# Effects of prenatal exposure to nanoparticles titanium dioxide and carbon black on female germline DNA stability

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## Preface

This PhD project is the result of two large animal studies on *in vivo* mutations in expanded simple tandem repeat (ESTR) DNA loci in female mice prenatally exposed to nanoparticles titanium dioxide ( UV-Titan) and carbon black (Printex 90).
The breeding of F2 generation mice from female F1 mice that were prenatally exposed was performed at the National Research Centre for the Working Environment from November 2008-January 2010.
The ESTR analysis was performed at the laboratory of Dr. Carole Yauk at the Environmental Health Centre, Health Canada, Ottawa in Ontario, Canada from February 2010-August 2010.
This PhD was funded by, The National Food Institute, Technical University of Denmark (DTU) and the National Research Centre for the Working Environment (NRCWE).

Supervisors were:

Christine Nellemann, Dep. of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark
Håkan Wallin, National Research Centre for the Working Environment
Ulla Vogel, National Research Centre for the Working Environment

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Without the invitation from Carole Yauk to stay in her laboratory at the Environmental Health Centre at Health Canada, Ottawa this PhD project would not have been possible. I am grateful for the experience and for the opportunity to work with expanded simple tandem repeat mutations (ESTR). I also wish to thank the staff and students at Health Canada for making me feel welcome during my 6 month stay. Especially, I would like to thank Andrea Rowan-Carroll and Caitlin Ritz for introducing me to the ESTR method and Thomas Shipley for probing the remaining Southern blots when my work visa expired. Also, I would like to thank Breanne Swayne for volunteering to help me with the digestion of DNA samples and for saving them after the earth quake (!) I am also grateful to Colin Davis for volunteering at Health Canada and for making agarose gels and TBE buffer for me like there was no tomorrow.

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Anne Mette Zenner Boisen

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## Summary

Particulate air pollution has been associated with an increased risk of cardiovascular disease and cancer in humans. Air pollution may also adversely affect pregnancy outcome and the integrity of sperm cells DNA. Animal studies have shown that inhalation of air particulates can induce mutations in premeiotic sperm cells. The investigation of potential mutagenic risk is of outmost importance, as it may lead to cancer. Furthermore, heritable mutations may be passed on to descendents and thereby pose a permanent genetic risk to the population.

The nanosized fraction of particulate air pollution has recently come into focus. Inhaled nanoparticles are cleared very slowly from the lungs and a small fraction may translocate into the bloodstream and compartments of the body. In the airways nanoparticles can induce a high degree of pulmonary inflammation and oxidative stress. Nanoparticles are more reactive than larger sized particles and may have unique properties as a result of their size. The exposure to nanoparticles in the occupational setting and from consumer products will most likely increase greatly in the near future and thorough investigations of their potentially hazardous effects are needed.

Expanded simple tandem repeat (ESTR) loci in mice are sensitive markers of mutagenic effects resulting from environmental exposures; Studies on adult mice have revealed that while particulate air pollution induced ESTR mutations in premeiotic sperm cells, the female germline was not affected. Unlike sperm cells that are continuously developed in adulthood, the majority of oocytes are in a dormant state during long periods of adult life and may therefore be less sensitive to mutations. However, female germ cells may be vulnerable during pregnancy when the female germ cells of the fetus are actively dividing.

The aim of this PhD study was to determine if two widely used nanoparticles titanium dioxide UV-Titan and carbon black Printex 90 induce ESTR mutations in the germ cells of prenatally exposed females. Pregnant generation P mice were exposed to ~42 mg UV-Titan/$m^3$/1 h/d during gestation days 8-18 or carbon black Printex 90 at gestation days (7,10,15 and 18) (total dose of 268 µg/animal) by intratracheal instillation. Maternal inflammation and DNA damage were assessed in order to assess the potential for indirect effects on offspring during pregnancy. Prenatally exposed F1 females were grown to maturity and mated with unexposed males. The ESTR mutation rate in F2 offspring was estimated from full pedigrees (mother, father, offspring). ESTR mutation rates of 0.029/0.025 (maternal allele) and 0.047/0.053 (paternal allele) in UV-Titan/Printex 90-exposed F2 offspring were not statistically different from those of F2 controls: 0.037/0.024 (maternal allele) and 0.061/0.038 (paternal allele). UV-Titan and Printex 90 exposure induced pulmonary inflammation in pregnant generation P mothers as well as changes in hepatic gene expression in the F1 prenatally exposed females. However, ESTR mutation rates were not increased by UV-Titan or Printex 90 in the germ cells of prenatally exposed F1 females.

# Dansk resumé

Partikler i luftforurening er blevet associeret med en øgning i risiko for kræft og cardiovaskulære sygdomme hos mennesker. Luftforurening kan have en negativ påvirkning på graviditetsparametre og sædcelle-DNA-integritet. Dyrestudier har desuden vist at inhalation af partikelforurening kan inducere mutationer i premeiotiske sædceller. Det er yderst vigtigt at få undersøgt denne potentielle mutationsrisiko da mutationer kan føre til kræft. Arvelige mutationer kan også videreføres til kommende generationer og på den måde udgøre en permanent risiko for genetiske påvirkninger i befolkningen.

Der er på det sidste kommet en øget fokus på fraktionen af partikelforurening i nanostørrelse. Inhalerede nanopartikler fjernes langsomt fra lungerne og en lille andel kan muligvis translokere over i blodbanen og videre ud i kroppen. I luftvejene kan nanopartikler generere betændelsestilstande samt oxidativt stress. Nanopartikler er mere reaktive end større partikler og kan have unikke egenskaber på grund af deres lille størrelse. Eksponeringen for nanopartikler i arbejdsmiljøet samt som forbruger vil sandsynligvis stige kraftigt i den nærmeste fremtid og en grundig undersøgelse af deres potentielt skadelige effekter er påkrævet.

Ekspanderede simple tandem DNA gentagelser (ESTR) i mus er følsomme markører for mutagene effekter forårsaget af en miljøpåvirkning. Studier på voksne mus har vist, at mens partikelforurening inducerer ESTR mutationer i premeiotiske sædceller, bliver hunlige kønsceller ikke påvirket. I modsætning til sædceller, som dannes løbende gennem hele voksenlivet befinder størstedelen af ægceller sig i et hvilestadie gennem lange perioder af kvindens liv og ægceller er derfor muligvis mindre følsomme overfor mutationer. Ægceller kan imidlertid være følsomme overfor mutationer under fosterudviklingen, hvor de kvindelige kønsceller udvikles.

Formålet med denne PhD var at undersøge om to hyppigt brugte nanopartikler titanium dioxid UV-Titan og carbon black Printex 90 kan inducere ESTR mutationer i prenatalt eksponerede hunlige kønsceller. Gravide generation P mus blev eksponeret for ~42 mg UV-Titan/$m^3$/1 t/d fra gestationsdag 8-18 eller for carbon black Printex 90 ved gestationsdag (7,10,15 og 18) (total dosis 268 µg/dyr) ved intratracheal instillation. Maternel inflammation og DNA skade blev undersøgt for at vurdere den potentielle indirekte effekt på afkom under graviditeten. Prenatalt eksponerede F1 hunmus blev ved kønsmodning parret med ueksponerede hanmus. ESTR mutationsraten i F2 afkom blev estimeret fra avl af (mor, far, afkom). ESTR mutation rater (0,029/0,025 for den maternelle allel og 0,047/0,053 for den paternelle allel) for UV-Titan/Printex 90-eksponerede F2 afkom var ikke statistisk signifikant forskellige fra mutationsraterne i F2 kontroller: (0,037/0,024 for den maternelle allel og 0,061/0,038 for den paternelle allel). Eksponering for UV-Titan og Printex 90 inducerede lungeinflammation i gravide generation P mus samt ændringer i genekspressionen i leveren hos F1 prenatalt eksponeret hunligt afkom. Der var i midlertidig ikke nogen ændring i ESTR mutationsraten af kønsceller ved UV-Titan eller Printex 90 eksponering af hunligt F1 afkom i fostertilstanden.

## Abbreviations and definitions:

**BAL:** Broncoalveolar lavage

**BaP:** benzo(a)pyrene

**CB:** Carbon black

**DEP**: Diesel exhaust particles

**DSB**: Double strand break

**DTU:** Technical University of Denmark

**ENU:** N-nitroso-N-ethylurea

**ESTR:** Expanded simple tandem repeats

**ETOP:** Etoposide

**FPG:** Formamidopyrimidine-DNA glycosylase

**Generation P**: The **P**arental generation, parental dams are directly exposed.

**Generation F1**: The first **F**ilial generation of the P generation. F1 females are exposed *in utero via* the generation P dams.

**Generation F2**: The second filial generation of the P generation. Produced by gametes from F1 females exposed *in utero* during gametogenesis.

**iPMS:** methanesulfonate

**NM:** Nanomaterials

**OA:** okadaic acid

**NP:** Nanoparticles

**NRCWE:** National Research Centre for the Working Environment

**PAH:** Polyaromatic hydrocarbons

**PCB:** polychlorinated biphenyls

**PM:** Particulate matter

**PMN:** Polymorphonuclear leukocytes

**RA:** Retinoic acid

**ROS:** Reactive oxygen species

**SM-PCR:** Single molecule PCR (polymerase chain reaction)

**SB:** Strand breaks

**SSB:** Single strand breaks

**TCDD**: 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin;

**$TiO_2$**: Titanium dioxide

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# Introduction

Mutations in the male and female germline may lead to adverse effects being inherited in future generations. Human exposure to particulate air pollution has been linked to cancer and has been shown to negatively affect pregnancy outcome and the integrity of sperm cell DNA. Moreover, animal studies have demonstrated that inhalation of particulate air pollution can induce mutations in tandem repeat DNA in male germ cells. Very few studies on the mutagenic potential of chemicals and particles in the female germline have been performed. Germ cells in females are generally believed to be less susceptible to mutagens than germ cells in males. Male germ cells are developed continuously throughout adult life. However, all germ cells in females are developed before birth and may therefore be vulnerable to prenatal exposure.

Nanoparticles measure less than 100 nm in at least one dimension and posses unique properties associated with their nanostructure. The exposure to nanoparticles in the occupational setting and from consumer products will most likely increase greatly in the near future. Inhaled nanoparticles are cleared very slowly from the lungs and a small fraction may translocate into the bloodstream. In the airways nanoparticles can induce a high degree of pulmonary inflammation and oxidative stress, which may indirectly affect the fetus during maternal exposure. As a result the effects of airborne nanoparticles may be more severe than those of larger sized particles.

## Aim:

The primary aim of this thesis was to determine if nanoparticles are able to induce expanded simple tandem repeat (ESTR) mutations in the germ cells of prenatally exposed females.
To investigate this I conducted two ESTR mutation studies on female mice exposed *in utero* (gestation days (7)8-18) and assessed the number of mutations in their offspring:

Study I: Prenatal exposure to Titanium dioxide UV-Titan by inhalation
(~42 mg UV-Titan/$m^3$/1h/d)
Study II: Prenatal exposure to carbon black Printex 90 by intratracheal instillation
(268 µg/animal, total dose)

Maternal inflammation and DNA damage were assessed in order to assess the potential for indirect effects on offspring during pregnancy.

**Overview of study design:**

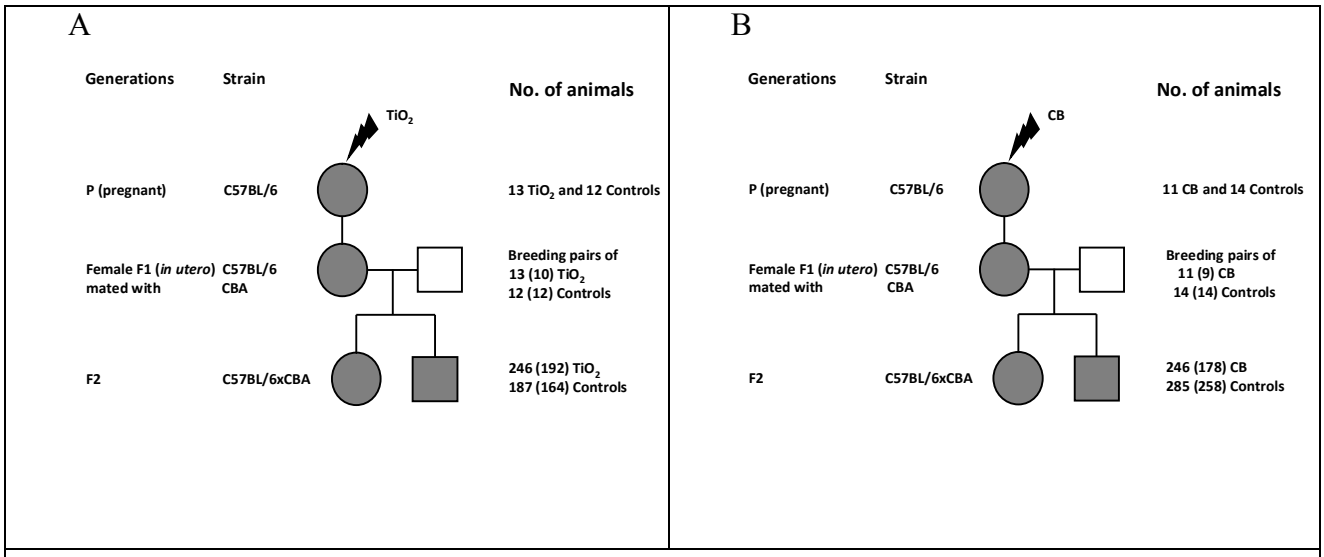



**Figure 1. Overview of pedigree study design.**

Circles and squares represent female and male mice respectively. Grey symbols represent exposed animals and their descendants. White squares represent non-exposed CBA breeding mates. Generation P pregnant mothers were directly exposed to either $TiO_2$ UV-Titan L181/clean air (A) or carbon black Printex 90/vehicle (B). The F1 female offspring were exposed *in utero*. F1 female offspring (C57BL/6) were mated at maturity to (CBA) male mice. ESTR Mutations were scored in the tail tissue of F2 offspring (C57BL/6xCBA). The number of animals exposed or collected is given. The number of successfully analyzed animals is in parentheses.

**Particulate air pollution**

Exposure to airborne particles in the urban air has been linked to adverse health effects such as lung cancer, cardiovascular disease and chronic obstructive pulmonary disease (COPD) in several large epidemiological studies (Samet et al. 2000; Pope et al. 2002; Brunekreef et al. 2009; Delfino et al. 2005; Krewski et al. 2009; Pope and Dockery 2006). The World Health Organization estimates that 2 million premature deaths worldwide can be attributed to indoor air pollution from the burning of solid fuels and outdoor urban air pollution (WHO Global update 2005). The risk of heart attack has been correlated with an increase in particulate matter $\leq 2.5$ μm ($PM_{2.5}$) the day before and 2 hours prior to the heart attack (Peters et al. 2001). Moreover, ambient air pollution can affect reproduction in humans: Epidemiological studies have shown that exposure to ambient air pollution during pregnancy is linked to premature birth, low birth weight, small for gestational age (Shah and Balkhair 2011), stillbirths (Pope et al. 2010) and postnatal mortality (Sram et al. 2005); as well as an increase in DNA damage in placenta tissue and umbilical cord blood (Sram et al. 1999; Neri et al 2006; Kannan et al. 2007). Male exposure to air pollution has been linked to abnormal sperm morphology, motility and DNA integrity (Sram et a. 1999; Rubes et al. 2005).

**Animal studies of air pollution-induced mutations in the germline**

Animal species have been used as early warning sentinels for environmental health hazards since the days of placing canaries in coal mines (O'Brien et al. 1993; Somers 2011). Experimental studies where sentinel species such as herring gulls or laboratory mice are exposed *in situ* to ambient air pollution has proven to be a valuable tool for assessing genotoxicity in the germline by measuring mutations at non-coding tandem repetitive DNA sequences in their offspring or directly in the gametes (Yauk and Quinn 1996; Yauk et al. 1998; Yauk et al. 2000; Somers et al. 2002; Somers et al. 2004; Yauk et al. 2008a).
ESTR *loci* are a special class of tandem repetitive DNA found in mice. Mutations in ESTR *loci* are believed to be induced by a non-targeted mechanism such as DNA damage or epigenetic modifications. Radiation and a number of chemicals and airborne particles have been shown to increase ESTR mutations in the male germline (Dubrova et al. 1998; Liu et al. 2009; Yauk et al. 2007; Polyzos et al. 2006x). Spontaneous mutation rates in ESTR loci are very high, which means that only a small number of animals and a low exposure dose are needed to see an effect (Singer et al. 2006). This makes ESTR analysis an invaluable tool for assessing DNA mutation rates in real life exposure scenarios. In a classic pedigree study one or both parents are exposed to a potential mutagen. The mice are then bred to

generate offspring, that are produced by the gametes that where developing during exposure. Studies have shown that ESTR mutations are primarily induced in mitotically active cells (Barber et al. 2009; Dubrova et al. 1998). Because of the high spontaneous mutation rate, alleles from outbred individuals are highly heterogeneous. In inbred strains allele size differs between strains. If two different inbred strains are mated, the hybrid offspring will possess ESTR alleles of different sizes depending on parental origin. Both methods can be used to investigate maternal/paternal mutation rates (see figure 1 and 2).

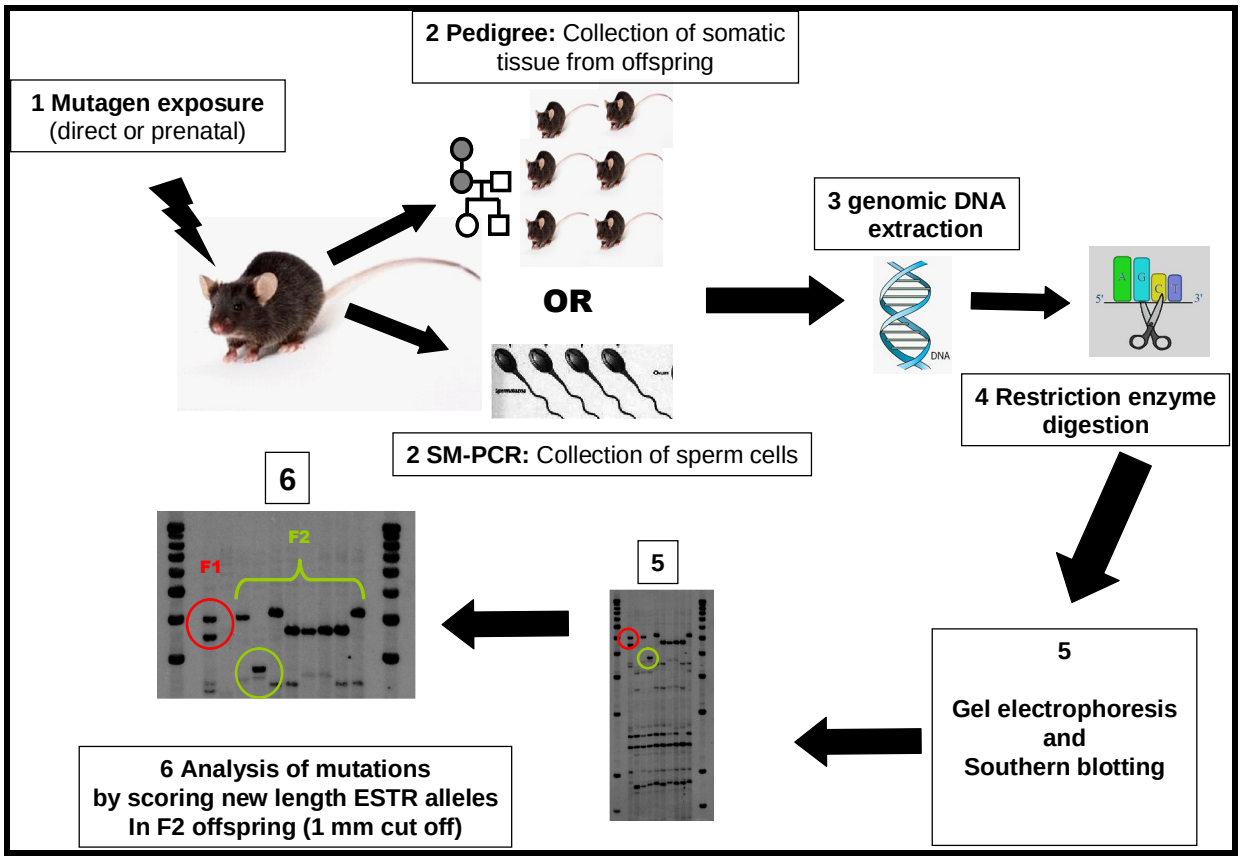



**Figure 2**. Overview of the ESTR method. 1 Mice are exposed to a potential mutagen directly or *in utero*. 2 The exposed mouse is mated and DNA from offspring is collected **or** 2 sperm cells are collected directly from the exposed male. 3 genomic DNA is extracted 4 DNA is digested and 5 electrophoresed to separate DNA fragments according to size. DNA from gels are blotted onto membranes, hybridized with ESTR radioactive probes and images from autoradiographs are developed. 6 The number of mutations is assessed by counting the number of new length alleles in F2 offspring or F1 gametes compared to the parental alleles.

Studies have shown that, when adult mice of both sexes are exposed to particulate air pollution, only the male germline is affected: In a study by Somers et al. 2002 male and female outbred Swiss-Webster mice were placed in cages in situ close to industrial (steel mills) and rural sites for 10 weeks. Males and females within exposure groups were mated to each other 6 weeks after exposure (to ensure that offspring was generated from pre-meiotic sperm cells) and the mutation rates of the paternal and maternal alleles assessed in their offspring. There was a 1.6-fold increase in the ESTR mutation rate in the paternal allele of offspring born to parents exposed to ambient air at the industrial site *loci* compared to those at rural sites. There was no difference in the maternal allele. To further investigate what components of air pollution induced the tandem repeat mutations another *in-situ* study of male and female outbred Swiss-Webster mice was performed at the same industrial site: Mice in the control group were placed in HEPA-filtered cages along side the exposed group. The HEPA-filters removed 99.97% of particles > 0.1 μm. The reduction in particulate matter in air pollution by the HEPA filter caused a 52 % decrease in the mutation rate in the paternal allele of the filtered air group compared to the whole air group. Mutation rates in alleles from adult females were unaffected (Somers et al. 2004). Within the same study male mice exposed to ambient air in industrial and rural sites were mated to unexposed females. Male exposure to ambient air from industrial sites resulted in a 2.8-fold increase in the mutation rate in the paternal allele compared to rural controls (Somers et al. 2004).

