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A critical review of studies on the reproductive and developmental toxicity of nanomaterials

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Disclaimer

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A critical review of studies on the reproductive and developmental toxicity of nanomaterials

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Table of Contents

Table of Figures

Table of Tables

Abbreviations

AlNP:	Aluminium nanoparticles
AgNP:	Silver nanoparticle
AuNP:	Gold nanoparticle
CB:	Carbon black
CdONP:	Cadmium oxide nanoparticle
CNS :	Central Nervous System
CNT:	Carbon nanotube
CuNP:	Copper nanoparticle
ENM:	Engineered nanomaterial
FSH:	Follicle stimulating hormone
FeNP:	Iron nanoparticles
GD:	Gestation day
GFAP:	Glial fibrillary acidic protein
GLP:	Good laboratory practice
Inh.:	Inhalation
i.p.:	Intraperitoneal
i.v.:	Intravenous
K-score:	Klimisch score
LH:	Luteinizing hormone
MCF-7 cells:	Human breast adenocarcinoma cell line
MN:	Manufactured nanomaterial
Mn_2O_3NP:	Mangan oxide nanoparticle
MWCNT:	Multiwalled carbon nanotube
N-score:	Score for nano-characterisation
NP:	Nanoparticle
NiNP:	Nickel nanoparticle
NM:	Nanomaterial
OECD:	The Organisation for Economic Co-operation and Development
PEG:	Polyethylene glycol
PVP:	Polyvinyl pyrrolidone
QD:	Quantum dot
R-score:	Relevance score
Resp.:	Respiratory
SAS:	Synthetic amorphous silica
s.c.	Subcutaneous
SeNP:	Selenium nanoparticles
SiO_2NP:	Silicon oxide nanoparticles
SOD:	Superoxide dismutase
SWCNT:	Singlewalled carbon nanotube
TEM:	Transmission electron microscopy
TG:	Test guideline
TiO_2NP:	Titanium dioxide nanoparticles
ZnONP:	Zinc oxide nanoparticles

Abstract

The scope of this project was to perform a critical review of the current knowledge from studies with testing of manufactured nanomaterials for reproductive and developmental toxicity.

Relevant literature databases and relevant search terms were identified, and a literature search was conducted in order to identify publications with *in vivo* data where manufactured nanomaterials have been tested for reproductive and developmental toxicity using an exposure route relevant for human exposure.

As a result, 111 publications covering 19 nanomaterials were identified. The publications were further assessed in full text and key information was extracted and transferred into tables for the specific nanomaterial. In order to identify the most relevant studies, each publication was scored for nano-characterisation, reliability/quality of the study, and the relevance in the context of the project.

A common overview of the data is given with respect to type of data (kinetic/ distribution data, fertility data, developmental data) and with respect to test species and exposure route used for the testing of each nanomaterial.

Based on the data a series of key questions regarding the reproductive and developmental toxicity is -as far as possible- answered and some recommendations for future work are given.

Executive summary

Objective

Reproductive and developmental toxicity are critical adverse effects that can occur upon systemic uptake of a substance. There is a concern that nanomaterials may cause such effects due to their ability to pass cell membranes and biological barriers in the human body.

The scope of this project was to perform a critical review of the current knowledge on the reproductive and developmental effects that have been reported with nanomaterials. Thus, the current project intends to provide:

An overview of available data with relevance for human toxicity based on a structured literature search.

A presentation and evaluation of the available data.

A scientific discussion of the data to provide answers to a series of key questions in relation to:

