stress and pulmonary inflammation have been suggested [2,3]. Prenatal and post-natal exposure to industrial or traffic-related air pollution have been linked with adverse effects on cognitive development [4,5], and it has been reported in several animal studies that exposure to diesel exhaust causes pathological changes in the brain and altered neurofunction [6–9]. Air pollutants are further linked to effects on sperm quality in men [10], and prenatal exposure to diesel exhaust has endocrine-disrupting properties in animal models [8,11–14]. Ambient air pollution and diesel exhaust are complex mixtures, and especially, ultrafine particles are suspected to have negative health effects [15]. Nanotechnology introduces a new source of exposure to airborne ultrafine particles [16]. Little is known about effects of nanoparticle exposure during prenatal life. It is possible that uncontrolled release of engineered nanoparticles may predispose humans to disease.

During maternal pulmonary exposure to nanoparticles, the majority of nanoparticles deposit deep in the airways and clear away slowly. Lungs of time-mated females exposed to surface-coated nanosized titanium dioxide particles (UV-Titan) by inhalation still contained 24% and 21% of the predicted pulmonary deposition 5 and 26–27 days after exposure, respectively [17,18]. A small fraction of nanoparticles crosses beyond the lung cavity [19] and a few may cross the placental barrier [20,21], depending on the nanoparticle size and properties. Nanoparticles have been found in the brain and testes of male offspring 6 weeks after prenatal exposure [22].

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We have previously reported that pulmonary exposure to nanoparticles, carbon black (Printex 90) or titanium dioxide (UV-Titan), during gestation, did not significantly affect traditional gestational parameters. However, the exposures did induce persistent maternal lung inflammation [17,23] and acute phase response in the lungs of exposed females [24]. These findings suggest that maternal pulmonary exposure to nanoparticles induces a pathological state in the mother, lasting throughout gestation and lactation. Maternal inflammatory cytokines can pass through the placenta and affect the developing brain [25,26]. We have assessed whether maternal pulmonary exposure to Printex 90 nanoparticles affects sexual development and neurofunction in the prenatally exposed offspring, similar to previously published studies [22,27–29].

Materials and Methods

The particles, exposure monitoring and exposure were described previously [23]. As summarized in [23], carbon black is a relatively clean particle, with <1% organic and inorganic impurities, and it has intrinsic potential to generate reactive oxygen species, is inflammogenic and mutagenic and was recently classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans. Measured in the Printex 90 particle suspensions for exposure, the average zeta-size was ~140 nm and the hydrodynamic number size distribution had the major mode at 50–60 nm. Printex 90 particle suspension was also analysed by the Limulus Amebocyte Lysate (LAL) Test for endotoxins, and the levels in the high-dose particle suspension (0.14 EU/mg Printex 90) were below the endotoxin tolerance limit for human and veterinary drugs (5 EU/kg BW/hr or 330 pg/kg BW/hr). A single high-dose instillation was calculated to contain 0.4 EU/kg BW or 28.9 pg/kg BW (Anne Mette Madsen, personal communication).

Eighty time-mated, nulliparous, young adult mice (C57BL/6BomTac; Taconic Europe, Ejby, Denmark) were received on gestation day 3 (GD 3) and housed under controlled conditions [17]. The chosen mouse strain is extensively used in nanotoxicology and therefore allows for interstudy comparison. On GD 7, 10, 15 and 18, the mice