A method for assessing ESTR mutations directly in single cells was developed by Yauk et al. 2002: In this study genomic DNA from isolated sperm tissue was digested and single Ms6-hm molecules amplified by single molecule PCR (SM-PCR) and subsequently analyzed for mutants by electrophoresis and Southern blotting (Southern et al. 1979). The SM-PCR approach was compared to a pedigree analysis within the same study. The spontaneous and radiation-induced mutation rates assessed directly in male germ cells were indistinguishable to those estimated by a traditional pedigree analysis in the same male mice (Yauk et al. 2002).

In 2008 Yauk and co-authors exposed C57BL/6xCBA male hybrids to whole ambient air at the same industrial site as in (Somers et al. 2002; Somers et al. 2004). After 16 weeks (10 weeks of exposure followed by 6 weeks in the laboratory to obtain the pre-meiotic sperm cells that were exposed) they found a 1.6-fold increase in the ESTR mutation rate (detected directly in pre-meiotic sperm cells by SM-PCR) compared to that of HEPA-filter controls. At earlier time points no elevation in ESTR mutation rate was found. After 3 weeks of exposure the amount of bulky DNA adducts in lungs were increased in the whole air exposure group. No increase in bulky adducts was found in testes at any time points, which suggests that DNA reactive chemicals did not reach the male germline. However, DNA strand breaks (SBs) were detected in the testes after 3 and 10 weeks of exposure, possibly resulting from particle-induced oxidative stress. Mice exposed to ambient air pollution had hyper-methylated sperm DNA

compared to HEPA-filter controls. This implies that epigenetic modifications may play a role in the generation of ESTR mutations.

A study by Barber et al. 2009 where male and female F1 offspring were exposed *in utero* to 1 Gy of acute gamma radiation at gestation day 12 gave proof-of-principle that female germ cells are indeed susceptible to induced ESTR mutations, when they are exposed prenatally during mitotic cell division. The ESTR mutation rate in the germline of BALB/c strain male and female F1 mice (assessed by a pedigree study in their F2 male offspring) were equally elevated approximately 2-fold by the exposure.

To investigate if prenatal exposure to particles could induce ESTR germline mutations Ritz et al. 2011 exposed male and female C57BL/6 mice *in utero* to 19 $mg/m^3$ diesel exhaust particles (standard reference material NIST 2975 generated by an industrial fork lift) during GD 7-19 for 1h/day and assessed the level of ESTR mutations in the C57BL/6xCBA F2 offspring: In male F1 a statistically significant 2-fold increase in ESTR germline mutations was seen. However, the 1.98-fold increase seen in the germline of F1 females was not statistically significant ($p= 0.1588$). The effect of prenatal exposure of male mice to diesel exhaust particles on ESTR germline mutations (Ritz et al. 2011) was similar to that of 1 Gy acute radiation (Barber et al. 2009). This exposure was performed at the National Research Centre for the Working Environment, Denmark and the analysis of ESTR mutations in the group of Carole Yauk, Health Canada, Ottawa.

As described above particle exposure has been shown to induce mutations in the developing germline in male mice and may pose a heritable, permanent risk to the integrity of the genome. Not a lot is known about mutagenesis in the female germline, but the study of Barber et al. 2009 showed that mutations can be induced while the female gametes are developing. The study by Ritz et al. 2011 suggested that mutations in the female germline may be induced by particles.

The nanosized fraction of air pollution, and engineered nanoparticles have recently come into focus because nanoparticles (NPs) may be more reactive and induce stronger effects during exposure than particles of a larger size (Donaldson et al. 2004). Furthermore, the effects of many NPs are still inadequately characterized (Card et al. 2010; Warheit et al. 2008). The aim of this PhD project was therefore to investigate the potential mutagenic effect of two widely used NPs on the germline of female mice.

## Introduction to nanotechnology

The applications of nanotechnology are many and diverse: Highly targeted drug delivery for cancer treatment, building materials with super strength and durability, and socks that never need to be washed are just a fraction of the possibilities in the nano era. Nanomaterials can be defined as materials that measure less than 100 nm in at least one dimension and which exhibit biological, physical and chemical characteristics associated with their nanostructure (Stone et al. 2007). The term nanomaterials include nano-objects (such as carbon nanotubes), where at least 2 dimensions are below 100 nm and nanoparticles (NPs), where all 3 dimensions are below 100 nm (Stone et al. 2010).

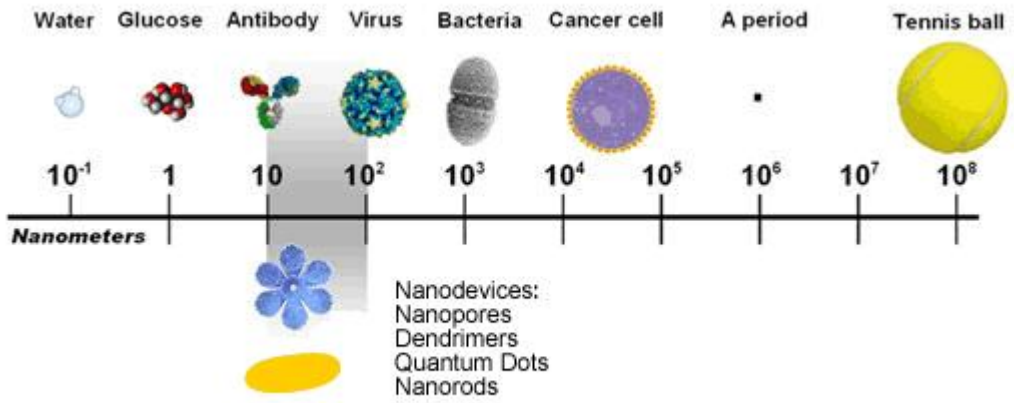



**Figure 3.** The nanorange (1-100 nm) in comparison to smaller and larger objects.
(source: National Cancer Institute, Bethesda, USA, http://nano.cancer.gov/learn/understanding)

## Origin and history of nanoparticles

Naturally occurring NPs such as in desert dust, volcanic ash and particles from forest fires have always been present in our environment. Mankind has unknowingly used NPs since antiquity, when the Romans used nano-sized gold particles for multi-colored stained glass (Freestone et al. 2007). During the industrialization byproducts from conventional processes such as diesel exhaust particles, welding fumes and combustion soot increased the amount of man-made NPs dramatically. Moreover, conventional products such as paints and printing ink also contain a fraction of pigment particles in the nano-range. The era of nanotechnology was launched in the 1980s with the invention of the scanning tunneling microscope and the discovery of fullerenes. But it wasn't until the early 2000s that engineered nanoproducts became commercially available on a larger scale (Aitken et al. 2006; McWilliams et al. 2010). The global market value of nanotechnology was estimated at 11.6 billion USD in 2009 .The nanotechnology-based economy is expected to grow by 11% annually until 2015 (McWilliams 2010),

and by the year 2015, 2-10 million people are expected to work with the use and development of nanotechnology (National Science Foundation 2001 (OSHA) European Agency for Safety and Health at Work). The increased production of nanomaterials has raised concerns about the potential risks to humans and the environment from this new technology.

**Nanotoxicology**

In 2004 Donaldson and co-workers addressed the need for the new discipline of nanotoxicology, which would address the potential hazards of the emerging industry of nanotechnology (Donaldson et al. 2004). Nanomaterials such as NPs belong to a very diverse group and little is known about the potential hazard of nanomaterials. The only denominators are the size criteria and by most definitions the possession of new properties when compared with their bulk counterparts. In order to assess potential toxicity of nanomaterials key physico-chemical properties such as size distribution, shape, agglomeration and/or aggregation, composition, surface coating, purity, relative surface area and surface reactivity, surface chemistry, surface charge, stability, crystalline phase, water solubility/dispersibility, dustiness and radical formation potential also need to be taken into account (Card et al. 2010; Warheit et al. 2007; Warheit et al. 2008). The European Union Regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) addresses the potential impact on human health and the environment of the production and use of chemical substances within the EU. There is no specific regulation that deals with nanomaterials under REACH. Chemical substances, including those at the nanoscale, which are manufactured or imported in volumes of ≥1 tonne per year, have to be registered under REACH. A chemical safety report based on a chemical safety assessment has to be included in the registration of chemicals in volumes of ≥10 tonnes per year. As a result of the extremely small size and low weight of NPs, the tonnage rule in reality exempts most nanomaterials from this requirement. There are ongoing discussions on whether NPs should be exempt from the tonnage rule and be fully tested at much lower tonnage limits in accordance with the precautionary principle (European Commission –Enterprise and Industry).

Due to their small size NPs have a larger relative surface area per mass than bulk size particles of the same material; this often makes NPs more toxic and inflammogenic (Duffin et al. 2007). In accordance with this many studies have shown that low toxicity, low solubility NPs can generate more reactive oxygen species than larger particles (Stone et al. 2007). Their extremely small size also enables some NPs to cross cell membranes and translocate from the environment into the organism.

**Deposition of particles in airways**

The main route of exposure to airborne particles in the occupational setting is through the respiratory tract: Where in the airways inhaled particles deposit is dependent on particle size (Nicod et al. 1999): Coarse particles > 10 µm in diameter deposit in the upper airways (nose and pharynx) while smaller particles 2-10 µm in diameter deposit in the bronchi and bronchioles and are cleared by the ciliary cells of the mucociliary escalator, which move particles trapped in mucus upwards into the pharynx, where they may be expelled or ingested and thereby reach the gastrointestinal tract. Particles < 2 µm are able to reach the alveoli, which lack ciliary cells. The clearance of these particles is therefore dependent on the phagocytosis of particles by alveolar macrophages. The clearance mediated by macrophages is slower (months-years) than ciliary clearance (hours-days). If the capacity of the macrophages is surpassed, as in the case of particle overload, particles can accumulate in the interstitium (Oberdörster et al. 2005). The particle deposition pattern differs between humans and rodents (Morrow et al. 1996) as well as between different rodent species (Raabe et al. 1996). However, the deposition patterns between humans and rodents may be more similar in the nanorange (Menache et al. 1995).

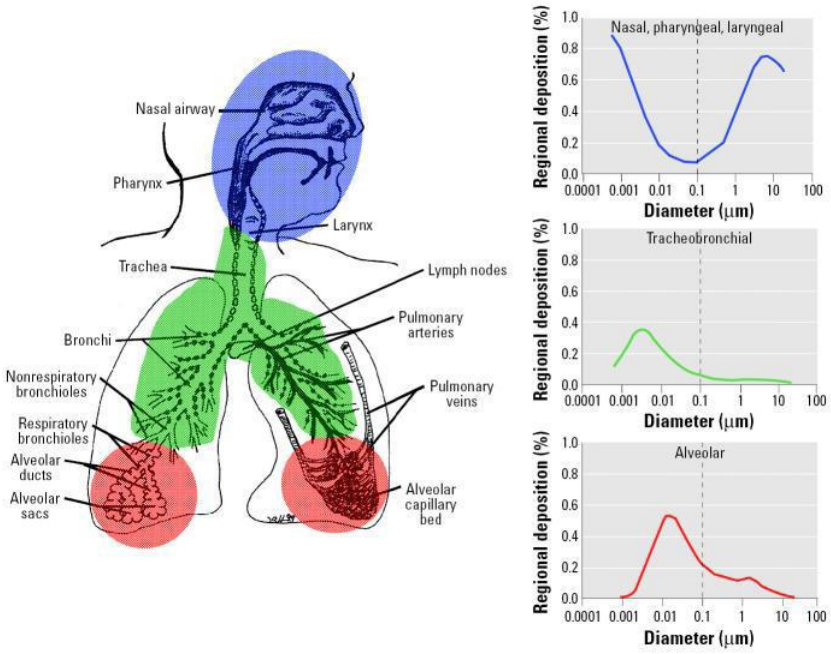



**Figure 4. Size-dependent deposition of particles in the airways.**
The majority of large particles ~10 µm deposit in the upper airways (nasal, pharyngeal and laryngeal region). Smaller particles deposit in the tracheobronchiolar region ( particles 2-10 µm) and in the alveolar region(particles <2 µm), modified from (Oberdöster et al. 2005).

**Translocation of nanoparticles**

The potential translocation of NPs is dependent on composition and other physico-chemical properties. Animal studies have shown that NPs are able to translocate across the blood-lung barrier and enter the lymphatic or circulatory system and secondary target organs via the pulmonary interstitium (Saduaskas et al. 2009; Oberdörster et al. 2002). The extent of NP translocation in humans is currently being debated (Nemmar et al. 2002; Mills et al. 2006; Möller et al. 2008). The translocation of NPs is considered to be very low. Less than 1 % of NPs are able to translocate beyond the lung and regional lymphatic system (Kreyling et al. 2010; Kreyling et al. 2002; Kreyling et al. 2009; Sadauskas et al. 2009; van Ravenzwaay et al. 2008). Studies in animals and ex-vitro studies on perfused human placentas have shown that NPs may be transferred from pregnant mothers to their fetuses across the placental barrier (Myllynen et al. 2008; Wick et al. 2010; Yamashita et al. 2011). NPs may have a direct negative effect on placental function as well as fetuses (Karttunen et al. 2010; Wick et al. 2010; Yamashita et al. 2011).

**Particle-induced inflammation**

Inflammation is part of the innate immunity mechanism and a crucial way of eliminating pathogens. Alveolar macrophages, which constitute the first line of defense, phagocytize foreign bodies that enter the lungs and produce early pro-inflammatory mediators such as cytokines, which recruit leukocytes (primarily neutrophils) to the site of inflammation (Toews 2001). The acute inflammatory response ceases when the stimulus is removed, but if the situation causing the inflammation cannot be resolved, as in the case of particle overload, the inflammation can become persistent (Azad 2008). The presence of activated macrophages in chronic inflammation results in the release of reactive oxygen species. Persistent inflammation has been linked to carcinogenesis (Knaapen et al. 2004; Borm et al. 2004) and it has been hypothesized that there is a close link between the ability of a particle to induce inflammation and genotoxicity (Greim et al. 2001). Inflammatory cytokines in pregnant mothers may cross the placenta and affect the fetus or activate production of cytokines in the placenta itself (Hougaard et al. 2011). Studies have shown that maternal inflammation during pregnancy may affect the immune function in offspring (Hodyl et al. 2008; Surriga et al. 2009).

**Particle-induced generation of reactive oxygen species (ROS)**

Many NPs are able to generate reactive oxygen species (ROS) (Oberdörster et al. 2005). ROS are unstable oxidants that can cause damage to cellular biomolecules such as lipids, proteins and DNA during states of imbalance between the production of ROS or reactive nitrogen species and antioxidants. This imbalance is called oxidative stress. ROS is produced endogenously by the natural intracellular metabolism of oxygen in mitochondria and on cell membranes (Donaldson et al. 2010).

The particle-induced primary pathway to ROS formation is driven directly by the particles and can take place without the presence of cells or by particle-related mitochondrial activation (Knaapen et al. 2004). Important particle parameters that influence primary ROS formation include size, surface area and the presence of metal ions and organic compounds (Møller et al. 2010).

The secondary pathway to particle-induced ROS formation is generated by inflammation induced by particles. Inflammatory pathways activate phagocytes such as neutrophils and macrophages and ROS is then generated by these activated phagocytes (Nicod et al. 1999; Knaapen et al. 2004). DNA damage by ROS can result in oxidized bases, base pair mutations and DNA deletions or insertions (Wiseman et al.1996), which in turn may lead to carcinogenesis. Oxidative stress may also promote carcinogenesis by influencing regulation and proliferation of cells (Knaapen et al. 2004).

**Particle-induced genotoxicity and mutagenicity**

Genotoxicity refers to potentially harmful effects to genetic material, which are not necessarily linked to mutagenesis, but may lead to it, for instance through errors during DNA replication or repair. Examples of genotoxicity include oxidation of DNA, DNA adducts and DNA strand breaks (SBs) (EC 2003). The possible mechanisms by which NPs may induce genotoxicity are reviewed in Donaldson et al. 2010: The direct interactions of NPs with DNA or cellular components, which govern cell division, can result in direct primary genotoxicity. Indirect primary genotoxicity may be caused by an enhanced ROS production through the interaction of NPs with mitochondria or membrane bound NADPH oxidases. Secondary genotoxicity may be elicited by the secondary pathway to particle-induced ROS formation generated by inflammation described above (Knaapen et al. 2004).

Mutations are permanent, transmissible changes in the amount or structure of genetic material involving single genes, gene segments or a block of genes or whole chromosomes (EC 2003). Mutations can either be inherited or arise by faulty DNA repair or incorrect nucleotide pairing during replication. Damage to DNA such as DNA adducts or oxidized bases may lead to point mutations: An example of this is the

oxidation of guanosine by ROS, which produces 8-oxo-7,8 dihydro-2'deoxyguanosine (8-oxodG). If 8-oxodG is not repaired it can mispair with dA and cause G:C to T:A transversions (interchanges of purine for pyrimidine bases) (Klungland et al. 1999). Another type of point mutations is transitions (purine to purine and pyrimidine to pyrimidine interchanges). In addition to point mutations, larger sections of DNA can be deleted or inserted as in the case of mutations in ESTR *loci*. Rodent assays for assessing germline mutations such as the specific *locus* test (which screens for phenotypic markers in offspring), the dominant lethal assay (which screens for post-implantation deaths *in utero*) and the heritable translocation assay (which screens for numerical and structural chromosome changes in offspring) have traditionally been used to monitor mutations in the germline. Alternatively, transgenic rodent models have also been used to investigate germline mutations (Wyrobek et al. 2007; Singer et al. 2006). It was previously believed that no mutagens acting exclusively on the germline existed (EC 2003). However, a study by Witt et al. 2003 showed that N-hydroxymethylacrylamide caused dominant lethal mutations, but did not elevate the number of micronucleus in bone marrow in mice (Witt et al. 2003).

Mutations in coding regions are rare events: The spontaneous mutation rate of the traditional germ cell mutation assay the specific *locus* test is approximately $6.6 \times 10^{-6}$. As a consequence of the low spontaneous mutation rate very large numbers of animals (1000-10000 per group) are needed to see an effect and the SLT as well as the heritable translocation assay (another traditional germline mutation test) are now rarely used (Singer et al. 2006). Due to the much higher spontaneous mutation rate of ESTR *loci* (in the order of 1-10% per gamete) a relatively small number of animals and low exposure doses are sufficient to observe an effect (Somers et al. 2006).

**Female gametogenesis and oogenesis**

Premordial germ cells in the mouse are derived from the embryonic ectoderm and become lineage restricted at about gestation day (GD) 7.2. By GD 8.5 the migration of primordial germ cells towards the genital ridges of the fetus begins. The genital ridges start to form at GD 10.5 (McLaren 2003). Mitotic divisions in primordial germ cells occur during migration and continue after the cells have reached the germinal ridge at GD 10-11 (McLaren 2000). At GD 13.5 murine oogonia start to cease mitotic division and progress through the stages of prophase I of meiosis until they arrest in the diplotene stage. By GD 15.5 most oogonia have entered prophase I of meiosis and are now called oocytes. Evidence suggests that a small population of oogonia do not enter meiosis until right after birth (Pepling 2006). Oogonia that do not enter premeiotic prophase are eventually degenerated. Spontaneous degeneration (atresia) occurs at very high frequencies throughout oogenesis (Russell and Russell 1992). After reaching the diplotene stage oocytes are gradually covered with a layer of granulosa cells, which together with the

oocytes, form follicles (Russell and Russell 1992). The oocytes remain dormant from before birth until puberty when the oestrus cycle evokes the maturation process in a small number of primordial follicles in the adult female. Meiosis I is completed in the oocyte hours before its ovulation from the mature (Graafian) follicle. Meiosis I produces one haploid secondary oocyte and a haploid polar body, which eventually degenerates. Fertilization of the oocyte induces meiosis II, which produces the mature ovum as well as a 2nd polar body which also degenerates. Oocytes complete meiosis I approximately half a day prior to ovulation (Russell and Russell 1992).

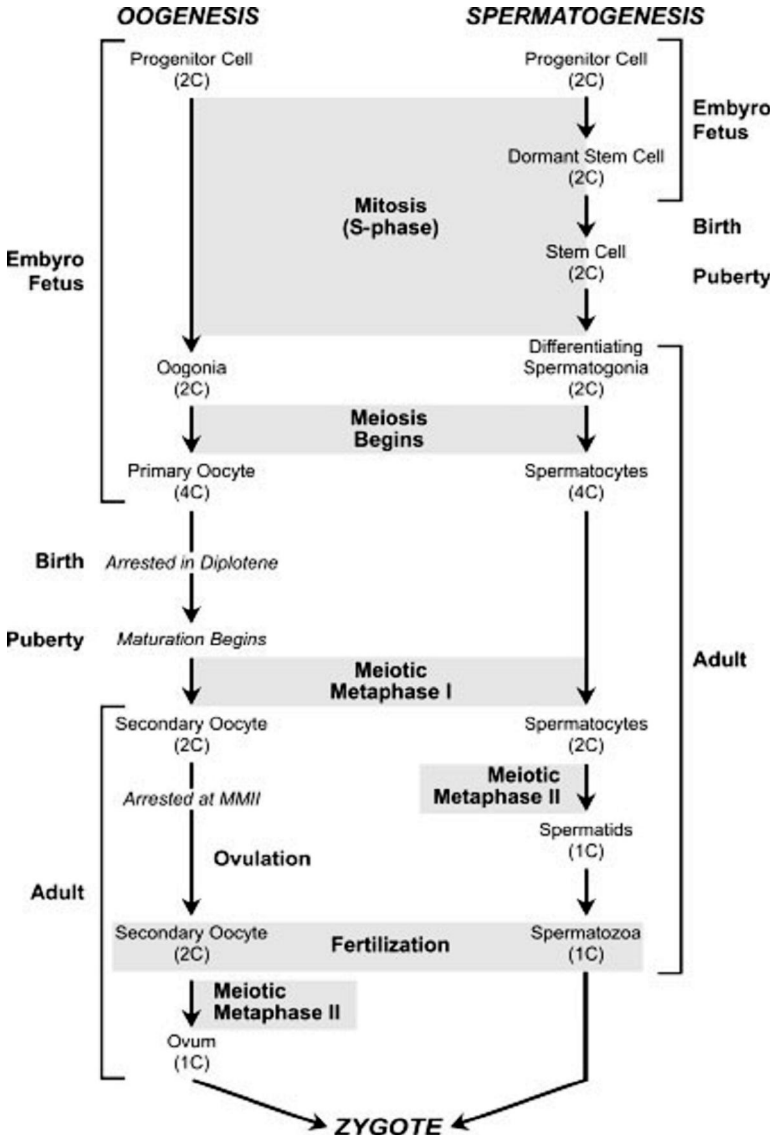



**Figure 5. Time-line of cell stages of oogenesis and spermatogenesis compared.**
From Wyrobek et al. 2007.

**Female germ cells as a target for mutations**

Female germ cells are overall less sensitive to ionizing radiation and chemical mutagens than their male counterparts (Adler et al. 2007). The exposure of a sufficient number of oocytes in mutation analyses has proven difficult due to the cyclic nature of oocyte maturation and the result has been that the majority of germ cell studies have been done on males. More than 30 specific locus test studies on chemically-induced germline mutations in males exist compared to only 5 in females. However, radiation-induced germline mutations in females have been studied more extensively using the specific locus test (Russell and Russell 1992; Searle and Phillips 1971; Selby et al. 1991). These results show that the spontaneous mutation frequency of oocytes is much lower than in sperm cells. No radiation-induced mutations have been seen in oocytes in meiotic arrest, but interestingly, high-dose-rate radiation produced more mutations in mature and maturing oocytes than in spermatogonia (Russell and Russell 1992).