- *Which type of data is available, quality and relevance?*

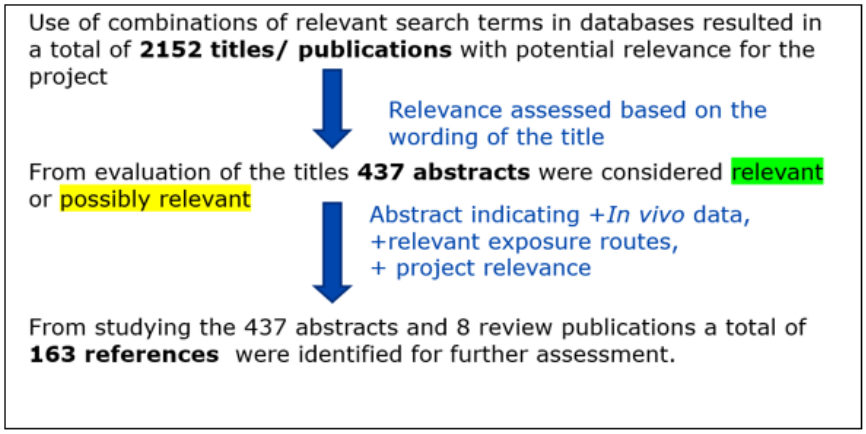
- *What does the data indicate with regard to kinetic properties of the nanomaterials (e.g. penetration into gonads and across placenta and into foetus and critical organ systems)?*

- *Which types of adverse effects on fertility have been observed?*

- *Which types of adverse effects on developmental effects have been observed?*

Literature search

Based on a structured literature search using STN (Scientific and Technical information Network) 2152 publications were identified using search terms relevant for identifying publications on manufactured nanomaterials in combination with a range of search terms relevant for description of reproductive and developmental toxicity. The retrieved publications were further screened for relevance as indicated in the figure below



During examination of the 163 references in full text, further references were included based on expert assessment, so that a total of 177 references were covered in the full text analysis. However, when screening these references for relevant data specifically to the project, the number of references were reduced to 111 references, as some studies did not fulfil the inclusion criteria after all or were reviews.

Data availability, quality and relevance

The 111 studies considered relevant for examining the reproductive and developmental toxicity of manufactured nanomaterials covered *in vivo* testing using rats or mice as test species. The selected studies used oral or respiratory tract exposure (inhalation or instillation), which were considered relevant routes of exposure for the human situation. A screening for the most relevant information from each of the 111 publications was made. The extracted data is reported in the nanomaterial-specific appendices to this report.

Testing of nano titanium dioxide and nano silver was most frequent and together contributed with 48% of the studies, whereas nano zinc oxide, nano silicon oxide and carbon-based nanomaterials together contributed with 34 % of the studies. 13 other nanomaterials contributed with the remaining 18% of the studies.

A total of six OECD TG studies were identified (three OECD TG 414 with TiO2NP, ZnONP, SiO2NP; one OECD TG 422 with AgNP; one OECD TG 416 with SiO_2NP and one OECD TG 415 with NiNP). All studies were conducted in rats using exposure by oral gavage.

For the studies that did not adhere to OECD guideline testing, many can be considered proof-of-concept and hypothesis generating studies, rather than conclusive studies, as often only a single to few target organs or bioindicators of effects were studied (e.g. mechanistic or functional parameters, for example gene expression in gonadal tissue or vascular reactivity in placenta) rather than commonly accepted end-points for adverse effects on reproduction and development.

Testing substances in bulk form alongside nanoforms to uncover dependence of particle size for toxicity were rather seldomly performed, and only few studies were identified.

It was apparent that very different levels of particle characterisation were provided for the studies, and only a minority of the references provided data on all of the key parameters: chemical composition, particle size, shape/aspect ratio and surface chemistry, indicated as toxicologically relevant parameters in the guidance for REACH registration of a nanomaterial.

Kinetics

Keeping in mind that the route of administration was oral or inhalation, the particles would have to cross the intestinal barrier or the lung before reaching the blood-testes or the placental barrier.

For nano titanium oxide, distribution of elemental Ti to the placenta was found only at a very high oral exposure level of 1000 mg/kg bw/day (no data on foetal concentration given) whereas translocation across the placenta and distribution to foetuses was observed at lower levels (≤ 280 mg/kg bw/day) of oral exposure for nano zinc oxide and nano silver (measured as elemental zinc and silver).