were anaesthetized with 3% Isoflurane and intratracheally instilled as described [30] with vehicle Nanopure water (0.2-μm-filtered γ-irradiated Nanopure Diamond UV water filtered by Thermo Scientific Barnstead Nanopure purification system, Copenhagen, Denmark) or carbon black (Printex 90, Degussa-Hüls, Frankfurt, Germany) dispersed in Nanopure water (total doses 11, 54 or 268 μg/animal). The highest instilled dose was similar to the estimated pulmonary deposition of Printex 90 in a previous inhalation study with daily exposure 42 mg Printex 90/m^3 for 1 hr/day on GD 8–18 and daily exposure corresponding to 12 hr at the time weight average Danish occupational exposure level for carbon black 3.5 mg/m^3 [23]. After the last exposure on GD 18, the time-mated mice were housed alone and monitored for birth. The expected day of delivery, GD 20, was assigned as post-natal day zero (PND 0) for the offspring. Maternal inflammation was assessed by monitoring neutrophil influx in the lungs as described [23]. At weaning, offspring were randomly distributed into balanced experimental groups with one female and one male per litter (where possible): a group for collection of organs on PND 23 (data presented elsewhere), a group for sexual development data (all dose groups) and a group for behavioural testing (control and 268 μg Printex 90/animal). Females and males were housed separately in cages of four or five (extra animals were added when needed).

Anogenital distance (AGD) was measured with a slide gauge in all offspring at weaning. Relative AGD was calculated (AGD/cube root of body-weight) [31]. Results are reported as litter average separately for female and male offspring. The onset of puberty (vaginal opening in females and pre-putial separation in males) was recorded three times a week, between PND 26 and 40. The offspring were weighed on PND 32, 39 and 47.

Behavioural testing was performed as described [17,18] on PND 72–75, during the light period with experimenters blinded to exposure status. The same observer was used within tests, and exposed and control animals were tested alternately. Animals were transferred to the experimental room 1 hr before the first test. Activity was assessed for 3 min in a circular (∅ = 1 m) Open field. Total ambulation and ambulation in 1-min time bins (to test for habituation) were calculated. Duration in each of the Open field zones (central and peripheral) and the number of zone crossings were extracted. Acoustic startle reaction (ASR) and pre-pulse inhibition (PPI) were tested as described [17,18]. The offspring group assigned to behavioural testing was weighed on PND 39, 53, 67 and 81.

The accepted level of statistical significance was 0.05. Litter was considered the statistical unit, where relevant. Data were analysed by analyses of variance (ANOVA), with treatment and sex as factors. When relevant, analysis of repeated measures was applied (days of sampling in PND, trials and time bins). Significant results from overall analyses were analysed by pairwise comparisons. Analyses were performed in SYSTAT 9. Puberty start was analysed by log rank test in SAS 9.2. All the procedures complied with the EC Directive 86/609/EEC and the Danish law regulating experiments on animals (The Danish Ministry of Justice, Animal Experiments Inspectorate, Permission 2006/561-1123).

Results

Pulmonary exposure to Printex 90 by four intratracheal instillations during gestation induced persistent lung inflammation in the exposed time-mated mice (the number of neutrophil cells in bronchoalveolar lavage fluid was increased 28-fold 3 days after exposure and 61-fold 26 days after exposure). No effects on gestation, lactation and offspring development were observed [23]. Weight gain after weaning was similar in exposed offspring and their controls, both in the groups observed for sexual maturation and in the group designated for behavioural testing (table 1).

Relative AGD measured on all offspring at weaning did not differ between offspring prenatally exposed to Printex 90 and their controls (table 1). Overall analysis of onset of puberty (vaginal opening) in females indicated statistically significant differences between groups. Female offspring prenatally exposed to 11 μg Printex 90/animal entered puberty significantly earlier (time of vaginal opening, $p = 0.01$) compared with controls, while higher-dose groups compared with the controls (table 1). As data did not indicate dose response,

Table 1.

Parameters of sexual maturation for offspring exposed prenatally to vehicle (Nanopure water) or Printex 90.