The majority of results from the dominant lethal assay show that post meiotic germ cells in males are more susceptible to mutagens than maturing oocytes (Adler et al. 2007). However, some chemicals, such as adriamycin, cisplatinum and other intercalating agents have shown specific female germline sensitivity (Adler et al. 2007). Intercalating agents only induce dominant lethals in maturing oocytes and not in any male germ cell stage (Adler et al. 2007; Eichenlaub-Ritter et al. 2007). This could be caused by variations in DNA repair mechanisms or checkpoint control (Cohen et al. 2006; Adler et al. 2007). Male germ cells seem to have a more stringent checkpoint control at meiosis than female germ cells (Adler et al. 2007).

Female germ cells have a high capacity for DNA repair throughout development. In contrast, male germ cells loose DNA repair ability in the post-meiotic stages of development (Wyrobek et al. 2007). In the female, DNA repair proteins and mRNA are stored in oocytes and enable DNA repair during and after fertilization (Wyrobek et al. 2007). However, the ability of oocytes to repair damage in the paternal genome may vary with genetic background (Marchetti and Wyrobek 2005) and some studies have suggested that the maternal genome may be more vulnerable than the paternal at a critical time during the first few divisions of the zygote, when oocyte mRNA is degraded (Gibbs et al. 1993). Accordingly, the alkylating agent ethylnitrosourea (ENU), a very potent mutagen, is more mutagenic in the maternal genome than the paternal within the zygote (Adler et al. 2007).

## Introduction to repetitive DNA

Repetitive DNA sequences are found throughout the genome in most eukaryotic organisms. In humans they account for 40-50 % of the genome (Lander 2001). They do not encode genes and have a high mutation rate. In 1985 Sir Alec Jeffreys showed that tandem repeat polymorphisms in hypervariable minisatellite DNA in humans could be used to identify individuals genetically via 'genomic fingerprints'. This technique has since been widely used in the forensic sciences and to verify family relations (Jeffreys 1985a; Jeffreys et al. 1985b Jeffreys et al. 1985c; Jeffreys et al. 1987; Jeffreys et al. 1991 and Gill et al. 1987). Tandem repeat mutations were first used as a biomarker of germline genetic effects in 1993 by Dubrova and co-authors, who demonstrated that ionized radiation induced germline mutations in male adult mice. Dubrova et al. also showed that minisatellite germline mutations had been induced in humans exposed to radiation after the Chernobyl accident (Dubrova et al. 1996; Dubrova et al. 1997; Dubrova et al. 2002).

## Microsatellites and minisatellites

Repetitive DNA micro- and minisatellites are distinguished by the number of base pairs (bp) in their repeat units. Microsatellites, also known as simple tandem repeat or short tandem repeats (STRs) consist of 1-5 bp repeat units, typically repeated 5-30 times (Tamaki et al. 2005). Unstable GC-rich minisatellites or variable number tandem repeats (VNTR) in humans have repeat units from 10 to more than 100 bp in length. The DNA sequence in repeat units varies within arrays (Jeffreys et al. 1990). Tandem repeats have been associated with several human disease loci: Mutations in trinucleotide repeats have been linked to Fragile X Syndrome and other diseases (Wenstrom et al. 2002) and human minisatellites play a role in the regulation of important genes including: the HRAS1 protooncogene (Krontiris et al. 1993), the myoclonus epilepsy type I (Lafreniére et al. 1997), the human serotonin transporter gene (Heils 1996) and the IDDM2 gene associated with type I diabetes (Vafiadis et al.1997). Mutations in human minisatellites occur mainly in the germline and are caused by a complex process based on meiotic recombination, whereas the somatic mutation rate is low and mainly based on intra-allelic mechanisms (Yauk et al. 2002; Shanks et al. 2008). Human minisatellite germline mutations are in the order of 1 % per gamete (Armour 2006) and are believed to occur via an untargeted mechanism (Yauk 2004). No appropriate animal model has been found for human minisatellites: Pigs, rats and mice do not have any highly unstable minisatellites (Yauk 2004). Minisatellites in mice have a low germline

mutation rate (below $10^{-3}$ per gamete) (Bois et al. 1998) and their mutation mechanism is fundamentally different from that of human minisatellites (Bois et al. 2002).

**Expanded simple tandem repeats (ESTR)**

Expanded simple tandem repeats (ESTR) were at first believed to be murine minisatellites, but are now identified as their own distinct class of tandem repeats. ESTR have only been found in mice, intensive searches for unstable ESTR in other species have not been carried out (Yauk 2004). ESTR have a high spontaneous mutation rate both in germline and somatic cells (Kelly et al. 1989; Gibbs et al. 1993).

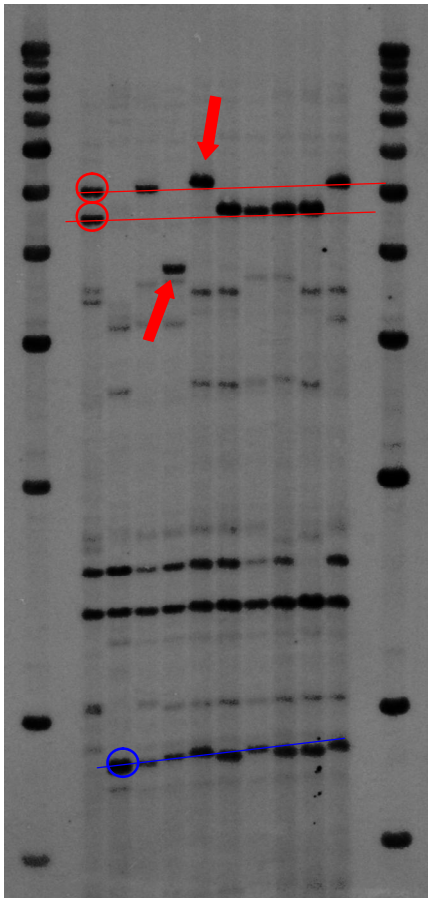


**Figure 6. Blot probed with Ms6-hm.**

First lane from the left is the F1 mother, second lane the F1 father. Lanes 3-7 are F2 offspring. The 2 maternal C57BL/6 Ms6-hm bands ( between 5-6 kb) are marked with red circles. Maternal mutations are indicated by red arrows (one large deletion and a small insertion).

The 2 paternal CBA Ms6-hm alleles ( between 1.6-2 kb) are marked with a single blue circle. F2 offspring for this family do not have any paternal Ms6-hm mutations.

ESTR consist of long homogeneous arrays (up to 20 kb) of short (<10 bp) repeat units (Gibbs et al. 1993; Yauk 2004). The size and mutation rate of ESTR loci are strain specific. The highest spontaneous mutation rate in inbred mice is found in BALB/c (~12% for Ms6-hm and Hm-2 combined) and decreases BALB/c>CBA>C57BL/6 (Barber et al. 2002). The variation in mutation frequency within inbred strains is low (Yauk et al. 2002). Initially some differences in stage specificity in male germ cell development were reported (Niwa et al. 1996). However, thorough studies have resolved that ESTR mutations are almost exclusively induced in premeiotic, mitotically dividing germ cells or mitotically active somatic cell (Dubrova et al. 1998; Barber et al. 2000; Barber et al. 2009). The ESTR mutation rate varies significantly between different tissues. Tissue with a high turn-over rate (high mitotic index) has a higher ESTR mutation rate, which supports the link to mitotic division (Barber et al. 2009; Dubrova et al. 1998). Mutation frequencies of ESTR increase in the following manner: Brain <spleen<sperm in adult mice. In proliferating brain tissue the ESTR mutation rate is high (Barber et al. 2009). Several studies on radiation-induced ESTR mutations have been performed (Dubrova et al. 1998; Yauk 2004; Somers et al 2006). High linear energy transfer (LET) radiation has a higher relative biological effectiveness than low LET radiation. At radiation doses <1 Gy ESTR mutations in premeiotic male germ cells are induced linearly in a dose-dependent manner. At doses > 1 Gy there seems to be a saturation effect (Somers et al. 2006). Radiation-induced ESTR mutation level in male germ cells correlates well with the specific locus tests, traditionally used for assessing mutations in the germline (Dubrova et al. 1998).

The ESTR Ms6-hm locus, also called PC-1 consists of a homogeneous array of (GGGCA)n repeats. It maps on chromosome 4, close to the brown coat color locus (Kelly et al. 1991). Most alleles exceed 400 repeat units (2 Kb) (Kelly et al. 1989; Kelly et al. 1991). The ESTR Hm-2 locus consists of (GGCA)n repeats. It is located on chromosome 9 and can contain up to 5000 repeat units (20 Kb) depending on mouse strain (Gibbs et al. 1993).

**Mechanisms of ESTR mutations**

No mechanism has been found to link exposure and induction of ESTR mutations. This makes results obtained by ESTR analysis harder to interpret. It has been suggested that the relatively small ESTR sites (~2-22 kb) are not a direct target for mutations. The mechanism is believed to be untargeted with ESTR mutations occurring far from the area of the genome directly affected. The damage signal is amplified by unknown processes and causes instability at ESTR loci (Somers et al. 2006). This is supported by the fact that directly targeted mutation events would require an unrealistically high number of radiation-induced DSBs in the genome. It has been calculated in several studies that the number of radiation damage events were too few (by a factor of ~100) to have caused the elevation in ESTR mutations

directly (Dubrova et al. 1998; Schiestl et al. 1994; Somers et al. 2006). The induction of ESTR mutations is believed to be a replication driven process, most likely caused by polymerase pausing or slippage (Barber et al. 2004; Yauk et al. 2002; Shanks et al. 2008a; Hardwick et al. 2009) promoted by the presence of hairpin structures (Weitzmann 1998). These secondary structures could cause misalignments between repeat units, which would result in gains or losses of repeats (Barber et al. 2006). Inflammation, ROS and epigenetic signaling causing DNA damage elsewhere in the genome could lead to delays in replication. (Somers et al. 2006; Yauk et al. 2008a). This process is similar to the way that mutations are believed to be induced in human microsatellites (Barber et al. 2006). It is likely that both spontaneous and induced ESTR mutations are caused by the same unknown mecanism: The ESTR mutation spectrum (pattern of the number of repeat units gained or lost) is identical for spontaneous and induced ESTR mutations. The induced ESTR mutation spectra between radiation and chemicals/particles are also identical (Dubrova et al. 2005; Yauk et al. 2008b). Studies on ESTR mutation rates in mice with impaired repair mechanisms for DNA double strand breaks (DSBs) and single strand breaks (SSBs) have shown that the spontaneous mutation rate is elevated 1.4-4.4 fold compared to wildtype mice of the same genetic background (Barber et al. 2004; Yamauchi et al. 2002). This suggests that DNA strand breaks may play a role in the development of ESTR mutations (Somers et al. 2006).

A number of chemicals have been shown to induce ESTR mutation (see table 3 in the discussion). Chronic exposure in C3H/10T1/2 mouse embryonic fibroblast cells to six chemicals exhibiting different types of genotoxicity: N-nitroso-N-ethylurea (ENU); benzo(a)pyrene (BaP); etoposide (ETOP); okadaic acid (OA); cisplatin (CisPt); and 5-azacytidine (5azadC). Induced ESTR mutation from 2-fold (ENU, BaP, ETOP), to 1.3-1.4 fold (OA, 5azadC), to nonresponsive (CisPt). All chemicals except the noninducer (CisPt) induced changes in global DNA methylation (Yauk et al. 2008b). Alkylating chemicals such as, ethylnitrosourea (ENU) and isopropyl methanesulfonate (iPMS) have been shown to induce ESTR mutations in premeiotic male germ cells. The topoisomerase II inhibitor, etoposide only caused ESTR mutations at meiotic stages (Vilarino-Guell 2003). A study by Hedenskog et al. indicated that polychlorinated biphenyls (PCB) may induce ESTR mutations in male germ cells (Hedenskog et al. 1997). Mainstream tobacco smoke (Yauk et al. 2007) and indoor air have also induced ESTR mutations in male germ cells (Zhou et al. 2009).

**ESTR mosaics and transgenerational instability**

Germline mosaics are common in ESTR loci (Gibbs et al. 1993) A germline mosaics is caused by a mutation that occurs very early in the diploid germ cells, which means that several offspring inherit the same mutant band. Somatic mosaics also occur frequently in ESTR loci. These are caused by mutations

in somatic cells in early embryogenesis and may result in offspring having additional bands, not present in either of their parents (Gibbs et al. 1993; Kelly et al. 1989). In the Ms6-hm allele mosaics are common with at least 3 % of mice having an additional mutant allele present in 60 % of somatic cells (Gibbs et al. 1993). Another occurrence observed in ESTR loci is transgenerational instability, which is a continued elevation in ESTR mutation rate several generations after the genomic insult took place (Barber et al. 2002; Barber et al. 2006; Barber et al. 2009).

# Methods

### Particles used in this thesis

The effects on germline mutations in prenatally exposed female mice were investigated using two engineered NPs that are used extensively worldwide: Titanium dioxide UV-Titan L-181 and carbon black Printex 90. Both are low solubility particles that were previously believed to be inert, but have now been classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (Baan et al. 2007). The characterization of UV-Titan and Printex 90 was performed by Keld A Jensen, Renie K Birkedal and Anni Vibenholt NRCWE. The analysis of distribution of titanium in tissue was performed by Erik H. Larsen and Katrin Löschner, DTU FOOD.

### Titanium dioxide (UV-Titan)

The titanium dioxide used in the inhalation study was UV-Titan L181 (Kemira, Pori, Finland) was supplied by The Danish Association of the Paint and Lacquer industry. Titanium dioxide ($TiO_2$) is a white pigment, which is produced by the industry in large quantities to be used in paints, plastics, cosmetics, food and other products. The worldwide production of $TiO_2$ was estimated at 4.4 million tonnes in 2004 (IARC Monographs, volume 93, 2010). Inhaled $TiO_2$ dust has been classified as possibly carcinogenic to humans (Baan et al. 2007): Sufficient evidence for carcinogenicity has been found in studies in rats exposed by inhalation and intratracheal instillation (IARC Monographs, volume 93, 2010). Evidence for carcinogenicity in humans is inadequate (IARC Monographs, volume 93, 2010) and there is currently no evidence of any link between $TiO_2$ exposure and cancer in the occupational setting (Boffetta et al. 2004; Ellis et al. 2010). Large amounts of nano$TiO_2$ are also used globally in different products such as paints and cosmetics. Nano$TiO_2$ is believed to be carcinogenic ( Mohr et al. 2006). However, results on nanoTiO2 induced mutations and genotoxicity are conflicting ( Trouiller et al. 2009; Johnston et al. 2009; Jackson et al. 2011b). $TiO_2$ toxicity depends on particle size, crystalline form and surface modifications (Johnston et al. 2009). Pulmonary exposure to nano$TiO_2$ generates inflammation in rodents (Bermudez et al. 2004; Manuscript I Hougaard et al. 2010). A single UV-Titan instillation of 54 µg induced an inflammatory response in mice after 1 day (Saber et al. 2011a) In the present study UV-Titan particles remained in lungs 4 weeks after inhalation, causing long-lasting inflammation (manuscript I Hougaard et al. 2010).

**Carbon black (Printex 90)**

The carbon black (CB) used in this study was Printex 90 (Degüssa, Frankfurt Germany), which is marketed as printing ink pigment. Carbon black is produced in large quantities worldwide, estimated to more than 10 million tonnes in 2005 (IARC Monographs, volume 93, 2010). The majority of CB is used as reinforcement in tires and other rubber products; CB is also used as a pigment in plastics, inks and paints (IARC Monographs, volume 93, 2010). CB Printex 90 consists of 99% pure carbon with less than 1% organic and inorganic impurities (Jacobsen et al. 2007), accordingly adverse health effects reported after Printex 90 exposure are assumed to be caused by the insoluble particle core rather than by associated compounds. CB is a well-characterized reference material in particle toxicology as a model of NP exposure and the carbon core of diesel emission particles. CB induces reactive oxygen species (ROS) (Jacobsen et al. 2007; Jacobsen et al. 2011), DNA strand breaks and DNA damage generated by oxidative stress (Jacobsen et al. 2008; Jacobsen et al. 2009). CB has been shown to be mutagenic *in vitro and in vivo* (Driscoll et al. 1996; Driscoll et al. 1997; Jacobsen et al. 2007; Jacobsen et al. 2010). Studies have shown that CB is carcinogenic in rats exposed by inhalation and intratracheal instillation and mice exposed by subcutaneous injection. Evidence for carcinogenicity in humans is inadequate (IARC Monographs, volume 93, 2010). It is therefore uncertain whether occupational exposure to CB is linked to risk of cancer (Morfeld and McCunney 2007).

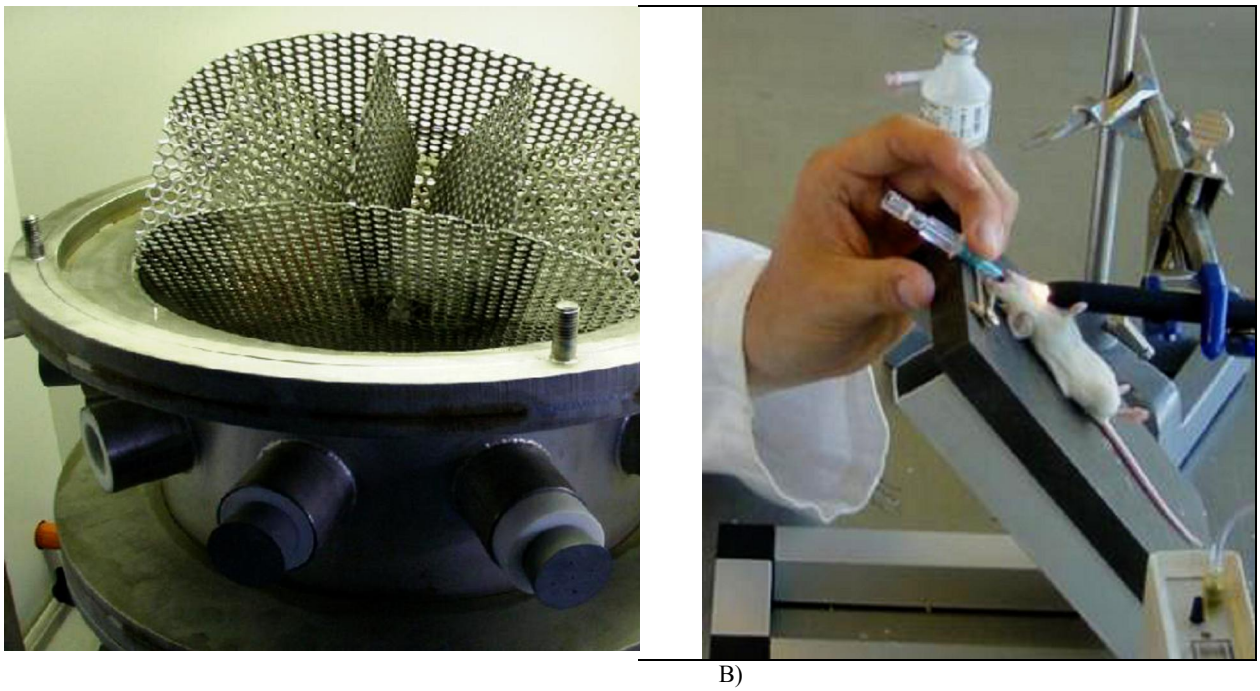
**Animal model**

Two strains of inbred mice were used in the present study: The C57BL/6JBomTac and the CBA/J. Time-mated C57BL/6JBomTac were used for the exposures. For the breeding of the F2 generation F1 C57BL/6JBomTac were mated with CBA. The C57BL/6JBomTac is an established model in the NRCWE. This makes it possible to directly compare results from this study to other particle exposures performed at the NRCWE. The C57BL/6JBomTac and CBA strains have a high fecundity (Silver 1995). This is an important parameter, when using a pedigree approach where ~200 offspring per group were needed. C57BL/6, CBA and C57BL/6JxCBA hybrids have been used in many ESTR studies because of the size difference between the Ms6-hm allele in the C57BL/6 and CBA strain (see figure 6), which makes it possible to determine the parental origin of ESTR mutations. The Ms6-hm allele for C57BL/6 was 5-6 kb long. A subgroup had Ms6-hm alleles at close to 7 kb. For the CBA strain the Ms6-hm allele was between 1.6-2 kb long. The Hm-2 alleles (4-5 kb) for C57BL/6 and CBA were not very different in

size in the present study. However, in the majority of cases it was still possible to determine parental origin of mutations in the Hm-2 allele.

**Exposure route -inhalation and intratracheal instillation**

Inhalation mimics the normal exposure route to particles in the occupational setting the best. In the present UV-Titan study pregnant mice were exposed by whole-body exposure. This was in order to avoid the added stress of restraining the mice. Whole-body exposure results in a considerable amount of particles being deposited in the fur of the animals as well. Due to grooming and fur licking the mice may be exposed to a higher dose than anticipated through the gastrointestinal tract. It is difficult to determine the precise dose that a mouse is exposed to during inhalation as it depends on the breathing pattern and deposition in the airways of the individual mouse. Intratracheal instillation is less time-consuming and more cost-effective than inhalation and the dose is delivered more precisely. Furthermore, the risk of laboratory contamination is minimized. The procedure of intratracheal instillation on pregnant mice did not adversely affect gestation parameters or offspring (Jackson et al. 2011a). The delivery of the instillation bolus into the lungs is checked with a pressure transducer to ensure correct delivery (Jacobsen et al 2009; Jackson et al. 2011a).The deposition pattern in instillation may be unnatural since the particles suspended in liquid is forced into the lungs of anesthetized mice.



B)

**Figure 7. Exposure methods.**

A) Inhalation chamber with steel cage.

B) Demonstration of intratracheal instillation (photo A from Petra Jackson photo B from Kenneth Klingenberg Barfod).

Particles are deposited deeper in the lungs compared to inhalation and particle-interaction with the upper airways, which may be important sites for toxicity, is bypassed (Oberdörster et al. 2004). However, potential exposure through the gastrointestinal tract is avoided. The choice of vehicle and the interaction of particles with the chosen vehicle should also be taken into consideration when interpreting instillation data. Studies have shown that the effects of the same particle given by inhalation and instillation can be more severe by inhalation (Driscoll et al. 2000). The inhalation and instillation exposures in the present study were performed by Petra Jackson, NRCWE.

**Cellular composition of bronchoalveolar lavage fluid**

Bronchoalveolar lavage (BAL) fluid was analyzed to determine the number and types of cells present (Hunninghake et al. 1979). The influx of inflammatory cells (e.g. neutrophils) into the lung lining fluid is a sign of inflammation. Maternal lung inflammation in generation P was assessed for both exposure studies. BAL fluid was collected from anaesthetized mice (Hypnorm-dormicum) by washing the lungs with 0.9% sterile NaCl through the trachea. The number of macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells were determined by differential cell count and presented relative to the total cell number in the BAL fluid. The BAL collection and analysis were done by Gitte Kristiansen and Lourdes Pedersen.

**The comet assay**

The Single Cell Gel Electrophoresis Assay (COMET) is a micro-electrophoretic assay that assesses DNA damage at the single cell level. It is a versatile technique that can be used to detect DSB, SSB and other kinds of DNA damage in a number of different tissues. Depending on the pH of the electrophoresis buffer, many different forms of DNA damage can be expressed. Cells are embedded in agarose and lysed. In the alkaline Comet assay the DNA is subjected to alkaline conditions in order to unwind the DNA and is then electrophoresed. The DNA strands with the most breaks will be smaller and thus migrate faster. After staining the DNA can be visualized in a fluorescence microscope. The extent of the damage is evaluated by the length of the tail of smaller DNA strands. (McNamee et al. 2000; Collins et al. 2009). DNA strand breaks are temporal occurrences that may be rejoined quickly by cellular repair mechanisms. The interpretation of DNA strand breaks as an endpoint is therefore highly dependent on the time of investigation (Collins et al. 2009).

A number of adaptations of the Comet assay exist: Following cell lysis the nucleotides are incubated with endonucleases that recognize specific types of non-strand break DNA damage/damaged bases such as oxidized purines and pyrimidines and cyclobutane pyrimidine dimers. The endonucleases convert the damage into SSB, which can then be estimated by the comet tail length. One such adaptation is the formamidopyrimidine-DNA glycosylase (FPG) for detecting oxidized purines (Karlsson et al. 2010).
The alkaline comet assay (pH >13) was used to evaluate the amount of strand breaks (DSB, SSB, excision repair sites, cross links, alkali labile sites) in BAL fluid and livers of time-mated females (generation P) and livers of F1 offspring in the Printex 90 exposure study (manuscript III Jackson et al. 2011d) and in the UV-Titan exposure study (Jackson 2011b, not part of this thesis). The level of oxidative damage to DNA in the liver of offspring exposed to carbon black by inhalation was assessed as FPG enzyme sensitive sites. The Comet analyses were performed by Petra Jackson, NRCWE.

**Methylation microarrays**

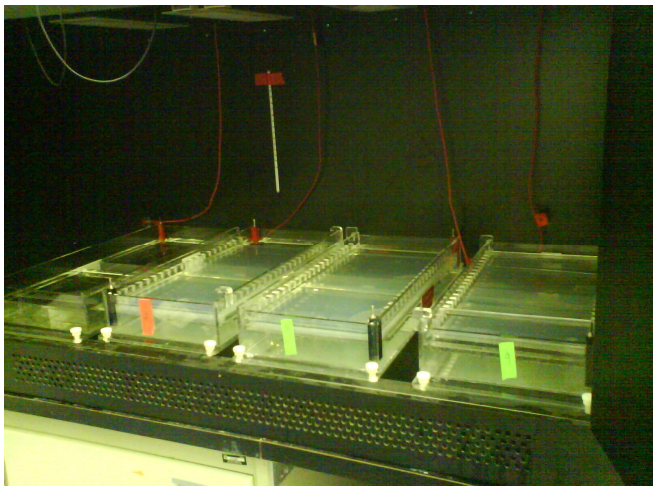
Epigenetics is the study of heritable changes in gene expression that are caused by functional modifications of the genome that do not involve changes in the underlying DNA sequence. DNA methylation, and histone deacetylation are examples of epigenetic mechanisms (Jirtle and Skinner 2007; Wadhwa et al. 2009; Hamilton et al. 2011).
To assess NP-induced changes in methylation patterns in the present set-up the genome wide methylation pattern of 6 Printex 90-exposed F1 females was compared to 6 F1 female controls. DNA extraction was performed on liver samples from F1 females with a kit from QIAGEN. Genomic DNA fragmentation was done by sonication and the enrichment of methylated DNA according to the Nimblegen protocol. Zdenka Orabi Kyjovska performed the DNA extractions. Sonication and enrichment of methylated DNA was done by Tomasz Wojdacz, University of Aarhus and the DNA methylation microarrays were done at Roche Nimblegen in Iceland. The preliminary results from the methylation microarray still need to be validated.

**Time-to-first-litter and number and sex of F2 offspring**

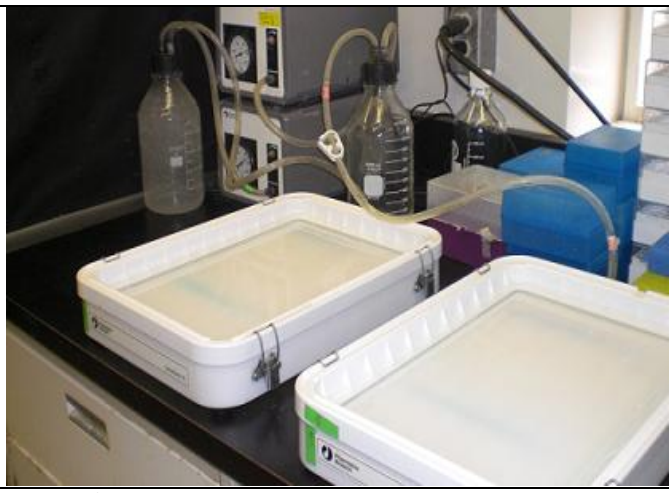
The time from when the F1 breeding pairs were mated until the birth of the first F2 litter was recorded as time-to-first-litter. The number of total offspring was determined on post natal day (PND) 2. The F2 offspring were sexed after euthanasia by looking at internal sex organs.

**Southern blotting and ESTR analysis**

Tail tissue from F1 parents and F2 offspring from the UV-Titan and Printex 90 studies was collected and DNA was extracted by phenol-chloroform extraction. Genomic DNA was digested to completion with restriction enzyme Alu1 to generate small fragments of DNA. digestions were checked on a short gel.



A Long gel chamber.



B) Vacugene transfer

**Figure 8. ESTR electrophoresis overview. (**Photos by Sanna Lemming).

Southern blotting (Southern et al. 1979) was performed by running digested DNA samples from F1 parents and F2 offspring on a 40 cm long 0.8 % agarose gels (SeaKem LE) in TBE buffer with ethidium bromide for 48 hours in a cooled chamber (air fans) at 130 Volts along with a 1 Kb ladder (Invitrogen, Burlington, Ont.) until the 1 Kb marker was off the gel. Gels were depurinated, denatured and neutralized and single strand DNA was transferred to a nylon membrane (GE Osmonics, Minnetonka, MN) by vacuum blotting using a VacuGene™ unit (see figure 8). The nylon blot was exposed to UV radiation to permanently attach the transferred DNA to the membrane. Nylon blots were soaked in hybridization solution at 65ºC and Ms6-hm probe + 1 Kb ladder/ Hm-2 + 1 Kb ladder labelled with freshly made radionucleotide $\alpha^{33}$P-dCTP and allowed to hybridize overnight. Blots were then washed with high stringency wash solution to get rid of excess probe. Washed blots were placed in a phosphoimage container with a phosphoimage plate. The autoradiograph was allowed to develop for 3 days before scanning with a Typhoon™ scanner to visualize the pattern of hybridization (size pattern of ESTR alleles). After scanning Ms6-hm probed blots were stripped with SDS before being reprobed with Hm-2. Blot image files were generated in Adobe Photoshop and printed for manual scoring of mutations. The Ms6-hm and Hm-2 alleles were scored relative to the 1 Kb ladder. Size differences in F2 alleles compared to F1 progenitor alleles of more than 1 mm was used as a cut off value to score mutations in

both parental alleles for both ESTR loci. This generic cut off point has been used successfully in other ESTR analyses (Somers et al. 2002; Yauk et al. 2008a; Ritz et al. 2011). ESTR bands were scored by 2 (Printex 90 study) or 3 (UV-Titan study) independent observers who were unaware of the exposure status of the families on the blots. In the current study identical mutant bands present in several offspring (germline mosaics) were scored as a single mutation event (see figure 9). Somatic mutations (new bands seen in tail tissue of F2, but not in the F1 progenitors) were not scored as mutations. Mutation rates were determined as number of mutant bands per total number of bands scored and compared using a one-tailed Fisher's test.

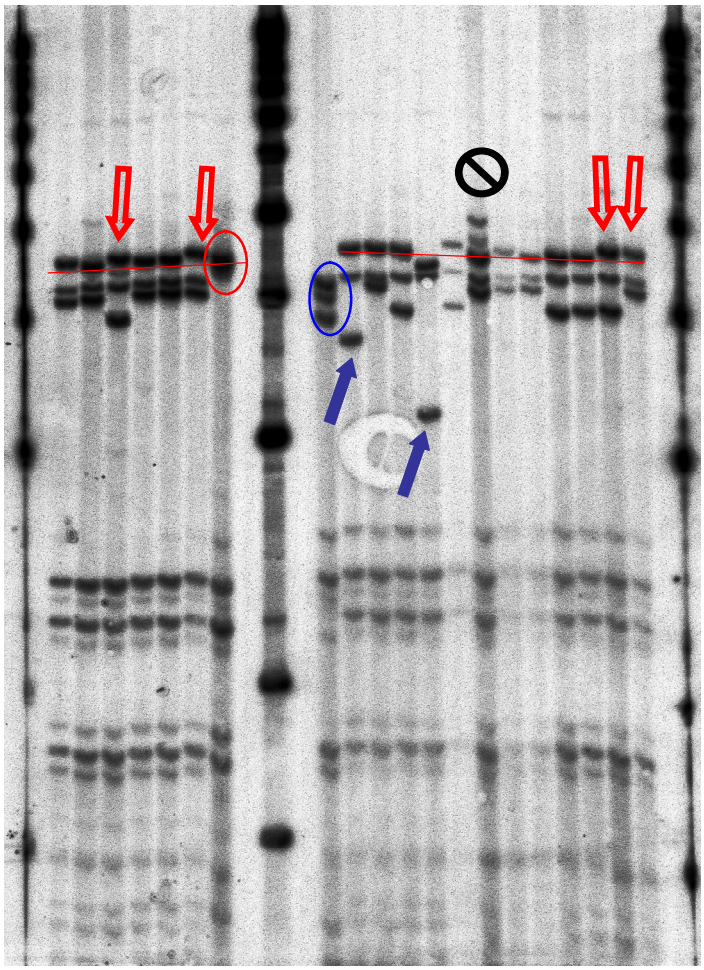


**Figure 9. Hm-2 probed blot.**

Hm-2 alleles are between 3-5 kb.

Maternal C57BL/6 and paternal CBA alleles are marked with red and blue circles respectively.

Blue arrows indicate singleton paternal mutations (two large deletion). Arrows outlined in red indicate a maternal germline mosaic mutation inherited by 4 offspring and scored as a single mutation event. The No Sign marks a lane with an incomplete digestion.

# Results

## Summary of manuscripts

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

Karin S Hougaard, Petra Jackson1, Keld A Jensen, Jens J Sloth, Katrin Löschner, Erik H Larsen, Renie K Birkedal,
Anni Vibenholt, Anne-Mette Z Boisen, Håkan Wallin and Ulla Vogel

**Hougaard et al. Particle and Fibre Toxicology 2010, 7:16**

**Aim:** To assess gestational and litter parameters, neurofunction and fertility in F1 offspring from time-mated female C57BL/6JBomTac mice exposed to UV-Titan by inhalation (1h/day to 42 mg/m$^3$ ($1.7 \cdot 10^6$ n/cm$^3$; peak-size: 97 nm) on GD 8-18.
To determine physicochemical particle properties and distribution of UV-Titan and maternal lung inflammation in time-mated females.

**Endpoints:** Gestation length. Weight of time-mated females and F1 offspring. Sex-ratio and viability of F1 offspring. Uterine implantations in time-mated females. Behavioral tests on F1 offspring. Fertility of male and female F1 offspring: time-to-first F2 litter. Physicochemical properties of UV-Titan. Distribution of UV-Titan in maternal and offspring tissues. Differential cell count of BAL in time-mated females.

**Results:** No differences in gestational and litter parameters were seen between the exposed group and controls. Male and female F1 offspring showed neurobehavioral alterations in adulthood. Prenatally exposed male F1 offspring displayed a non-significant delay in time-to-first-litter. UV-Titan particles consisted of mainly elongated rutile $TiO_2$ with an average crystalline size of 21 nm and were modified with Al, Si and Zr. In time-mated females 38 and 33 mg Ti/kg was detected in lungs at 5 and 26-27 days post-exposure, respectively. Thus, ~21-24% of predicted pulmonary deposition of UV-Titan could be accounted for. Titanium in livers of time-mated females and offspring was below the detection limit. Inhalation exposure to UV-Titan induced inflammation in time-mated female mice still present 26 days post exposure.

**Manuscript II: ôNano$TiO_2$ (UV-Titan) does not induce ESTR mutations in the germline of prenatally exposed female miceô**

Anne Mette Zenner Boisen, Thomas Shipley, Petra Jackson, Karin Sørig Hougaard, Håkan Wallin, Carole L. Yauk, Ulla Vogel

**Submitted to Particle and Fibre Toxicology 09-11-2011.**

**Aim:** To investigate the potential of UV-Titan of causing germline mutations in female F1 offspring from pregnant C57BL/6JBomTac mice exposed to UV-Titan by inhalation 1h/day to 42 mg/$m^3$ ($1.7 \cdot 10^6$ n/$cm^3$; peak-size: 97 nm) on GD 8-18 (Manuscript I).

**Endpoints:** ESTR mutation analysis in F2 offspring (pedigree analysis). Sex-ratio of F2, and F2 litter size.

**Results:** There was no difference in ESTR mutation frequency between the exposed group and controls. No difference was observed in sex-ratio or litter size between groups.

**Manuscript III:ôPulmonary exposure to carbon black in pregnant mice: Effects on DNA strand breaks in dams and offspringô**

PETRA JACKSON, KARIN SØRIG HOUGAARD, ANNE METTE Z. BOISEN, NICKLAS RAUN JACOBSEN, KELD ALSTRUP JENSEN, PETER MØLLER, GUNNAR BRUNBORG, KRISTINE BJERVE GUTZKOW, OLE ANDERSEN, STEFFEN LOFT,
ULLA VOGEL, & HÅKAN WALLIN

**Nanotoxicology, 2011; Early Online, 1ï 15**

**Aim:** To assess the effect on time-mated females and their prenatally exposed offspring of carbon black Printex 90 exposure by inhalation (42 mg/m$^3$/h/d on GD 8-18) or instillation (4 doses on GD 7,10,15,18. Total doses 11, 54 and 268 µg/animal).

**Endpoints:** For inhalation and instillation exposure: Gestational and lactational parameters. Lung inflammation in time-mated females (BAL), DNA strand breaks in BAL fluid (time-mated females) and livers (time-mated females and their offspring). For inhalation only: Oxidative DNA damage in F1 offspring exposed to Printex 90 (FPG sensitive sites).

**Results:** Exposure to Printex 90 by inhalation and the highest instilled dose of 268 µg/animal caused long-term maternal lung inflammation. Inhalation exposure to Printex 90 increased DNA strand breaks in time-mated females and offspring. There was no increase in DNA strand breaks in mice exposed to CB by instillation. There was no oxidative DNA damage in F1 offspring exposed to Printex 90 by inhalation assessed by FPG sensitive sites,

**Manuscript IV: ôNanosized carbon black (Printex90) does not induce ESTR mutations in germ cells of female mice exposed *in uteroô***

Authors: Anne Mette Zenner Boisen, Thomas Shipley, Petra Jackson, Karin Sørig Hougaard, Håkan Wallin, Christine Nellemann, Carole L. Yauk, Ulla Vogel.

**Draft manuscript not yet submitted.**

**Aim:** To investigate the potential of carbon black to cause germline mutations in female F1 offspring from pregnant C57BL/6JBomTac mice exposed to carbon black Printex 90 by instillation (4 doses on GD 7,10,15,18. Total dose 268 µg/animal) (Manuscript III).

Endpoints: ESTR mutation analysis in F2 offspring (pedigree analysis). Sex-ratio and litter size of the F2 generation.

Results: There was no difference in ESTR mutation frequency between the prenatally exposed F1 females and controls. There was no differences in sex-ratio or litter size between groups.

**Other results not yet reported:**

**Time to first F2 litter from F1 fathers Printex 90 study:**

Fertility of F1 males prenatally exposed to Printex 90 did not differ between groups.

**Epigenetic data from the Printex 90 study:**

Preliminary data from a micro array based genome wide methylation study of the F1 female offspring prenatally exposed to Printex 90 showed that 270 regions of the genome display different methylation status when comparing controls and Printex 90 exposed mice. These data still need to be validated.

**Table 1. Summary of effects in Generation P dams and females and F1 females**

| | $TiO_2$ UV-Titan inhalation<br>42 mg/m$^3$<br>1 h/day x 11 days | CB Printex 90 (instillation)<br>268 µg/animal |
|---|---|---|
| Generation P | **Lung inflammation**[a)]<br>19-fold ↑PMN [1)]<br>3-fold ↑PMN [2)]<br><br>**Gene expression** [b)]<br>Lungs –inflammation,immune and acute phase response [1)]<br>Liver – minor changes [1)]<br><br>**Ti in tissue**[a)]<br>38 mg/kg and <0.5 mg/kg Ti in lung and liver<br>33 mg/kg and 0.5 mg/kg Ti in lung and liver<br><br>**Genotoxicity**[c)]<br>BAL –none [1,2)]<br>Liver-none [1,2)] | **Lung inflammation**[d)]<br>29-fold ↑PMN [1)]<br>61-fold ↑PMN [2)]<br><br>**Gene expression**[e)]<br>Lungs-cytokines and chemokines<br><br>**Genotoxicity**[d)]<br>BAL –none [1,2)]<br>Liver-none [1,2)] |
| Generation F1 females | **Ti in tissue**[a)]<br><0.4 mg/kg Ti in liver [3,4)]<br><1 mg/kg and 0.5 mg/kg Ti in milk [3)]<br><br>**Gene expression**[c)]<br>Liver- Changes in RA Signaling in F1 females<br><br>**Genotoxicity**[c)]<br>Liver-none [3,4)] | **Gene expression**[e)]<br>Liver- Changes in cellular signaling, inflammation, cell cycle and lipid metabolism in F1 females[3)]<br><br>**Genotoxicity**[d)]<br>Liver-none [3,4,5]<br><br>**Gene methylation** [f)]<br>Liver-270 regions of genome differentially methylated in exposed group [6)] |

1) Generation P Females ( ~4 days post exposure) 2) Generation P Dams ( ~26 days post exposure) 3) F1 newborns 4) F1 at weaning 5) F1 adolescents 6) adults a) Hougaard et al. 2010 b) Halappanavar et al. 2011 c) Jackson et al. 2011b d) Jackson et al. 2011c e) Jackson et al. 2011 PMN: Polymorphonuclear leukocytes, RA: Retinoic acid.

# Discussion

The aim of this thesis was to investigate if prenatal exposure to the NPs UV-Titan and Printex 90 would induce mutations in the female germline by using a method that has proven to be very sensitive in germline studies of adult male mice (Yauk et al. 2008a; Somers et al. 2006). Dams of generation P (figure 1) were exposed by whole-body inhalation 1 hour a day to 42 mg/m$^3$ UV-Titan on GDs 8-18 or by intratracheal instillation to a total dose of 268 µg/animal on GDs 7,10,15 and 18. $TiO_2$ UV-Titan and CB Printex 90 did not induce ESTR mutations in the F2 offspring of prenatally exposed F1 female mice under the current conditions (manuscripts II, IV and table 4). Results from the exposure that predated the ESTR studies (directly exposed generation P and their F1 offspring) are presented in table 1.

### Characterization of UV-Titan and Printex 90

The analysis of particle characteristics is very important due to their potential influence on toxicity. Exposure to different types of $TiO_2$ and CB (Saber et al. 2011a; Saber et al. 2011b; Jiang et al. 2008; Warheit et al. 2007) has resulted in different toxic effects. Consequently, comprehensive particle characterization makes interpreting results from different laboratories easier. $TiO_2$ UV-Titan was characterized in manuscript I (Hougaard et al. 2010) and CB Printex 90 in manuscript III (Jackson et al. 2011d). Both the UV-Titan inhalation study and the Printex 90 inhalation study described in manuscript III Jackson et al. 2011d used a concentration of 42 mg/m$^3$. Due to the smaller particle size of Printex 90 more particles were estimated to deposit in the pulmonary region compared to UV-Titan (figure 4) 287 µg/animal for Printex 90 and 73 µg/animal for UV-Titan respectively (model revised from Jacobsen et al. 2009 result in manuscript I Hougaard et al. 2010 and manuscript III Jackson et al. 2011d). The Printex 90 instillation dose of 268 µg/animal for the ESTR study was chosen to be directly comparable to the Printex 90 inhalation study. Table 2 gives a summary of the main particles characterization results of the UV-Titan (inhalation) and Printex 90 (instillation) used for maternal exposure in the ESTR studies.