End-point	Control	Printex 90 11 μg/animal	Printex 90 54 μg/animal	Printex 90 268 μg/animal
Number of time-mated females	24	17	17	22
Number of litters PND 1	20 (−1[1])	10 (−4[1])	11 (−2[1])	20
Sex ratio[2]	0.46 ± 0.04	0.66 ± 0.06	0.56 ± 0.06	0.45 ± 0.04
AGD at weaning females (mm) (n = 17, 8, 8 and 14)	4.17 ± 0.10	4.81 ± 0.30	4.15 ± 0.18	4.40 ± 0.11
AGD at weaning males (mm) (n = 17, 8, 8 and 14)	6.68 ± 0.24	7.20 ± 0.41	7.16 ± 0.21	6.92 ± 0.08
Relative AGD at weaning females	2.12 ± 0.05	2.28 ± 0.14	2.06 ± 0.07	2.14 ± 0.04
Relative AGD at weaning males	3.28 ± 0.09	3.50 ± 0.13	3.42 ± 0.11	3.31 ± 0.04
Vaginal opening (PND) (n = 8, 8, 8, 4)	37.7 ± 2.29	35.4 ± 0.75**	37.6 ± 1.08	37.0 ± 1.80
Pre-putial separation (PND) (n = 10, 3, 7, 7)	34.7 ± 1.05	33.0 ± 0.00	33.8 ± 0.94	35.8 ± 0.61
Total weight gain females (g) (maturation group)	8.3 ± 0.4	8.0 ± 0.3	9.0 ± 0.6	8.4 ± 0.6
Total weight gain males (g) (maturation group)	13.0 ± 0.8	11.5 ± 0.1	11.9 ± 1.7	10.9 ± 1.0
Total weight gain females (g) (behaviour group)	11.2 ± 0.3	–	–	11.3 ± 0.3
Total weight gain males (g) (behaviour group)	17.9 ± 1.2	–	–	18.6 ± 0.4

PND, post-natal day (days after birth); AGD, anogenital distance.

We have previously reported that exposure did not statistically significantly affect gestation, lactation and offspring development [23]. AGD was measured in all offspring at weaning. Results are reported as litter average ± SEM for male and female offspring. Onset of puberty (vaginal opening in females and pre-putial separation in males) was recorded for one male and one female per litter, in litters where possible, between PND 26 and 40 (three times a week). The offspring assigned for maturation testing were weighed on PND 32, 39 and 47. The offspring assigned to behavioural testing were weighed on PND 39, 53, 67 and 81. $^{**}p < 0.01$.

[1]Died during instillation.

[2]Females in litter (%).

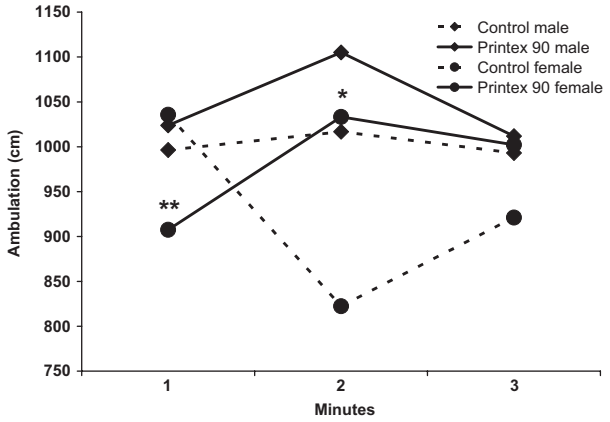

Fig. 1. Open field results for female and male offspring exposed prenatally to vehicle (Nanopure water) or Printex 90 (n♀ = 15, 10; n♂ = 12, 12). Analysis of habituation in Open field by tracking of movement by 'minute' intervals. The exposed females moved differently in the first 2 min, as compared to their control. Thus, the female offspring prenatally exposed to 268 μg Printex 90/animal displayed a different pattern of habituation. *$p < 0.05$, **$p < 0.01$.

this may be a chance finding. Puberty start in the male offspring (time of pre-putial separation) started at similar time in the exposed groups and in the controls (table 1).