Both $TiO_2$ and CB are classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (Baan et al. 2007). Evidence for carcinogenicity in humans for both NPs is inadequate. There is sufficient evidence for carcinogenicity in experimental animals for both $TiO_2$ (rats) and CB (rats and mice) (IARC Monographs volume 93 2010). CB Printex 90 is mutagenic in a transgenic epithelial mouse cell line (Jacobsen et al. 2007; Jacobsen et al. 2009).

**Table 2. Characterization of UV-Titan and Printex 90**

| Particles | $TiO_2$ UV-Titan | CB Printex 90 |
|---|---|---|
| Declared particle size from the manufacturers | 17 nm [a)] | 14 nm [c)] |
| Size measurements from the present studies | 97 nm geometric mean size [b)] | 50-60 nm hydrodynamic size distribution<br>139 nm average zeta-size [d)] |
| Surface area (BET) | 107.7 $m^2/g$ [b)] | 295-338 $m^2/g$ [e,f)] |
| Phase | Rutile [a,b)] | amorphous |
| morphology | Equidimensional to needleshaped aggregates and agglomerates of $TiO_2$ crystallites with diameters from <10 to >100 nm along the shortest and longest axes, 50 % < 97 nm. [b)] | Spherical to subspherical carbonaceous agglomerates and minor amounts of free single primary spheres [d)] |
| Chemical composition | $Na_2O$ 0.60 wt%<br>$SiO_2$ 12.01 wt%<br>$Al_2O_3$ 4.58 wt%<br>$ZrO_2$ 1.17 wt%<br>$TiO_2$* 70.81 wt%[b)] | 99% C<br>0.8% N<br>0.01% $H_2$<br>[f)] |
| Mass-size distribution | 75%>1600 nm and <1%<100 nm [b)] | Volume distribution in vehicle:<br>Peaks of 50-60 nm and 200-400 nm as well as minor amounts of µm-size particles [d)] |
| Particle number conc. | $1.70\pm0.20x10^6/cm^3$ [b)] | |
| Total inhaled/instilled dose | Inhalation: 840 µg/animal [b)] | Instillation: 268 µg/animal [d)] |
| Estimated deposition | 73 µg/animal in pulmonary region<br>315 µg/animal in extra-pulmonary region<br>365 µg/animal in gastro-intestinal tract [b)] | 268 µg/animal in pulmonary region [d)]<br>Comparable to total inhaled dose of CB in Jackson et al. 2011b |

* UV-Titan is coated with polyalcohol adding to the remaining wt%. a) Kemira b) Hougaard et al. 2010 c)Degussa-Hüls d) Jackson et al. 2011b e) Saber et al. 2005 f) Jacobsen et al. 2008

**Doses of UV-Titan and Printex 90**

The UV-Titan and Printex 90 doses in the present prenatal studies are comparable to doses of chemicals that have induced germline mutations in adult males (Vilarino-Guell et al. 2003) (see table 3). Time did not permit me to investigate ESTR mutations in the F1 male offspring, so it is unresolved if the present two exposure scenarios caused ESTR mutations in the male germline. Ritz et al. 2011 showed that prenatal exposure to 19 $mg/m^3$ diesel exhaust particles (DEP) induced ESTR germline mutations in males, but not females. The 1.98-fold increase observed in DEP-exposed females was not statistically significant. In the present study we used more than a 2-fold increase in concentration (42 $mg/m^3$ UV-Titan and a corresponding instilled dose of Printex 90). The daily exposures in the present study were low enough to be comparable to a real life situation and corresponded approximately to 0.5 day ($TiO_2$) and 1.5 days (CB) at the current 8-hr time weighed average for the Danish occupational exposure limit of 6 mg titanium/$m^3$ (=9.75 mg/ $m^3TiO_2$) and 3.5 mg CB/$m^3$ (The Danish Working Environment Authority 2007).

**Exposure routes and direct and indirect effects of nanoparticles**

Dams in the UV-Titan study were exposed by whole-body inhalation to avoid restraint-stress. This most likely increased the load of NPs to the gastrointestinal tract due to fur grooming. Exposure via the gastrointestinal tract may be able to induce ESTR germline mutations (Somers et al. 2008). Inhalation is a more realistic exposure route, but, unlike with intratracheal instillation, the precise amount of NPs that the animal is exposed to is uncertain. In the UV-Titan study we exposed the mice by intratracheal instillation to ensure that the potential effect was caused primarily by exposure via the airways.
Translocation of inhaled titanium to the liver of dams and the potential translocation to offspring was below the detection limit (manuscript I Hougaard et al. 2010). The extra-pulmonary Printex 90 concentration was not analyzed in view of the difficulties in measuring carbon-based particles in biological systems. Due to the very low amount of NPs that is believed to be able to translocate (Kreyling et al. 2002) it is more likely that the particle-induced SBs in secondary target organs (liver) and fetuses that we have shown in the Printex 90 inhalation study (manuscript III Jackson et al. 2011d) are caused by particle-elicited inflammation and oxidative stress (secondary genotoxicity). The differences in gene expression in F1 offspring in the UV-Titan and Printex 90 inhalation/instillation studies are also believed to be caused by indirect effects (Jackson et al. 2011b; Jackson et al. 2011c).

**Possible causes of induced ESTR mutations**

**Inflammation, ROS and DNA strand breaks**

ESTR mutations are believed to be induced via polymerase pausing resulting from a non-targeted mechanism resulting from epigenetic changes or DNA damage such as strand breaks, adducts or oxidative stress elsewhere in the genome (Yauk et al. 2008a; Somers et al. 2006). The UV-Titan and Printex 90 exposures produced a pulmonary inflammatory response in generation P. However, No SBs were seen in generation P and F1 at the time-points tested in the UV-Titan and Printex 90 studies predating the ESTR analyses (see table 1). Due to the transient nature of DNA strand breaks, it is possible that strand breaks occurred at earlier time points and were repaired. UV-Titan produces long-lasting inflammation in mice (manuscript I Hougaard et al. 2010) and Printex 90 is a potent inducer of inflammation, acute phase response, ROS and ROS-induced mutagenicity (Jacobsen et al. 2009; Jacobsen et al. 2011d; Saber et al. 2011a). Yauk et al. 2008a found that exposure to polluted air produced SBs in sperm collected 3 and 10 weeks after exposure. No SBs were found after 16 weeks, at the time

when ESTR mutations were significantly increased 1.6-fold. It is therefore possible that after 16 weeks SBs had been repaired, but that they may have elicited the original signal which caused the increase in ESTR mutations.

**Epigenetics**

It's possible that ESTR mutations are elicited through epigenetic signaling such as changes in methylation patterns, Yauk et al. 2008a found that exposure to polluted air caused global hypermethylation as well as ESTR mutations in sperm cells. Chemicals, which induced ESTR mutations *in vitro*, also changed methylation patterns (Yauk et al. 2008b). Preliminary data indicate that the Printex 90 exposure induced methylation pattern changes in 270 genomic regions in the liver of F1 females from the present study compared to controls (unpublished results). However, these epigenetic changes did not result in an increase in ESTR mutations, possibly because of erasure of epigenetic marks in F1 oocytes (Barber et al. 2009). Global methylation imprints in the maternal and paternal genome are erased during early embryogenesis in a sex-dependent manner (Adler et al. 2007). The case for an epigenetic mechanism in other studies is strengthened by the fact that germline instability can be transferred through both parental alleles, even though only the paternal progenitor was exposed originally (Barber et al. 2002; Niwa et al. 2000). Ritz et al. 2011 also observed a non-significant trend of elevated ESTR mutation rate in the unexposed maternal allele. The only study to date, which has shown induced ESTR mutations in female germ cells (Barber et al. 2009), used a pedigree approach, where mutations were scored in somatic tissue of F2 male offspring only. Studies have shown that epigenetic change can be sex-specific (Wijchers et al. 2011). I therefore recalculated the ESTR mutation rates for the UV-Titan study by F2 sex (mutations in F2 males/ total number of F2 males and mutations in F2 females/total number of F2 females) to investigate a possible sex-specific effect. There was no effect in offspring divided by sex. The mutation rates for both sexes combined (table 4) and sex-divided ratios were very similar (data not shown).

Barber et al. 2009 showed that prenatal exposure to radiation similarly elevated ESTR mutation rates 2-fold in both the male and female germ line. ESTR mutations were also induced in proliferating somatic tissues in both F1 males (bone marrow, brain, sperm) and females (bone marrow, brain) exposed prenatally. F2 descendants of F1 males displayed transgenerational instability with elevated ESTR mutation frequencies in bone marrow, brain and sperm compared to controls. However, in F2 descendants of F1 females there was no transgenerational instability and ESTR mutation rates were similar to those in controls. The authors suggest that this sex difference in transgenerational instability may have been caused by the passive erasure of epigenetic marks that takes place in the maternal genome (Barber et al. 2009). Prenatally exposed females were thus less sensitive than males in terms of

transgenerational instability and it is possible that differences in epigenetics between the sexes may play a role in the lack of sensitivity to ESTR mutations in females exposed to UV-Titan and Printex 90 seen in this study.

**The method of ESTR analysis**

**Technical considerations**

Gels were run at 130 V for 48 hours (3.25 V/cm) to obtain maximum resolution of the DNA fragments (Sambrook, E.F. Fritsch, T. Maniatis 1989). For the majority of the C57BL/6 the maternal Ms6-hm allele was 5-6 kb long. A subgroup had maternal Ms6-hm alleles at close to 7 kb. The Hm-2 allele was between 4-5 kb. The upper limit of efficient separation of linear DNA for a 0.8% agarose gel is somewhere between 7-10 kb (Sambrook, E.F. Fritsch, T. Maniatis 1989).

Mutation analysis is very dependent on the quality of the bands. Even though the 1 mm criterion was used as a general rule, the scoring of a mutation is subjective in many cases, especially in smaller length mutations, which are the most common (Kelly et al. 1991). All scoring was done blinded to exposure status and only when two (Printex 90 experiment) or three (UV-Titan experiment) independent observers had agreed on which offspring were mutants was the exposure status made known. Each gel was loaded with at least three lanes of 1 Kb ladder to aid in scoring. The use of in-lane standards would aid in making measurements more precise. If time had permitted more copies of parental samples could have been loaded on the gels to aid in the scoring. Test samples were run on different gels on different days and yielded the same result in terms of position of parental alleles and number of mutations in offspring.

**Comparing ESTR results from different laboratories**

Several studies have shown that spontaneous and induced ESTR mutation rates vary between strains (Barber et al. 2002; Dubrova et al. 2005). The highest mutation rate in inbred mice is found in BALB/c and decreases BALB/c>CBA>C57BL/6 (Barber et al. 2002). The Ms6-hm allele in the CBA strain is 1.6-2 kb compared to 5-7 kb in C57BL/6. For this reason the CBA strain may be a more sensitive model for scoring ESTR mutations than C57BL/6, which was used in the present study. Even though repetitive DNA is difficult to sequence the smaller allele size might make sequencing or high-resolution melting analysis of ESTR alleles possible (Vossen et al. 2009).

Properties of the electrophoresis step (gel length, voltage, agarose concentration) determines how well resolved changes in ESTR allele size are, which has an impact on the number of alleles identified as mutated (Dubrova et al. 2005; Somers et al. 2006). The criteria for scoring mutants also differs between

| Exposure | Model | Effect | study |
|---|---|---|---|
| ethylnitrosourea, (ENU) bleomycin, cyclophosphamide, mitomycin C, and procarbazine. | *In vivo* adult male germline | All chemicals induced ESTR mutations | Glen et al. 2008 |
| PCB and diesel | *In vivo* adult male germline | PCB alone and PCB + diesel induced ESTR mutations. Diesel alone did not. | Hedenskog et al.1997 |
| Formaldehyde | *In vivo* adult male germline | Induced ESTR mutations | Liu et al. 2009 |
| Tobacco smoke | *In vivo* adult male germline | Induced ESTR mutations | Yauk et al. 2007<br>Marchetti et al. 2011 |
| N-nitroso-N-ethylurea(ENU), benzo(a)pyrene (BaP), okadaic acid and etoposide | *In vitro* embryonic cell line | All except okadaic acid Induced ESTR mutations | Polyzos et al 2006a |
| TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin; | *In vivo* adult male germline | No statistically significantly induced ESTR mutations | Ryo et al. 2006 |
| N-nitroso-N-ethylurea (ENU);<br>benzo(a)pyrene (BaP);<br>etoposide (ETOP);<br>okadaic acid (OA);<br>cisplatin (CisPt);<br>5-azacytidine (5azadC). | *In vitro* embryonic cell line | All except (CisPt) Induced ESTR mutations | Yauk et al. 2008b |
| Mixed indoor pollutants from residential air | *In vivo* adult male germline | Induced ESTR mutations | Zhou et al.2009 |
| ethylnitrosourea (ENU) isopropyl methanesulfonate(iPMS) etoposide | *In vivo* adult male germline | Induced ESTR mutations | Vilarino-Guell 2003 |
| Ambient particulate air pollution | *In vivo* adult male and female germline | Induced ESTR mutations in the male germline. Not in the female. | Somers et al. 2002<br>Somers et al. 2004 |
| Ambient particulate air pollution | *In vivo* adult male germline | Induced ESTR mutations | Yauk et al. 2008a |
| 1 Gy of acute radiation | *In vivo* prenatal exposure of male and female germline | Induced equal levels of ESTR mutations in the male and female germline. Transgenerational instability induced in males, but not in females. | Barber et al. 2009 |
| Diesel exhaust particles SRM NIST 2975 | *In vivo* prenatal exposure of male and female germline | Induced ESTR mutations in the male germline. Not in the female. | Ritz et al. 2011 |

**Table 3. Examples of exposures that have induced ESTR mutations**

various groups. Cut-off values in the size difference between offspring and progenitors that is interpreted as a mutation range between 30-200 bp (Yauk et al. 2002; Niwa et al. 1996; Hedenskog et al. 1997). Direct comparisons of mutation rates should therefore only be done within the same laboratory group and mouse strain. A set of universal guidelines for the above considerations to make studies more comparable has been proposed (Somers et al. 2006). The 3 only studies on particle-induced ESTR

mutations in prenatally exposed females Ritz et al. 2011 and manuscript II and IV of this thesis have all been done in the same laboratory, during the same time period, using the same mouse strain, laboratory equipment, protocol and scoring criteria (see table 4). This set-up was sensitive enough to see a 2-fold increase in prenatally exposed male mice. In the present study the mutation rates between exposed groups and controls were very similar, which makes it unlikely that we failed to statistically detect a small fold induction in mutation rate. Furthermore, the groups of F2 offspring used in the present study were more than double in size compared to Ritz et al. 2011. To make scoring consistent between this study and Ritz et al 2011, the same individual (blinded to exposure status) was one of two (or three) people, who analysed all 3 studies.

**The significance of ESTR mutation analysis**

The spontaneous mutation rate of ESTR *loci* is considerably higher than the *loci* used in traditional germline mutagenesis tests such as the dominant lethal assay and the specific *locus* test (Somers et al. 2006). Consequently, a relative low number of animals is necessary and realistic exposure scenarios such as low doses, mixtures or in situ exposure to pollutants can be tested using ESTR analysis ( Zhou et al. 2009; Yauk 2004; Yauk et al. 2008a; Marchetti et al. 2011). Exposure to a diverse group of mutagens such as radiation, airborne particles and various chemicals with different modes of action seems to induce ESTR mutations by the same unknown mechanism (Somers et al. 2006; Singer et al. 2006; Yauk et al. 2004).

Even though ESTR *loci* do not code for genes and an elevated ESTR mutation rate does not seen to adversely effect mice, instability at ESTR *loci* are an indication that the germline is being affected. Furthermore, many studies have linked instability in DNA repeat *loci* with diseases in humans (Kronritis et al. 1993; Wenstrom et al. 2002). It is possible that *loci* similar to the ESTR are present in the human genome. If this is the case, new sensitive biomarkers, directly relevant to humans may be developed. However, more mechanistic knowledge on ESTR mutations is needed to interpret results obtained by this method. To this end correlative studies on other genetic endpoints and ESTR mutations may be undertaken (Somers et al. 2006).

**The female germline as a target for mutations**

The study of germline mutagens in females have been hampered by the limited amount of oocytes available and the low spontaneous mutation rate of oocytes, which mean that a very large number of offspring are required from exposed females for the traditional germline mutagenicity tests (Adler et al.

2007; Russell and Russell 1992). In contrast, all developmental germ cell stages can be obtained from adult male mice by controlling mating intervals post-exposure (Dubrova et al. 1998; Adler et al. 2007). Even though ESTR mutations may be induced in the female germline (Barber et al. 2009) the results of the present study and that of Ritz et al. 2011 indicate that premeiotic female germ cells are less susceptible than male to particle induced mutations. However, vulnerability to mutations has been shown to be cell stage specific in many tests: Some research suggests that the maternal germline is more vulnerable than the male during conception. The alkylating agent ethylnitrosourea (ENU) is more mutagenic in the maternal genome than the male within the zygote (Adler et al. 2007). Furthermore, in the event of fertilization by sperm with an elevated ESTR mutation rate the female genome may be exposed indirectly to genomic destabilization via the paternal genome (Niwa et al. 2000; Barber et al. 2002) and the resulting mutations may be transferred to future generation through both the male and female germline (Barber et al. 2002). NP exposure could thus result in genomic instability from the paternal genome being transmitted through female lines. To date only radiation, diesel SRM 2975, UV-Titan and Printex 90 have been tested for their ability to cause ESTR mutations in developing female germ cells. Before dismissing the potential of particles to cause ESTR mutations in the female germline a highly potent germ cell mutagen such as ENU should be tested.

**Time points chosen in the present study**

In the present ESTR studies we targeted GD 8-18 and 7-18. Female germ cells are mitotically dividing until GD 13.5 and may be the most susceptible to ESTR mutations at this time (Barber et al. 2009). However, the necessity of replication for mutation fixation in eggs has not been established for ESTR *loci* and we therefore targeted all stages of prenatal female gametogenesis. We estimated that a sufficient amount of inflammation and ROS would be present prior to GD 13.5 to induce ESTR mutations in females as Baber et al. 2009 showed acute radiation-induced ESTR in prenatally exposed females at GD 12.

Both Barber et al. 2009 and the present studies used a pedigree approach and estimated F1 germline ESTR mutations in somatic tissue of F2 offspring. This raises the possibility that exposure to UV-Titan and Printex 90 could have induced damage in germ cells and that this causes them to be degraded. Very little is known about the cause of oocyte atresia (Pepling 2006), but studies have indicated that oocytes may be susceptible to damage by ROS (Menezo et al. 2010).Exposure to cigarette smoke can induce loss of primordial follicles (Tuttle et al. 2009) and prenatal exposure to cigarette smoke has been shown decrease the number of granulosa cells critical for follicle development (Lutterodt et al. 2009). I did not find any decrease in litter size or time-to-first-F2-litter in the exposed groups in the two studies, which indicates that there was no effect on fertility.

**Table 4. Summary of ESTR mutation rates in F2 offspring of female C57BL/6 mice prenatally exposed to DEP, $TiO_2$ or CB**

| Groups | N (F2 offspring) | Mutant bands | | Mutation rate ± SEM (P value [a]) | |
|---|---|---|---|---|---|
| | | Paternal origin | Maternal origin | Paternal origin | Maternal origin |
| **1) DEP Female controls** | 79 | 9 | 5 | 0.057±0.026 | 0.032±0.020 |
| **DEP Female exposed** | 72 | 5 | 9 | 0.035±0.022 (P=0.261) | 0.063±0.029 (P=0.16) |
| **2 $TiO_2$ Female controls** | 164 | 20 | 12 | 0.061±0.00281 | 0.037±0.00297 |
| **$TiO_2$ Female exposed** | 192 | 18 | 11 | 0.047±0.01068 (P=0.84) | 0.029±0.01328 (P=0.79) |
| **3 CB Female controls** | 253 | 19 | 12 | 0.038±0.00150 | 0.024±0.00168 |
| **CB Female exposed** | 178 | 19 | 9 | 0.053±0.00250 (P=0.17) | 0.025±0.00157 (P=0.53) |

[a] Fisher's exact test 1-tailed

Groups: 1) Ritz *et al*. 2011 2) Boisen unpublished results 3) Boisen unpublished results.

However, in view of the high fecundity of rodents, the degradation of damaged oocytes may not have been large enough to have an impact on litter size. Several studies have shown that exposure to radiation has an impact on the duration of fertility in female mize, but little impact on litter size (Russell and Russell 1992). Moreover, exposure to 29 chemicals at the maximum tolerated dose in female mice did not affect the lifetime reproductive capacity in 41 % of cases (Adler et al. 2007). Studies using the SM-PCR method for analyzing ESTR mutations directly in the male germline have found the mutation rates to be similar to those found in offspring of exposed males (Yauk et al. 2002). However, in females this has never been investigated.

Another possibility is that the amount of accumulative damage in the UV-Titan and Printex 90 studies was not large enough to induce ESTR mutations. In Barber et al. 2009 a single dose of 1 Gy was delivered at GD 12. A dose protraction effect has been seen in mature and maturing murine oocytes exposed to several small versus 1 large dose of radiation (Russell and Russell 1992). Oocytes have a high capacity for DNA repair and the pre-mutational destabilizing events may have been repaired before or during fertilization.

**Types of nanoparticles used**

Few studies on ESTR mutations induced by particles or chemicals have been conducted (see table 3). It is possible that UV-Titan and Printex 90 do not induce ESTR mutations. It is not known if exposure to $TIO_2$ or CB affects ESTR mutation rates in the male germline. Not all substances tested have induced ESTR mutations: In Polyzos et al. 2006a okadaic acid, which is a kinase inhibitor and a potent tumour promoter failed to induce ESTR mutations *in vitro* (see table 3). However, the high reactivity and inflammogenic potential of NPs make them good candidates for the induction of ESTR mutations. Results from our group at the NRCWE showed that a single UV-Titan instillation induced an inflammation after 1 day (Saber et al. 2011a) and UV-Titan particles in the present study were retained in lungs of generation P mice 4 weeks after inhalation, causing long-lasting inflammation (manuscript I, Hougaard et al 2010). The change in hepatic gene expression seen in F1 offspring prenatally exposed to UV-Titan in the present exposure scenario (Jackson et al. 2011b) is likely to have been caused by this maternal inflammation (shown by neutrophil recruitment and increased expression of inflammation and acute phase response related genes (Manuscript I Hougaard et al. 2010; Halappanavar et al. 2011)). Prenatally exposed F1 females exhibited a larger transcriptional response than F1 males (Jackson et al. 2011b).

Our studies have also shown that Printex 90 is mutagenic *in vitro* (Jacobsen et al. 2011) and that it can produce long-lasting inflammation, acute phase response, retention of particles in lungs of exposed mice as well as oxidative stress (Jackson et al. 2011c; Jackson et al. 2011d; Jacobsen et al. 2009; Saber et al. 2011a). Hepatic expression in F1 offspring prenatally exposed to Printex 90 in the present instillation study revealed that considerably more genes were differentially expressed in exposed females (476 genes) than in exposed males (17 genes) (Jackson et al. 2011c) and preliminary data indicate that the same exposure induced methylation pattern changes in several hepatic genes in F1 females (unpublished results). Differentially expressed genes in females were related to inflammation and cell regulation and thus these data support the notion that Printex 90 NPs instilled in pregnant females lead to DNA damage and transcriptional activation in the exposed offspring. Even though, the tested NPs did not induce ESTR mutations, prenatally exposed females seem to be more sensitive than males to NP-induced effects on gene expression (Jackson 2011c, Boisen 2011 unpublished) and thus the potential of NPs to affect gene expression in prenatally exposed males and females should be investigated further. In view of the growing use and widespread distribution of products containing NPs with new and unknown properties the need for investigating their potential health effects is ever-increasing.

# Future research

### Ideas for improving ESTR analysis in female germ cells

One of the disadvantages of ESTR analysis is the large number of animals required. Although, this is far less than in the classical *in vivo* germline mutagenesis assays approximately ~150/animals per group are still needed to obtain a statistically significant result in ESTR analysis. An a priori power analysis showed that this group size in the present study provided a 90 % chance of detecting a 2-fold increase in ESTR mutation rate at the 5% significance level. SM-PCR ESTR analysis bypasses this need for a large number of animals by assessing the mutation rate directly in sperm cells. The Ms6-hm allele from a single cell is amplified by single-molecule PCR and the product electrophoresed and Southern blotted as in pedigree studies. Studies have shown that the results in sperm are comparable to result from using a traditional pedigree approach (Yauk et al. 2002). In the present set-up only 15 prenatally exposed male mice per group would be sufficient to examine germline mutation rate. An assessment of ESTR mutation rate directly in oocytes has not been attempted due to the technical difficulties in collecting a sufficient number of oocytes and distinguishing oocytes from the somatic granulosa cells.

However, protocols for collecting oocytes in mice do exist (De Felici and McLaren 1983; Sun et al. 2004) Sun and colleagues have developed a technique for harvesting pre-antral oocytes from C57BL/6xCBA hybrids. Using this technique 60-70 preantral follicles containing meiotic prophase stage oocytes could be harvested from the ovaries of each individual female, consequently 5 females per group should be sufficient. This would reduce the number of animals per group by more than a factor 30 in accordance with the 3 R principle (Russell and Burch 1959) of minimizing the use of laboratory animals. Furthermore, a recent study (Wojtasz et al. 2009) used flow cytometry to isolate live murine meiotic prophase oocytes from somatic cells based on size and other characteristics without using antibodies. Populations of >90% pure germ cells could be isolated from gonads of female fetuses at GD 18.5 within a day. By using one of these methods, isolated oocytes could be used in SM-PCR ESTR analysis or as a prescreening tool before moving on to a pedigree study. Alternatively, an assessment of genotoxicity directly in female germ cells could be made using the comet assay. By eliminating the need for breeding a large number of mice in the F2 generation, the time frame of the analysis would be shortened by approximately 7.5 months/study based on the present pedigree studies.. Due to the length of ESTR *loci* currently in use (< 2 kb-20 kb), it is not possible to assess mutation rates through DNA sequencing. Developing a panel of smaller *loci* might enable direct sequencing. Moreover, it has been proposed to use whole genome sequencing for assessing induced germline mutation frequencies, reviewed in Beal et al. 2012.

# Conclusion

This PhD project investigated nanoparticle-induced female germline mutations in expanded simple tandem repeat (ESTR) DNA *loci*. Elevated mutation rates at ESTR *loci* have proven to be a very sensitive endpoint to assess mutagenicity in the male germline at low, environmentally relevant levels of particulate air pollutants and chemical exposures. Male germ cells are primarily vulnerable to mutations in ESTR *loci* at the premeiotic stage. To my knowledge only 4 ESTR studies targeting premeiotic germ cells in females have been performed; of these only acute radiation exposure induced ESTR mutations in the female germline. More knowledge on the mechanistic link between environmental exposures and instability at ESTR DNA *loci* is needed to fully interpret results obtained by this method.

The nanoparticles UV-Titan and Printex 90 did not elevate ESTR mutation rates in prenatally exposed female mice at the tested time-points and concentrations: ESTR mutation rates of 0.029/0.025 (maternal allele) and 0.047/0.053 (paternal allele) in UV-Titan/Printex 90-exposed F2 offspring were not statistically different from those of F2 controls: 0.037/0.024 (maternal allele) and 0.061/0.038 (paternal allele). UV-Titan and Printex 90 exposure induced a neutrophil influx in lungs of generation P females and dams as well as changes in gene expression in lungs consistent with an inflammatory response. No effects were seen on reproductive parameters in the F1 generation (time-to-first F2-litter and sex-ratio and litter size of F2 litters). However, prenatal exposure to UV-Titan and Printex 90 had an effect on gene expression in F1 offspring. In both studies more genes were differentially expressed in F1 females than in F1 males. Prenatally exposed F1 females, thus seem to be more sensitive than prenatally exposed F1 males to NP-induced effects on gene expression. In support of this, preliminary data show a profound change differentially methylated regions of the genome in F1 females prenatally exposed to Printex 90.

Prenatal exposure of F1 female offspring to nanoparticles titanium dioxide UV-Titan or carbon black Printex 90 did not elevate the mutation rate in ESTR DNA *loci*, indicating that females may be less susceptible than males to particle-induced ESTR mutations. An estimation of the ESTR mutation rate of prenatally exposed F1 male offspring from the current exposures should be performed to assess if this is indeed the case.

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# Manuscript I: óEffects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in miceô

Karin S Hougaard, Petra Jackson1, Keld A Jensen, Jens J Sloth, Katrin Löschner, Erik H Larsen, Renie K Birkedal, Anni Vibenholt, Anne-Mette Z Boisen, Håkan Wallin and Ulla Vogel

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PARTICLE AND FIBRE TOXICOLOGY

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# 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]

## 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 $K_2$EDTA). 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%.

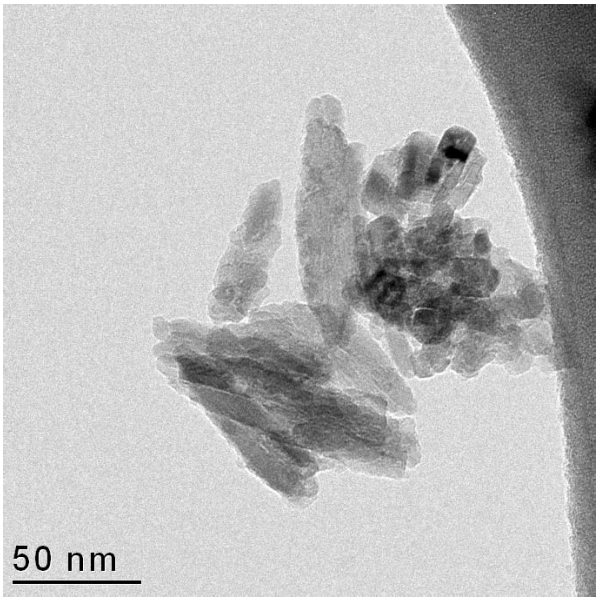


**Figure 1 TEM image of $TiO_2$ crystallites**. Transmission electron microscopy image showing the typical equidimensional to elongated morphology of the $TiO_2$ crystallites in UV-Titan L181. Bar = 50 nm.

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.

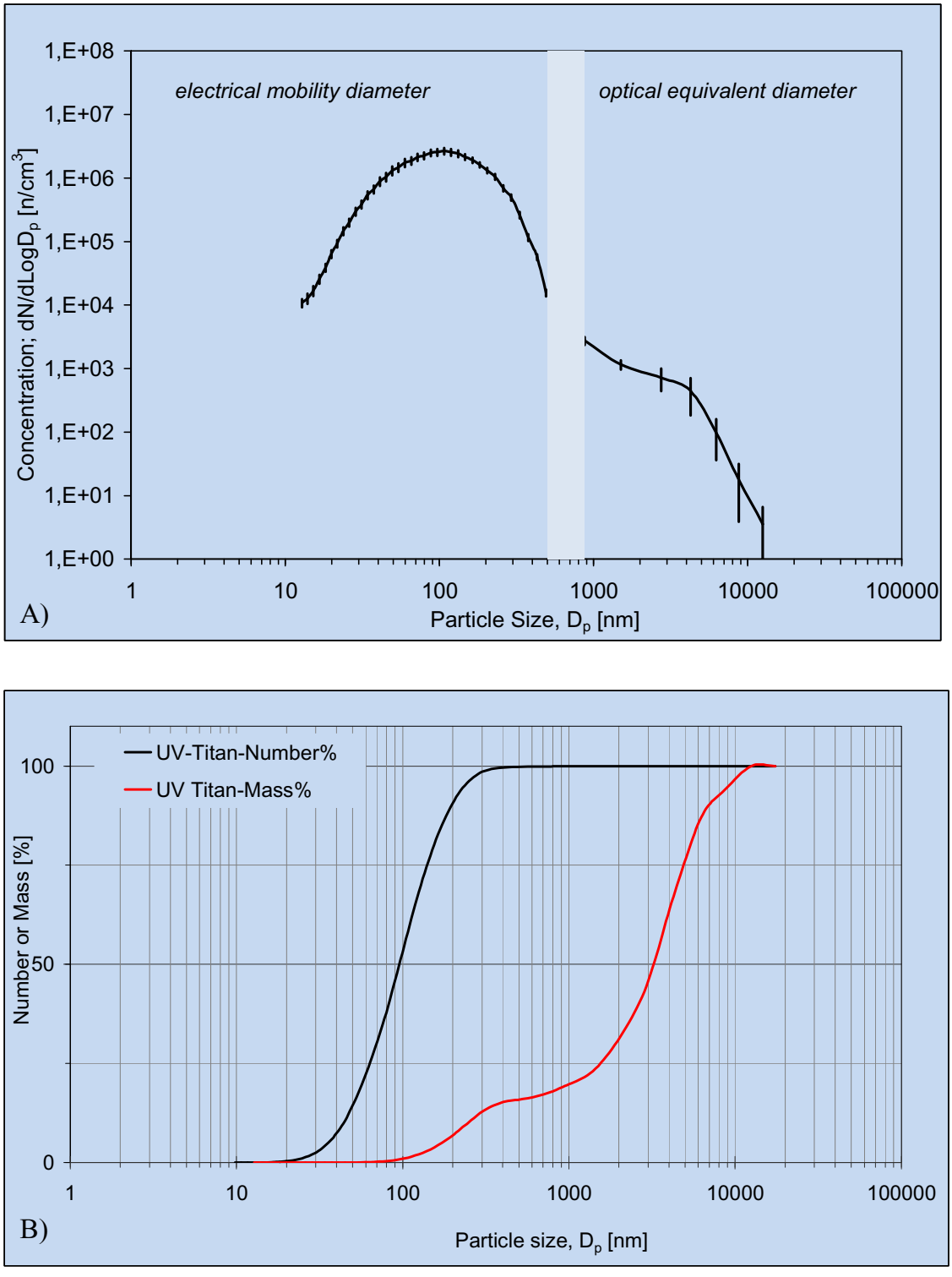


**Figure 2 Characteristics of the exposure atmosphere**. A) Particle number size distribution of the UV-Titan L181 in the exposure chamber. Data are based on nine one-hour exposure measurements. Mean ± SD. B) Accumulated number and mass concentration of particle concentrations in the exposure chamber. 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.

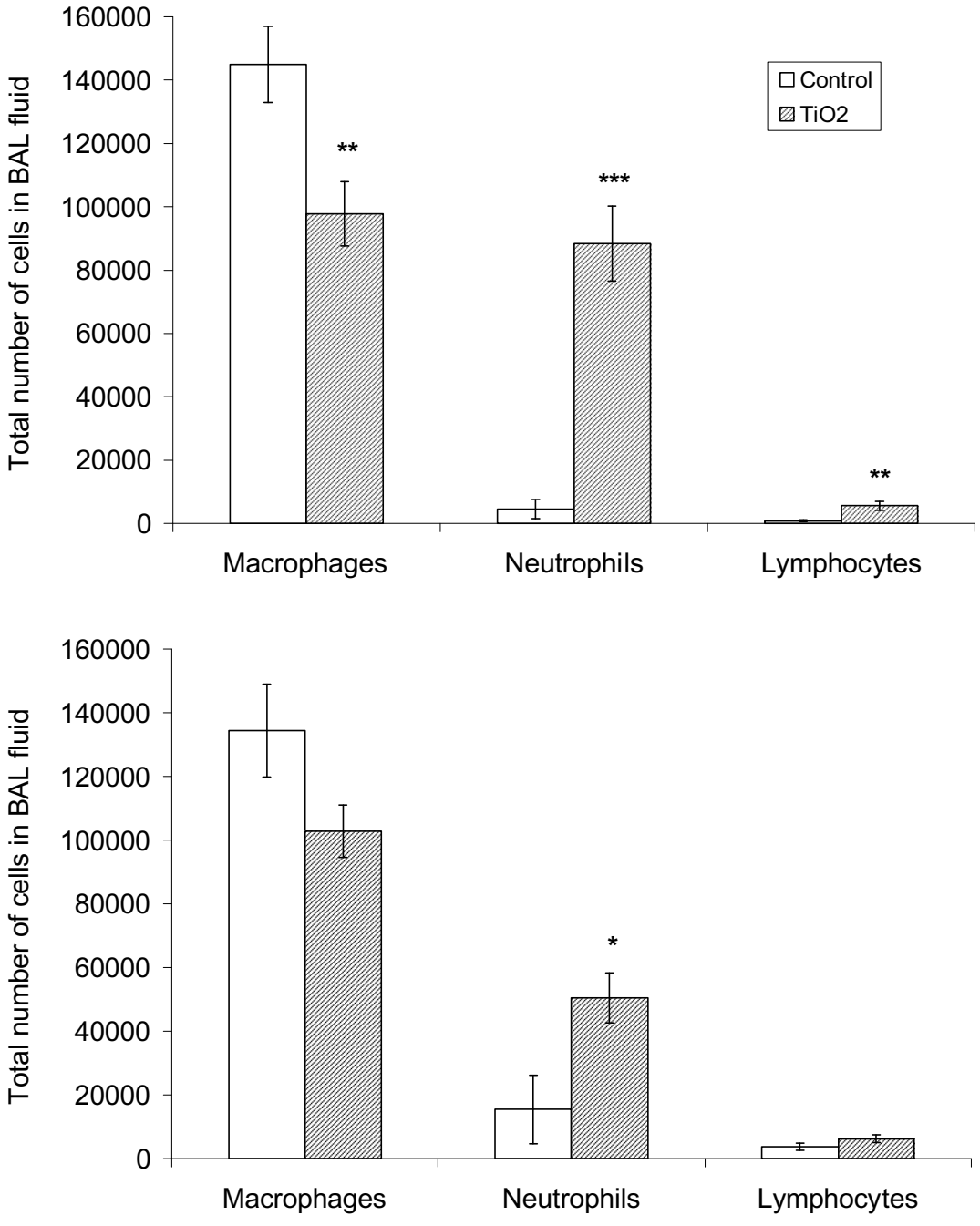


**Figure 3 Differential cell count in bronchoalveolar lavage fluid**. The total number of cells in BAL subdivided by cell type. A: time-mated mice that had not achieved pregnancy, 5 days after termination of exposure (n = 8-9). B: littering time-mated dams after weaning, 26-27 days after exposure (n = 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

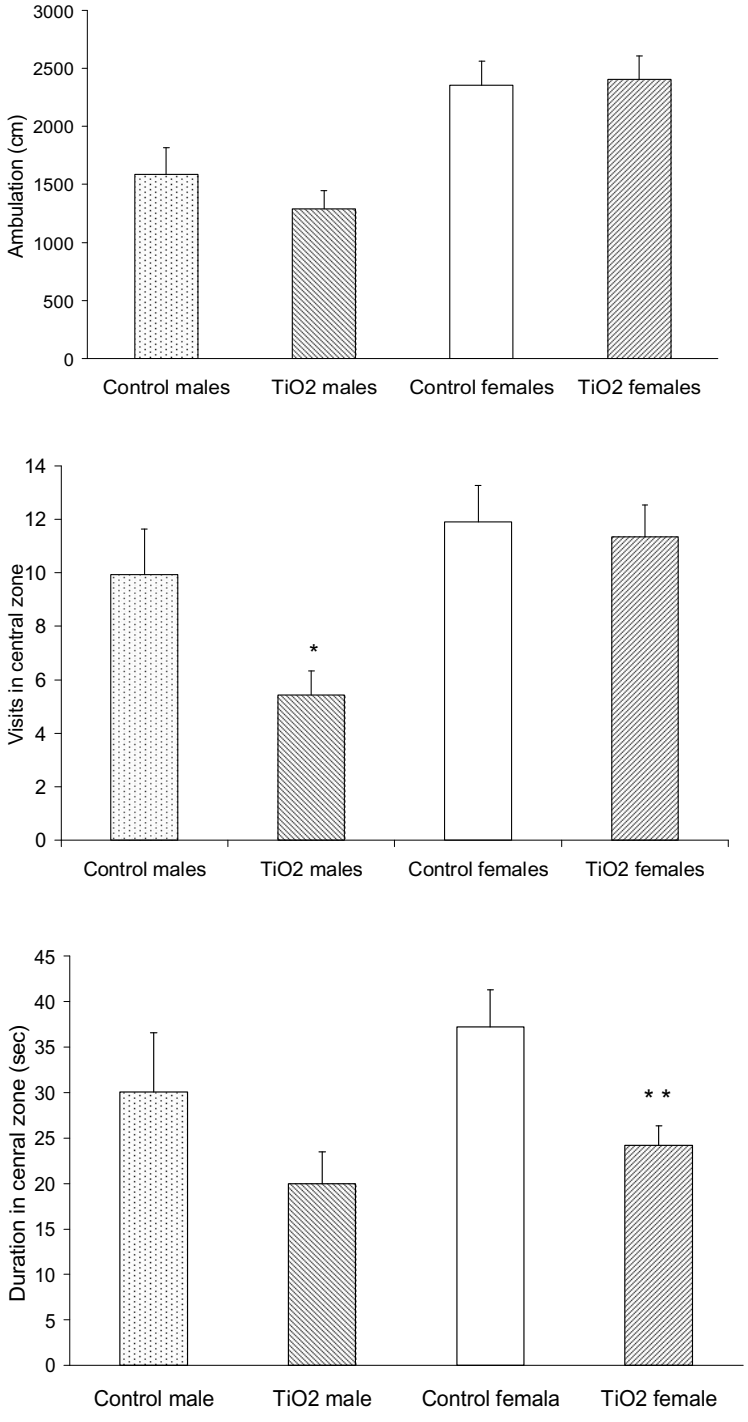


**Figure 4 Open field**. Open field performance during a 3-min observation period in male and female offspring from dams exposed to ambient air or $TiO_2$ during gestation. (A) Ambulation. (B) Visits to the central zone of the open field. Time spent in central zone of the open field (C). Mean ± SEM,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

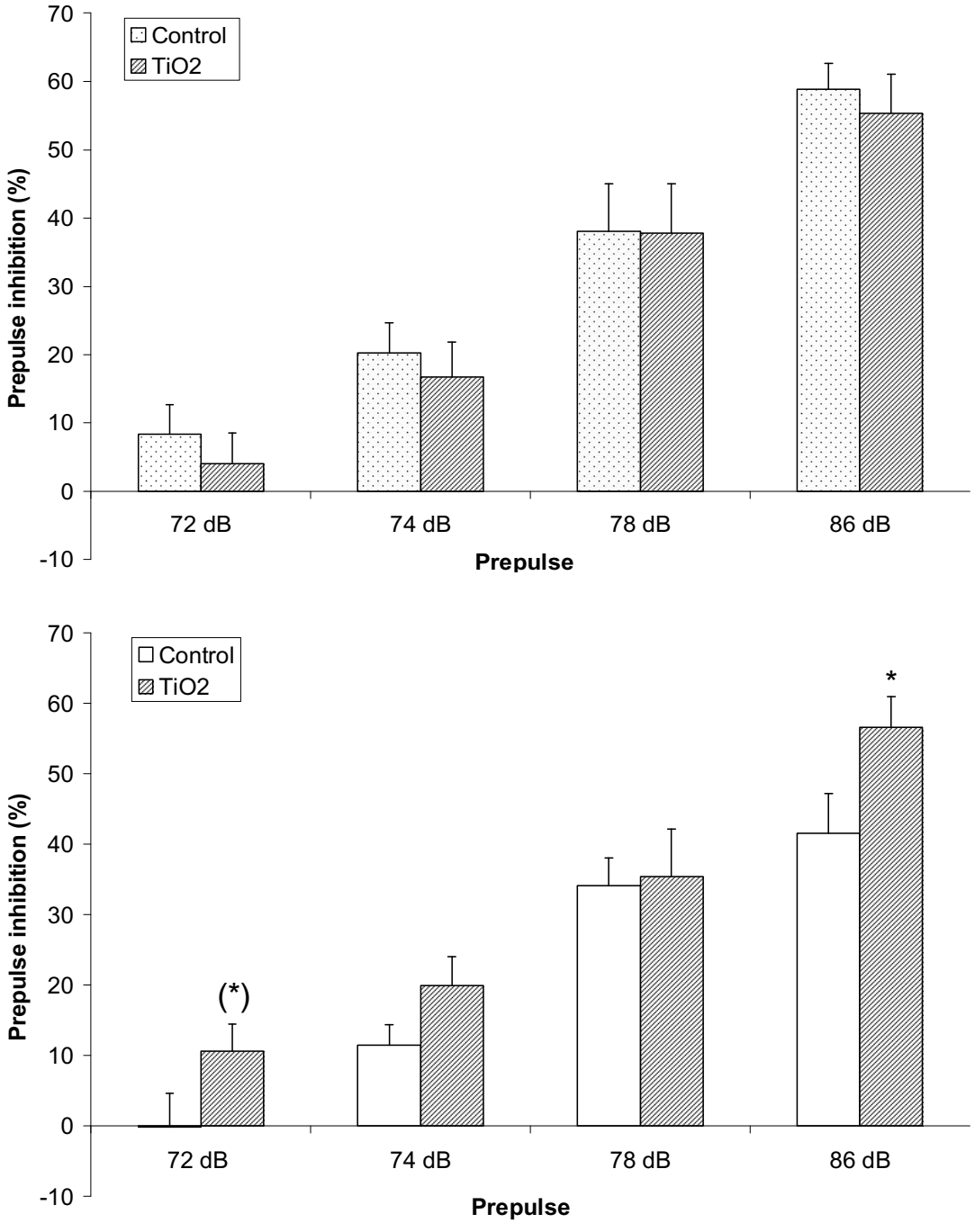


**Figure 5 Prepulse inhibition**. Prepulse inhibition in male (A) and female (B) offspring from dams exposed to ambient air or $TiO_2$ during gestation, at four different levels of prepulse. Mean ± SEM, n = 10-14. (*) $0.05 < p < 0.1$; *$p < 0.05$ vs. controls at same level of prepulse.