The basal acoustic startle response was of similar magnitude in exposed and control offspring, and the offspring reacted similarly to PPI. For locomotor activity investigated in the Open field, the overall statistical analysis indicated no difference in the total distance moved during the 3-min observation. However, analysis of offspring habituation in minute intervals as repeated measure in the ANOVA indicated that the pattern differed between groups. The females moved differently during the first 2 min, compared with their control (fig. 1); total movement during the first minute was reduced compared with that of control ($p = 0.03$); and total movement during the second minute was increased compared with that of control ($p = 0.03$). Thus, the female offspring prenatally exposed to 268 μg Printex 90/animal displayed a different pattern of habituation. There were no differences in Open field activity between the exposed and control male offspring (fig. 1).

Discussion

We report here that maternal pulmonary exposure to the nanoparticle Printex 90 had no effect on acoustic startle of exposed offspring. Exposure induced small changes in neurofunction of the prenatally exposed female offspring as assessed in the Open field, while behaviour of exposed male offspring compared to the controls. Developmental neurotoxicity of nanoparticles has little been studied and even less so in both female and male offspring. One study of maternal inhalation exposure to nanosized titanium dioxide particles (42 mg TiO_2/m^3 1 hr/day on GD 8–18 with an estimated total inhaled dose of 840 μg) reports changes in the Open field for both male and female offspring, but only changes in startle reactivity for female offspring [17,18]. In a study with maternal inhalation exposure of resuspended diesel exhaust particles (19 mg DEP/m^3 1 hr/day on GD 8–18 with an estimated total inhaled dose of 378 μg), female exposed offspring displayed a tendency towards increased activity, whereas exposed male offspring behaved as controls [32]. One possible cause of the observed sex differences is a sex-specific sensitivity to maternal lung inflammation [33].

Nanoparticles do not readily translocate from the lung cavity, and therefore, only a small fraction of the instilled Printex 90 would enter the blood stream and cross the placenta. A direct particle effect on the foetus is probably minimal. The change in neurobehaviour was more likely an indirect effect caused by maternal inflammation. Maternal inflammation after lipopolysaccharide exposure during gestation (500 μg/kg BW on GD 14, 16, 18 and 20) is reported to affect neurogenesis and recognition memory of exposed rat offspring [34]. In the present study, the maternal pulmonary exposure was performed by intratracheal instillation. Instillation exposure induces stronger inflammation than does exposure by inhalation [35]. The reported effects may therefore be overestimated in comparison with nanoparticle inhalation at the same pulmonary dose.

A few other studies have assessed the effects of prenatal exposure to nanoparticles on the nervous system. Titanium dioxide nanoparticles (100 μg/animal) were injected subcutaneously (SC) into pregnant mice on GD 3, 7, 10 and 14. Nanoparticles were found in the brain, and they induced apoptotic changes in the olfactory bulb in the exposed male offspring 6 weeks after the prenatal exposure [22]. A comparable exposure (100 μg/animal TiO_2 SC on GD 6, 9, 12 and 15) changed gene expression in the offspring brain [27], and another exposure (100 μg/animal TiO_2 SC on GD 6, 9, 12, 15 and 18) increased the levels of dopamine in the offspring brain [28].

We have reported previously that offspring prenatally exposed to UV-Titan exhibited changes in the Open field and Startle test [17,18] and female offspring prenatally exposed to diesel exhaust particles exhibited increased activity in the Open field [32]. In conclusion, prenatal exposure to nanoparticles has been repeatedly associated with changes in behaviour related to activity level. This suggests that maternal exposure to nanoparticles may impact development of the nervous system, and future studies ought to include offspring neurofunction as an end-point.

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Conflict of Interests

The authors report no conflict of interests.

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APPENDIX I

Effect of liver tissue quality and levels of DNA strand breaks in comet assay.

EFFECT OF LIVER TISSUE QUALITY AND LEVELS OF DNA STRAND BREAKS IN COMET ASSAY

The article is to be published.