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.

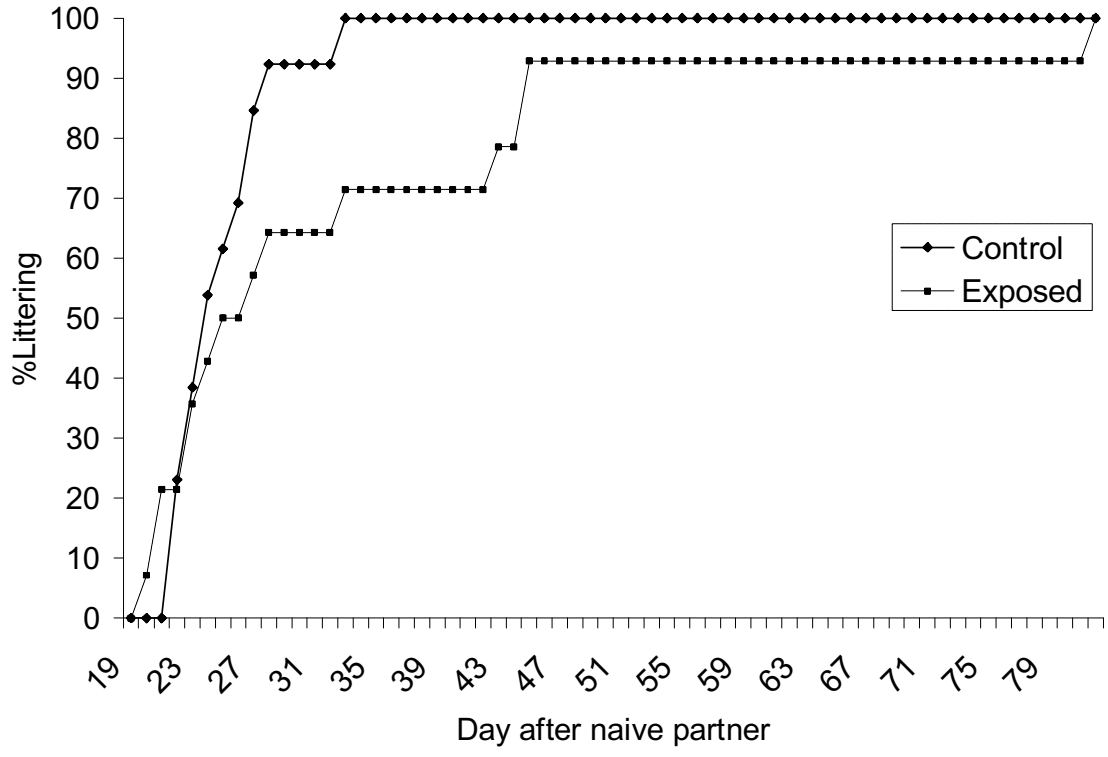


**Figure 6 Time-to-first F2 litter**. Littering curves for male offspring of control and UV-titan exposed pregnant mice. As adults, male C57BL offspring were mated to naïve CBA/J mice and time-to-first-delivery of F2 litter was recorded.

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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# Manuscript II: "Nano$TiO_2$ (UV-Titan) does not induce ESTR mutations in the germline of prenatally exposed female mice"

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**NanoTIO$_2$ (UV-Titan) does not induce ESTR mutations in the germline of prenatally exposed female mice**

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Abstract:

**Background:** Particulate air pollution has been linked to an increased risk of cardiovascular disease and cancer. Animal studies have shown that inhalation of air particulates induces mutations in the male germline. Expanded simple tandem repeat (ESTR) *loci* in mice are sensitive markers of mutagenic effects on male germ cells resulting from environmental exposures; however, female germ cells have received little attention. Oocytes may be vulnerable during stages of active cell division (e.g., during fetal development). Accordingly, an increase in germline ESTR mutations in female mice prenatally exposed to radiation has previously been reported. Here we investigate the effects of nanoparticles on the female germline. Since pulmonary exposure to nanosized titanium dioxide (nano$TiO_2$) produces a long-lasting inflammatory response in mice, it was chosen for the present study.

**Findings:** Pregnant C57BL/6 mice were exposed by whole-body inhalation to the nano$TiO_2$ UV-Titan L181 (~42.4 mg UV-Titan/$m^3$) or filtered clean air on gestation days (GD) 8-18. Female C57BL/6 F1 offspring were raised to maturity and mated with unexposed CBA males. The F2 descendents were collected and ESTR germline mutation rates in this generation were estimated from full pedigrees (mother, father, offspring) of F1 female mice (192 UV-Titan-exposed F2 offspring and 164 F2 controls). ESTR mutation rates of 0.029 (maternal allele) and 0.047 (paternal allele) in UV-Titan-exposed F2 offspring were not statistically different from those of F2 controls: 0.037 (maternal allele) and 0.061 (paternal allele).

**Conclusions:** We found no evidence for increased ESTR mutation rates in F1 females exposed *in utero* to UV-Titan nanoparticles from GD8-18 relative to control females.

**Keywords:** ESTR, nanoparticles, oogenesis, *in utero*

Introduction

Mutations in male and female gametes may lead to detrimental inherited effects in subsequent generations. Human exposure to particulate air pollution (PAP) has been shown to adversely affect germ cells in males [1]. Moreover, animal studies have demonstrated that inhalation of PAP can induce mutations in the male germline [2,3,4,5,6]**.** Airborne particles in the nanometer range deposit deep in the airways. These particles are cleared very slowly and a small fraction may translocate into the bloodstream [7,8]. Inhaled nanoparticles (NPs) are potent inducers of pulmonary inflammation and oxidative stress, which may affect the fetus indirectly during maternal exposure [9,10,11].

As a model of NP exposure we tested nanosized titanium dioxide (nano$TiO_2$) UV-Titan, which is used in the production of paints [9,12,13]. Large quantities of nano$TiO_2$ are used globally in a wide range of products. $TiO_2$ was previously believed to be inert, but inhaled $TiO_2$ has now been classified as possibly carcinogenic to humans by the International Agency for Research on Cancer [14]. $TiO_2$ toxicity depends on particle size, crystalline form and surface modifications [15]. Pulmonary exposure to nano$TiO_2$ causes inflammation in rodents [16,9] and we recently found that a single UV-Titan instillation induced an inflammatory response in mice after 1 day [12,17]. In addition, UV-Titan particles remained in lungs 4 weeks after inhalation, causing long-lasting inflammation [9] .

Expanded simple tandem repeat (ESTR) *loci* in mice exhibit high spontaneous mutation rates enabling the study of induced germline mutations following environmental exposures. Radiation, air particulates, and a number of chemicals have been shown to increase ESTR mutations in male germ cells [18,19,2,5]. Very limited data exist on induced mutations in female germ cells, which have previously been considered highly resistant to genotoxicity [20]. However, oocytes could be vulnerable during stages of active cell division, i.e. during fetal development [20,21]. A recent study showed that prenatal exposure to 1 Gy of acute irradiation on GD12 resulted in a 1.94-fold increase in ESTR mutations in the offspring of irradiated female mice [22,23].

We hypothesized that prenatal exposure to NPs will affect female germline ESTR mutation frequency during stages of active cell division, similar to what has been found for male germline cells [1]. The present study investigates $TiO_2$ nanoparticle-induced effects on female germline DNA by exposing pregnant female mice (P) to nano$TiO_2$ or clean filtered air *via* inhalation and subsequently mating their offspring (F1) with unexposed males. The observed F1 female germline

ESTR mutation frequency was calculated by comparing allele size in the F2 offspring to their mother's allele size to quantify repeat gains and losses.

## Materials and Methods

### Animals and exposure

All mice (figure 1) were housed under controlled environmental conditions [9]**.** Generation P consisted of time-mated, nulliparous mice (C57BL/6JBomTac) exposed by whole-body inhalation to UV-Titan L181 (Kemira, Pori, Finland), a rutile $TiO_2$ (70.8 wt%) modified with 1.17 wt% zirconium, 12.01 wt% silicon, 0.60 wt% sodium oxide and 4.58 wt% aluminium. UV-Titanium is coated with polyalcohol adding to the remaining wt%. Primary particle size was 20.6 nm and surface area (BET) 107.7 $m^2/g$. The particle number concentration in the exposure atmosphere was $1.70 \pm 0.20 \cdot 10^6/cm^3$. The major particle size-mode was ~100 nm (geometric mean number diameter 97 nm). 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 [9]. A detailed description of the physico-chemical characteristics of particle preparation, sample analysis and exposure monitoring of UV-Titan is reported in [9]. Mice were exposed to ~42.4 mg UV-Titan/$m^3$ or filtered clean air on GD8-18, one h/day as described [9]. Generation P gave birth to generation F1 (C57BL/6JBomTac). At 19 weeks of age, 26 prenatally exposed F1 females (13 controls and 12 $TiO_2$-exposed) were mated with unexposed CBA/J (Charles River, Sulzfeld, Germany) to produce generation F2 (C57BL/6 x CBA/J). A total of 450 F2 offspring (figure 1) were collected for the present study. Mutation analysis and scoring were successful for 388 offspring. Procedures complied with EC Directive 86/609/EEC and Danish regulations on experiments with animals (Permission 2006/561-1123).

### DNA extraction and mutation analysis

F1 parents were euthanized after breeding, F2 offspring on postnatal day (PND) 2-7 or at maturity (PND80). F1 and F2 tail tissue was flash frozen in cryotubes (NUNC) in liquid $N_2$ and stored at -80°C. DNA was extracted by phenol-chloroform extraction and ESTR analysis was performed as in [2]. Briefly, 25 μg of mouse tail DNA was digested with *AluI* (New England BioLabs, Pickering, Ont.) at $37^o$ C overnight. F1 and F2 DNA samples were run on 40 cm long 0.8% agarose gels (SeaKem LE) for 48 hours in a cooled chamber at 130 V along with a 1 Kb ladder (Invitrogen, Burlington, Ont.). DNA was transferred to a nylon membrane by vacuum blotting (GE Osmonics,

Minnetonka, MN) and hybridized to $^{32}$P-labeled *Ms6-hm* and *Hm2* probes [2]. F2 bands showing a shift of at least 1 mm relative to the F1 progenitor allele were scored as mutants. Bands were scored independently by 3 observers blinded to exposure status. Mutation rates were determined as the number of mutant bands per total number of bands scored (table 1) and compared using a one-tailed Fisher's exact test.

Results and discussion

F1 females were prenatally exposed to UV-Titan by maternal inhalation of 42.4 mg UV-Titan/$m^3$ 1 hour/day on GD8-18 (figure 1). 164 and 192 offspring from control and exposed females, respectively, were scored. Thus, a total of 328 and 384 inherited bands were scored per group. The observed mutation rate in germ cells of UV Titan-exposed F1 females was not significantly different from controls (table 1). The *Ms6-hm* and *Hm-2* mutation rates in control females were similar to those found for females in other studies using the same mouse strain [19,2]. Furthermore, the number of offspring, sex-ratio and time to birth of the first F2 litter did not differ between groups, suggesting that UV-Titan did not affect viability of the F2 offspring (data not shown). Absence of effect is therefore not due to lower viability of affected offspring. Mutations in ESTRs should not affect offspring fitness since these *loci* do not have known functions.

ESTR mutations have been suggested to be induced *via* polymerase pausing resulting from the presence of epigenetic changes or DNA damage such as oxidative stress, strand breaks or adducts elsewhere in the genome rather than by direct DNA damage [5]. We have reported that the inhalation of a total dose of 840 µg UV-Titan per animal at GD8-18 induced persistent inflammation in the lungs of the time-mated P generation (figure 1) [24,9]. Furthermore, 476 genes were found to be differentially expressed in the liver of newborn F1 generation females prenatally exposed to UV-Titan. We hypothesize that the transfer of inflammatory cytokines across the placenta may have caused this differential gene expression [10] since no $TiO_2$ was detected in maternal liver, mother's milk or offspring liver [9].

ESTR mutation analysis is a sensitive method, enabling analysis under realistic exposure scenarios. An *a priori* power analysis showed that group size in the present study provided a 77 % chance of detecting a 2-fold increase in ESTR mutations at the 5% significance level. The exposure and the estimated inhaled dose of 840 µg used in this study is comparable to the permissible exposure limit by Danish Regulation and the exposure route (inhalation) is also relevant to environmental exposure [9]. As little as 54 µg UV-Titan can induce inflammation in mouse lungs after one day [12,17].

Female germ cells enter meiotic prophase on ~GD13.5 [21]. In the present study female mice were prenatally exposed from GD8-18 ensuring that the period of mitotic germ cell division was targeted; these mothers were exposed to ~458 µg prior to GD13. Consequently, a high degree of inflammation was likely to be present at GD13.5, when oocytes cease to be susceptible to ESTR mutations [22,21].

In parallel with the present study (in the same laboratory and time period), ESTR germline mutations in male and female mice prenatally exposed to diesel exhaust particles (DEP) by inhalation were quantified [2]. Male germ cell mutation rates were significantly increased following exposure to DEP and may thus be regarded as a positive control for the ability to detect induced ESTR mutation. ESTR mutation rates were not significantly increased in germ cells of females prenatally exposed to DEP. To our knowledge, this is the only other study, which has investigated chemically induced ESTR mutations in prenatally exposed females. A recent study showed that dividing oocytes are susceptible to mutations *in vivo*. Prenatal exposure to 1 Gy of acute irradiation on GD12 resulted in a 1.94-fold increase in the ESTR mutation rate [22].

Nano$TiO_2$ can induce DNA strand breaks and carcinogenic effects *in vivo* [25,11,26]. We recently reported that UV-Titan inhalation did not increase DNA strand breaks in the P or F1 generations [10], suggesting that genotoxic effects in offspring are negligible. Correspondingly, in the study of prenatal DEP exposure by [2], which showed ESTR instability in male offspring, the exposure also failed to increase DNA strand breaks in liver from newborns [27]. Epigenetic changes have been suggested as the underlying mechanism of ESTR instability [22,5]. A recent study found DNA deletions in mice prenatally exposed to nano$TiO_2$ [26]. However, the small effective sample size and the very large maternal dose used in the study hamper interpretation. The results on nano$TiO_2$ induced mutations and genotoxicity are conflicting [10,15,26]. The various types of commercially available nano$TiO_2$ also make it difficult to generalize. It is possible that NPs with very active surface chemistry, which produce more reactive oxygen species (ROS) or a large inflammatory response, could induce germline mutations. In the present study we have only assessed the effects of a single type of $TiO_2$ NP. We are currently investigating the effects of prenatal exposure to nanosized carbon black Printex90, a more efficient generator of ROS than both DEP and nano$TiO_2$ [12] to further address the question of female susceptibility to NPs. The present study indicates that prenatal exposure to nano$TiO_2$ does not affect female germline ESTR mutation frequency.

Authors' contributions

AMZB was substantially involved in the design of the study, collected animal tissue, processed samples and performed the electrophoresis, blot probing, image processing, mutation scoring, statistical analysis and drafted the manuscript. TS re-probed and developed images for a large portion of blots and revised the manuscript. PJ exposed the P generation mice, assigned F1 offspring for the current study and revised the manuscript critically. KSH was project manager of the study and revised the manuscript critically. HW was substantially involved in the design of the study and revised the manuscript critically. CLY was substantially involved in the design of the study, scored mutations and revised the manuscript critically. UBV was substantially involved in the design of the study and revised the manuscript critically. All authors read and approved the final version of the manuscript.

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Table 1. Summary of ESTR mutation rates in F2 offspring of prenatally exposed female C57BL/6 mice

| **Group** | **probe** | **N (F2 offspring)** | **Mutant bands** | | **Mutation rate ± SEM (P value [a])** | |
|---|---|---|---|---|---|---|
| | | | **Paternal origin** | **Maternal origin** | **Paternal origin** | **Maternal origin** |
| Female controls | *Ms6-hm* | 164 | 11 | 5 | 0.0671±0.0002 | 0.0305±0.0002 |
| Female controls | *Hm-2* | 164 | 9 | 7 | 0.0549± 0.0004 | 0.0427±0.0004 |
| **Female controls** | **Total** | **164** | **20** | **12** | **0.0610±0.0028** | **0.0366±0.0030** |
| Female $TiO_2$ exposed | *Ms6-hm* | 192 | 10 | 4 | 0.0521±0.0004 | 0.0208±0.0002 |
| Female $TiO_2$ exposed | *Hm-2* | 192 | 8 | 7 | 0.0417±0.0004 | 0.0365±0.0003 |
| **Female $TiO_2$ exposed** | **Total** | **192** | **18** | **11** | **0.0469±0.0107 (P=0.84)** | **0.0286±0.0133 (P=0.79)** |

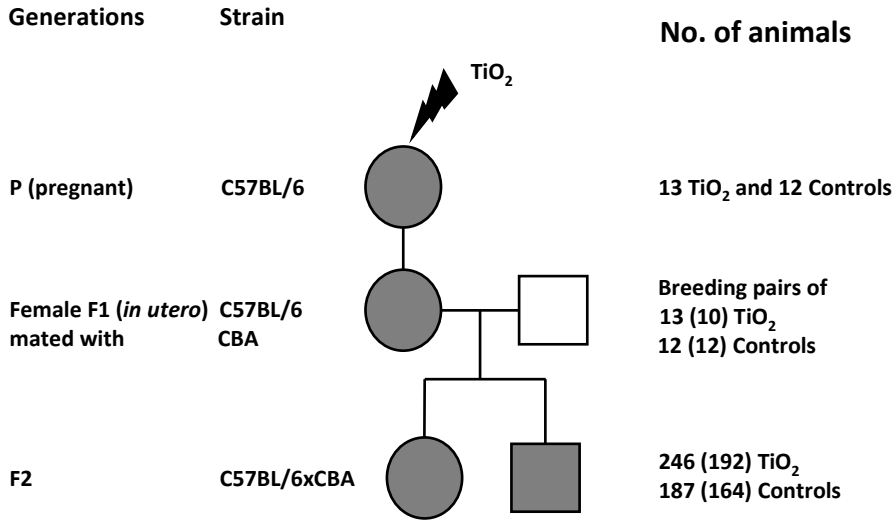
[a] Fisher's exact test 1-tailed.

## Figure Legend:

Figure 1. Overview of the pedigree study.

Circles and squares represent female and male mice respectively. Grey symbols represent exposed animals and their descendants. White squares represent non-exposed CBA mates. Generation P pregnant mothers were exposed: 13 $TiO_2$ exposed and 12 Controls. 246 F2 offspring were collected from $TiO_2$ and 187 from Controls (number of successfully analyzed offspring 192 and 164, respectively).

Figure 1. Overview of the pedigree study.



# Manuscript III: "Pulmonary exposure to carbon black in pregnant mice: Effects on DNA strand breaks in dams and offspring"

PETRA JACKSON, KARIN SØRIG HOUGAARD, ANNE METTE Z. BOISEN, NICKLAS RAUN JACOBSEN, KELD ALSTRUP JENSEN, PETER MØLLER, GUNNAR BRUNBORG, KRISTINE BJERVE GUTZKOW, OLE ANDERSEN, STEFFEN LOFT, ULLA VOGEL, & HÅKAN WALLIN

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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.

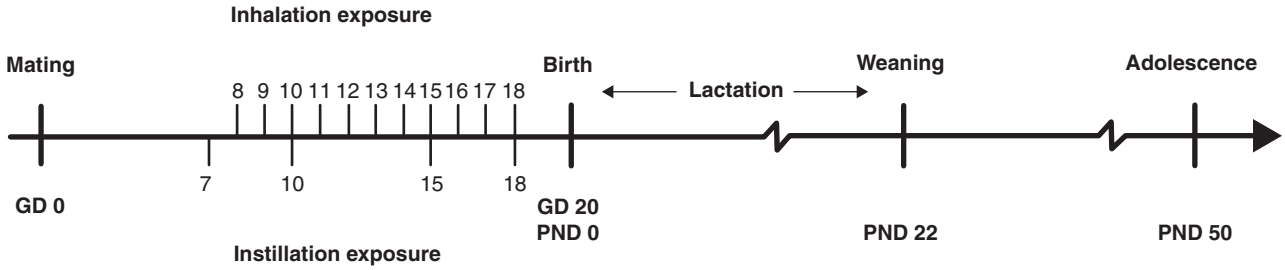
All the procedures complied with the EC Directive 86/609/EEC and the Danish law regulating experiments on animals (The Danish Ministry of Justice, protection of experimental animals (Dyreforsøgstilsynet) with Animal Experiments Inspectorate Permission 2006/561-1123).

### *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/$10^6$bp.

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/$10^6$bp 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

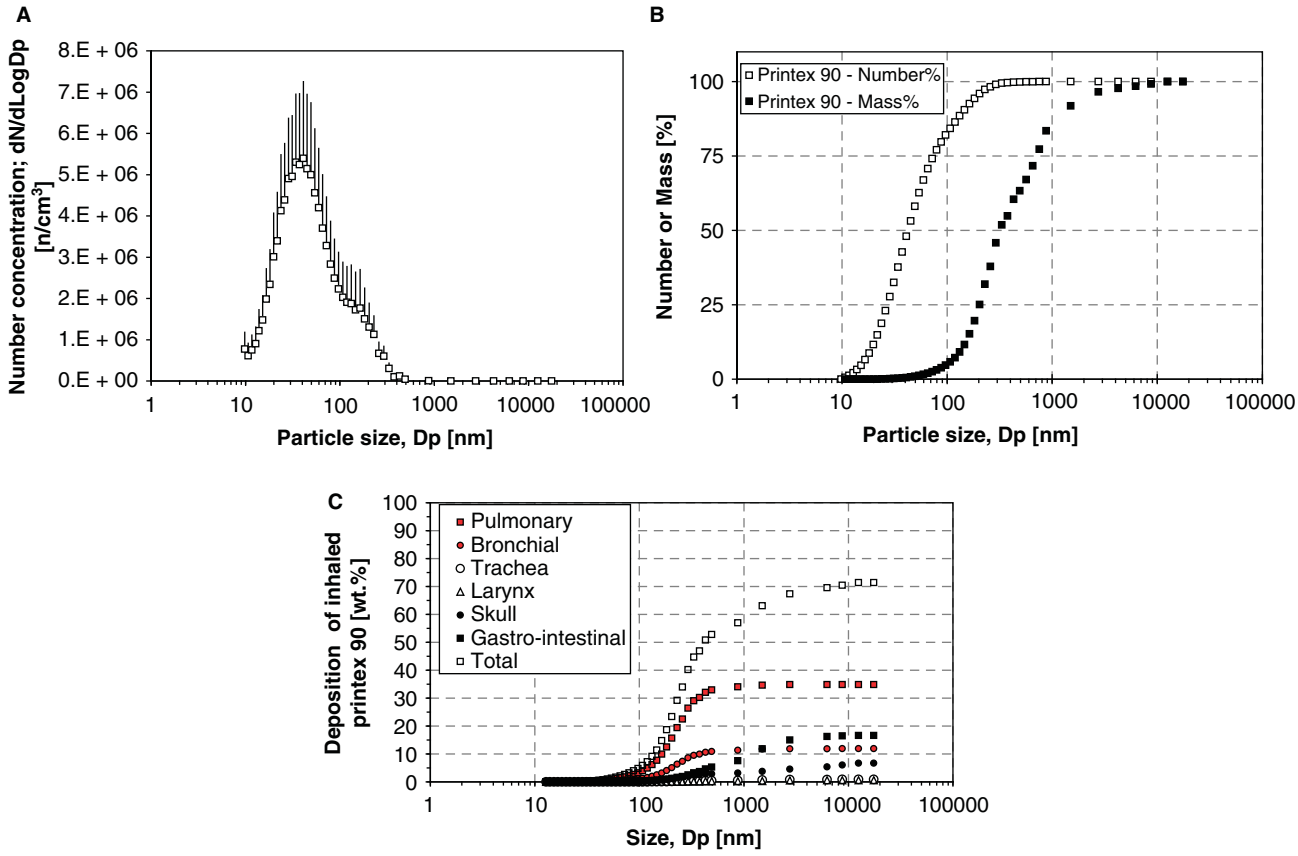



Figure 2. Size distribution data for the inhalation exposure and calculated deposition in mice. (A) Average number size distribution of Printex 90 dust in the inhalation exposure chamber. Error bars are the standard deviation of the different individual exposure runs. (B) Accumulated average number and 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

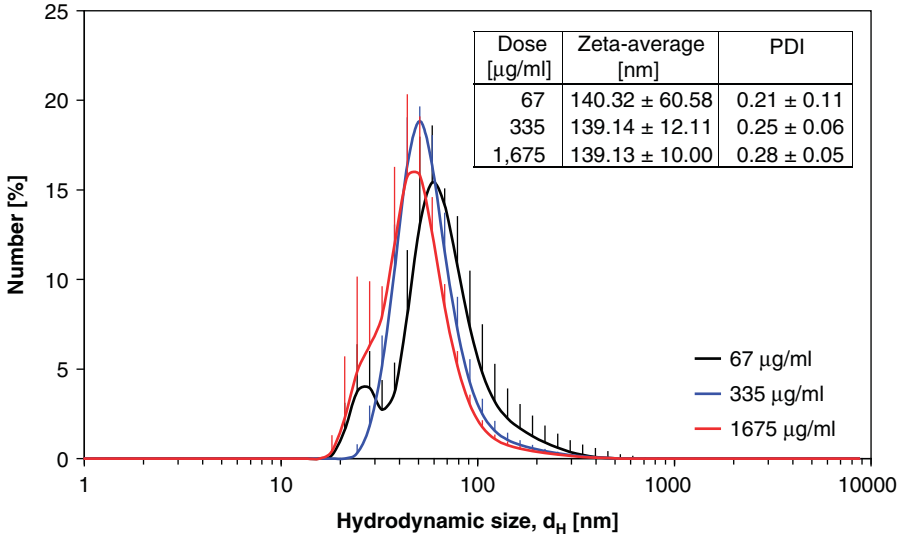



Figure 3. Hydrodynamic size distribution of intratracheally instilled Printex 90 dispersions. Particle size distribution at the three instillation concentrations for the intratracheal instillation exposure measured by Dynamic Light Scattering. Error bars show the standard deviation of six measurements.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

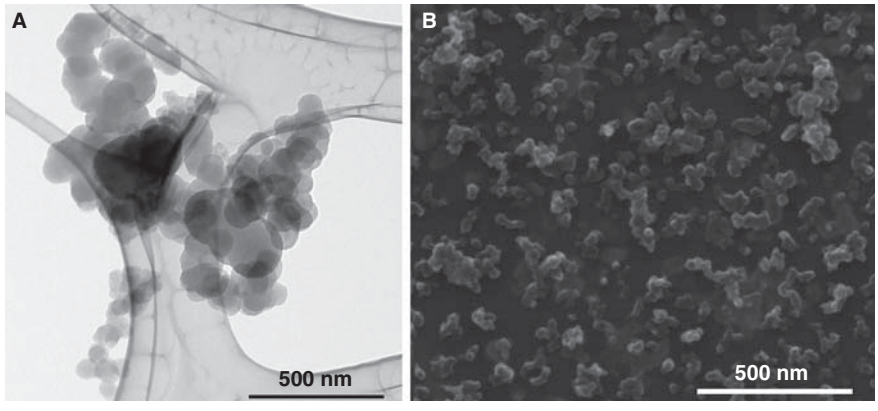



Figure 4. Printex 90 intratracheal instillation exposure characterization by TEM and SEM. (A) Transmission Electron Micrograph of agglomerated small- and medium size Printex 90 aggregates (67 µg/mL). (B) Scanning Electron Micrograph illustrating the overall size and morphologies of Printex 90 aggregates 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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# Manuscript IV: "Nanosized carbon black (Printex90) does not induce ESTR mutations in germ cells of female mice exposed in utero"

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**Nanosized carbon black (Printex90) does not induce ESTR mutations in germ cells of female mice exposed *in utero***

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Abstract:

**Background:** Particulate air pollution is associated with increases in cardiovascular disease and cancer in humans. In animal models, inhalation of particles also leads to induced tandem repeat mutations in the male germline. However, few studies have examined mutagenicity arising in female germ cells. Oocytes may be vulnerable to mutations during active cell division (e.g., during fetal development). Here we investigate the effects of nanoparticles on the female germline using highly unstable tandem repeat DNA markers. Nanosized carbon black (Printex90) is mutagenic in vitro and a potent generator of reactive oxygen species and inflammation in mice, and was therefore chosen for the present study.

**Findings:** Pregnant C57Bl/6 mice were exposed by intratracheal instillation to 268 μg/animal nanosized carbon black Printex90 or vehicle on gestation days (GD) 7, 10, 15 and 18. Female C57Bl/6 F1 offspring were raised to maturity and mated with unexposed CBA males. Expanded simple tandem repeat (ESTR) germline mutation rates in the resulting F2 generation were estimated from full pedigrees (mother, father, offspring) of F1 female mice (178 CB-exposed F2 offspring and 258 F2 controls). ESTR mutation rates of 0.025 (maternal allele) and 0.053 (paternal allele) in CB-exposed F2 offspring were not statistically different from those of F2 controls: 0.024 (maternal allele) and 0.038 (paternal allele).

**Conclusions:** We found no evidence for increased ESTR mutation rates in F1 females exposed in utero to CB nanoparticles relative to control females

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Keywords: ESTR, nanoparticles, oogenesis, in utero, germ cells

## Introduction

Heritable mutations may have harmful effects that extend beyond the exposed individual to their unexposed descendants. Human exposure to particulate air pollution can adversely affect sperm structure and function (Sram et al. 1999). Moreover, exposure of mature male mice to ambient levels of particulate air pollution in urban/industrial centres leads to increased rates of heritable tandem repeat mutation (Somers et al. 2002; Somers et al. 2004; Yauk et al. 2008a) Thus, exposure to low, environmentally relevant levels of particulate air pollutants appear to adversely affect male germ cells. Nanosized airborne particles deposit deep in the airways and are removed very slowly; a small fraction may translocate into the bloodstream (Kreyling et al. 2009; Sadauskas et al. 2009) to potentially be delivered systemically to other tissues. Inhaled nanoparticles (NPs) generate pulmonary inflammation and oxidative stress, which may affect the fetus indirectly during maternal exposure (Jackson et al. 2011a; Jackson et al. 2011b; Jacobsen et al. 2009; Saber et al. 2011a; Saber et al. 2011b).

Expanded simple tandem repeat (ESTR) loci in mice exhibit high spontaneous mutation rates enabling the study of induced germline mutations following environmental exposures. Induced ESTR mutations are believed to be caused by an indirect mechanism resulting from DNA damage, oxidative stress or epigenetic changes in the genome (Yauk et al. 2008a). Radiation, particulate air pollution and mutagenic chemicals can induce ESTR mutations in mouse sperm (Dubrova et al. 1998; Hedenskog et al. 1997; Ritz et al. 2011; Vilarino-Guell et al. 2003; Yauk et al. 2008b). Female germ cells have previously been considered very resistant to genotoxic insults and limited data on mutagenicity in oocytes exist (Adler et al. 2007). However, recent work demonstrates that exposure to acute radiation leads to induced ESTR mutations in mitotically dividing oocytes, i.e. during gametogenesis in the fetus (Barber et al. 2009a). The present study explores the mutagenicity of nanosized carbon black Printex90 (CB), which is 99% pure carbon and a well-characterized reference material in particle toxicology as a model of both NPs and combustion-related air pollution. CB is a widely used pigment and a potent inducer of reactive oxygen species (ROS) (Jacobsen et al. 2008). CB is classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (Baan et al. 2006).We hypothesized that NPs with very active surface chemistry and which generate a high amount of ROS could induce ESTR mutations in developing female germ cells. We investigated nanoparticle-induced effects by exposing pregnant mice (P) to CB or vehicle via intratracheal instillation and subsequently mated

their female offspring (F1) with unexposed males. The observed F1 female germline ESTR mutation rate was calculated by comparing allele sizes of the F2 offspring to their F1 mothers to quantify repeat gains and losses.

Materials and Methods

**Animals and exposure**

All mice (figure 1) were housed under controlled environmental conditions (Jackson et al. 2011a). Time-mated female mice (generation P) (C57BL/6JBomTac) were exposed on gestation days (GD) 7, 10, 15 and 18. Mice were anesthetized with 3% Isoflurane and instilled with either 67 μg carbon black Printex90 dispersed in 40 μL nanopure filtered water (vehicle) or, for the control group, with 40 μL of vehicle only. The total instilled dose was 268 μg/animal. Particle preparation, characterization and instillation procedures were as described previously (Jackson et al. 2011a; Jackson et al. 2011b). Printex90 was a gift from Degussa-Hüls, Frankfurt, Germany. Declared particle size was 14 nm and hydrodynamic size distribution peaked at ~50 nm in vehicle. Generation P gave birth to generation F1 (C57BL/6JBomTac). At 13 weeks of age, 25 prenatally exposed F1 females from separate litters (14 controls and 11 CB-exposed) were mated with unexposed CBA/J (Charles River, Sulzfeld, Germany) to produce generation F2 (C57BL/6 x CBA/J). Time-to-birth of the first F2-litter, sex-ratio and number of offspring in the first litter was analyzed. A total of 531 F2 offspring (figure 1) was collected. Mutation analysis was successful for 436 offspring. Procedures complied with EC Directive 86/609/EEC and Danish regulations on experiments with animals (Permission 2006/561-1123).

**DNA extraction and mutation analysis**

F2 offspring were euthanized on postnatal day (PND) 2-7 or at maturity (PND80). F1 parents were euthanized after a sufficient number of F2 offspring had been obtained. F1 and F2 tail tissue was flash frozen in cryotubes (NUNC) in liquid $N_2$ and stored at −80°C. DNA was phenol-chloroform extracted and ESTR analysis was performed as previously described [2]. Briefly, 25 μg tail DNA was digested with AluI (New England BioLabs, Pickering, Ont.) at 37$^{o}$C overnight. F1 and F2 DNA samples were run on 40 cm long 0.8% agarose gels (SeaKem LE) along with a 1 Kb ladder (Invitrogen, Burlington, Ont.) for 48 hours in a cooled chamber at 130 V. DNA was vacuum blotted onto a nylon membrane (GE Osmonics, Minnetonka, MN) and hybridized to $^{32}$P-labeled Ms6-hm

and Hm2 probes (Ritz et al. 2011b). F2 bands showing a shift of at least 1 mm relative to the F1 progenitor allele were scored as mutants. Bands were scored independently by 2 observers unaware of exposure status. Scoring was 99% concordant in the independent scoring. Mutation rates were determined as the number of mutant bands/total number of bands scored (table 1) and compared by a one-tailed Fisher's exact test.

Results and discussion

The observed mutation rate in germ cells of CB-exposed F1 females was not significantly different from controls (table 1). There was no CB-dependent effect on reproductive parameters; time to birth of first litter, sex-ratio and litter size in first F2 litter were unaffected, suggesting that CB did not impair viability and thereby conceal the true number of mutations (data not shown). ESTR loci have no known function and therefore should not affect offspring fitness.

The high spontaneous mutation rate of ESTR loci makes this a very sensitive assay for investigating heritable mutations in laboratory mice with the use of relatively few animals and low exposure doses. An a priori power analysis showed that group size in the present study provided a 77% chance of detecting a 2-fold increase in ESTR mutations (5% significance level). Different types of exposures can induce ESTR mutations in the male germline (Dubrova et al. 1998; Hedenskog et al. 1997; Ritz et al. 2011; Vilarino-Guell et al. 2003; Yauk et al. 2008b). To date only radiation exposure has induced ESTR germline mutations in females (Barber et al. 2009). We have previously shown that prenatal exposure to 19 mg/m$^3$ diesel exhaust particles (DEP) induced ESTR germline mutations in males, but not in females (Ritz et al. 2011). A 1.98-fold increase observed in DEP-exposed females was not statistically significant (table 1). In a subsequent study on female prenatal exposure to nano$TiO_2$, which used a higher exposure dose (42 mg/m$^3$) and a larger number of offspring, no increase was observed (table 1) (Boisen unpublished). Previous work on male mice exhibiting increased ESTR mutation rates resulting from exposure to urban air particulates did not reveal evidence of increased bulky DNA adducts in sperm of these animals(Yauk et al. 2008a). Increased sperm DNA strand breaks were hypothesized to result from oxidative stress. In addition, hypermethylation in sperm of these animals provide support for an epigenetic component to this effect. In general ESTR mutations are believed to be the result of non-targeted effect of epigenetic changes or cell cycle effects resulting from DNA damage such as adducts, strand breaks (SBs) or oxidative stress (Yauk et al. 2008a).

CB generates higher amounts of inflammation in mice than nano$TiO_2$ (Saber et al. 2011), as well as much higher levels of ROS than both nano$TiO_2$ and diesel (Saber et al. 2011; Jacobsen et al. 2008). Thus, CB provides an excellent model to explore the potential effects of NP-induced ROS-driven ESTR mutations. We have previously shown that inhalation or intratracheal instillation of CB causes long-lasting inflammation, acute phase response, oxidative stress and particle retention in lungs of exposed mice (Jackson et al. 2011a; Jackson et al. 2011b; Jacobsen et al. 2009; Saber et al. 2011a, Bourdon unpublished results). We have also shown that CB is mutagenic in vitro and that the mutation spectrum is compatible with mutations being caused by ROS production (Jacobsen et al. 2011). CB inhalation leads to SBs in the livers of dams and prenatally exposed offspring, while intratracheal instillation of a corresponding dose did not cause SBs (Jackson et al. 2011a). However, in another study intratracheal instillation of a similar dose of CB (Printex90) did induce SBs in the lungs and livers of adult mice (Bourdon unpublished results). Mice from the present study were previously used to explore hepatic gene expression in the F1 offspring exposed in utero to CB. This work revealed that 476 genes related to inflammation and cell regulation were differentially expressed in CB-exposed females, while only 17 genes differed between exposure groups for males. Thus, these data support the notion that CB nanoparticles instilled in pregnant females lead to DNA damage and transcriptional activation in the exposed offspring (Jackson et al. 2011b).

One study has shown that dividing oocytes are susceptible to ESTR mutations in vivo: Prenatal exposure to 1 Gy of acute irradiation on GD12 lead to a 1.98-fold increase in ESTR mutations (Barber et al. 2009). In the present study dams were exposed on GD 7,10,15,18 to cover the majority of fetal development and to target mitotic germ cell division (Jackson et al. 2011a). Dams were exposed to 134 μg CB prior to meiotic prophase on ~GD13.5 when oocytes may be most susceptible to ESTR mutations (McLaren 2000).. However, the necessity of replication for mutation fixation in eggs has not been established for ESTR loci. This dose should ensure a high degree of inflammation and ROS, effects that we have previously observed after one day exposure with as little as 18 μg CB by intratracheal instillation (Bourdon unpublished results). To maximize the chance of seeing an effect, the dose chosen in the present study is ~2-fold higher than in (Ritz et al. 2011). However, it is still comparable to the permissible occupational exposure limit by Danish Regulation for CB (Jackson et al. 2011a).

Our results on NP-induced ESTR mutations (Ritz et al. 2011; Boisen unpublished) (table 1) suggest that germ cells in females are less susceptible to mutations than in males, even during mitotic cell division.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AMZB was substantially involved in the design of the study, collected animal tissue, processed samples and performed the electrophoresis, blot probing, image processing, mutation scoring, statistical analysis and drafted the manuscript. TS probed and developed images for a large portion of blots and revised the manuscript. PJ exposed the P generation mice, assigned F1 offspring for the current study and revised the manuscript critically. KSH was project manager of the study and revised the manuscript critically. HW was substantially involved in the design of the study and revised the manuscript critically. CLY was substantially involved in the design of the study, scored mutations and revised the manuscript critically. UBV was substantially involved in the design of the study and revised the manuscript critically. All authors read and approved the final version of the manuscript.

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Table 1. Summary of ESTR mutation rates in F2 offspring of female C57BL/6 mice prenatally exposed to DEP, $TiO_2$ or CB

| Groups | N (F2 offspring) | Mutant bands | | Mutation rate ± SEM (P value [a]) | |
|---|---|---|---|---|---|
| | | Paternal origin | Maternal origin | Paternal origin | Maternal origin |
| **1) DEP Female controls** | 79 | 9 | 5 | 0.057±0.026 | 0.032±0.020 |
| **DEP Female exposed** | 72 | 5 | 9 | 0.035±0.022 (P=0.261) | 0.063±0.029 (P=0.16) |
| **2 $TiO_2$ Female controls** | 164 | 20 | 12 | 0.061±0.00281 | 0.037±0.00297 |
| **$TiO_2$ Female exposed** | 192 | 18 | 11 | 0.047±0.01068 (P=0.84) | 0.029±0.01328 (P=0.79) |
| **3 CB Female controls** | 253 | 19 | 12 | 0.038±0.00150 | 0.024±0.00168 |
| **CB Female exposed** | 178 | 19 | 9 | 0.053±0.00250 (P=0.17) | 0.025±0.00157 (P=0.53) |

[a] Fisher's exact test 1-tailed

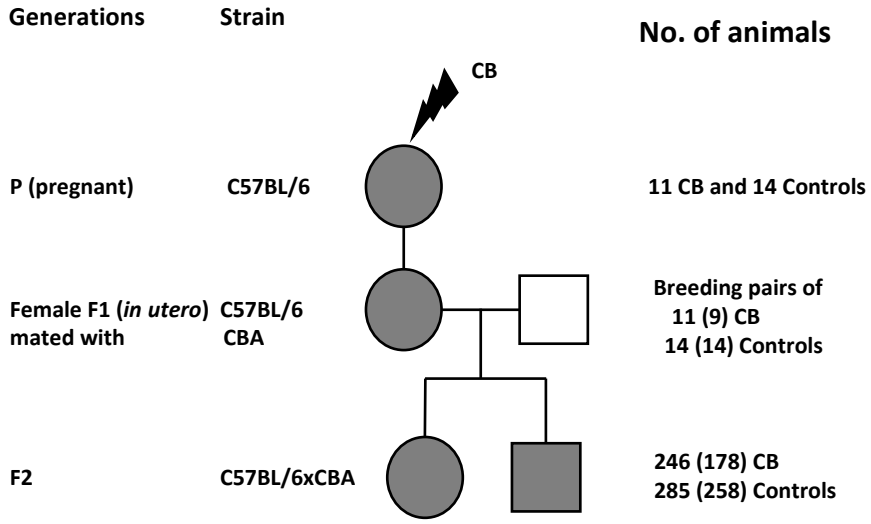
Groups: 1) Ritz *et al*. 2011 2) Boisen unpublished results 3) Boisen unpublished results.

Figure Legend:

Figure 1. Overview of the pedigree study.

Circles and squares represent female and male mice respectively. Grey symbols represent exposed animals and their descendants. White squares represent non-exposed CBA mates. Generation P pregnant mothers were exposed: 11 CB exposed and 14 Controls. 246 F2 offspring were collected from CB and 285 from Controls (number of successfully analyzed offspring 178 and 258, respectively).

Figure 1. Overview of the pedigree study.



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