These data suggest that nanoparticles can translocate across the placenta and reach the foetal compartment following maternal exposure by the oral route. The majority of the studies assessed tissue concentrations by chemical element analysis rather than the presence of nanoparticles. Two *in vivo* studies on placental transfer of silver do, however, assess transfer of the actual particles and observe the presence of silver particles in the foetus. Transport of polystyrene beads across the placenta was furthermore documented in the *ex vivo* placenta perfusion model. Several studies might not have been able to detect translocation of very low levels of particles due to too high limits of detection. It should be noted that translocation of even small proportions of the administered dose may still represent a considerable number of particles. More data is needed, with focus on detection of transfer of the particles across the placenta, especially following exposure by the oral and airway routes.

Transfer of nanoparticles across the blood-testis barrier was investigated in several studies with silver particles. A dose-dependent increase in tissue levels was observed in rats after repeated oral exposure to 56 nm particles from 30 to 500 mg/kg bw/day. For silicon dioxide nanoparticles, no increase in Si content was observed in testes and ovaries after oral exposure at up to 1000 mg/kg bw/day in rats. No distribution of Ti to testes was detected after oral or i.v. exposure of male rats to nanosized titanium dioxide particles up to a cumulated exposure of 62 mg/kg bw. In females, Ti distributed to the ovaries after i.v. but not when orally exposed. After 90 days of repeated oral exposure of rats to gold nanoparticles, particles distributed and accumulated in testicular tissue, at an exposure level of 20 µg/kg bw/day (determined by Transmission electron microscopy (TEM)).

These data suggest that nanoparticles can distribute to organs with relevance for reproduction and foetal development, but that the potential for transfer depends on the type of nanomaterial and the particle size. More data is needed to elucidate the distribution to testis and female reproductive organs.

Fertility

The majority of the identified studies examining effects on gonads investigated the effects in males, whereas females were much less studied. The studies are generally performed in adult or pre-pubertal/pubertal rats or mice. The main adverse effects reported for all the investigated nanoparticles are reduced sperm quality, daily sperm production as well as reduced weight of the testes and histopathological changes in the testes. The effects on reproductive performance has only been sparsely investigated. However, it should be noted that some of these data to some extent are not consistent with data available from OECD TG studies, reporting no reproductive effects of the same nanomaterial. Sperm parameters was however not investigated in the OECD GT 422 studies available in rats.

For some of the nanomaterials, the effects on testes and sperm parameters could probably be explained by the chemical exposure as such, rather than exposure to the nanoform of the chemical, as e.g. cadmium and cobalt are known reproductive toxicants.

In general, there are large variations in the study protocols among the studies focusing on effects on the sex organs and fertility in relation to timing and duration of exposure, as well as the level of nanoparticle characterization. This hampers comparison of studies and conclusion of the findings, as only fragmented observations are obtained for most particles. More systematic data is therefore needed to elucidate the effects on fertility and reproductive parameters of nanoparticles of different sizes and composition.

Development

In general, results from studies applying OECD TG (oral exposure in rats) indicated no to limited concern for developmental toxicity of TiO_2NP, ZnONP, SiO_2NP and AgNP.

Based on review of the non-OECD TG studies, which often investigate different outcomes compared to the OECD TG studies, some endpoints of concern can, however, be pointed out:

- TiO_2NP: effects on placenta and cardiovascular system in the offspring (rats, inhalation)
- AgNP: developmental neurotoxicity (rats, oral)
- ZnONP: effects on placenta, fetal growth and offspring viability (mice, oral)
- SiO_2NP: no specific concerns based on the present material
- MWCNT: no specific concern based on the present material
- Carbon black: developmental neurotoxicity (mice, inhalation and resp. tract exposure)
- AlNP: developmental neurotoxicity (mice, resp. tract exposure)
- CuNP: decreased offspring viability (mice, inhalation)
- FeNP: decreased offspring viability (mice, resp. tract exposure)
- NiNP: decreased offspring viability (rats, oral)

For TiO_2NP and ZnONP, effects on the placenta were reported after inhalation and oral exposure, respectively. Maternal exposure to CuNP, FeNP and NiNP and ZnONP was associated with decreased survival and/or growth and viability of the offspring pups after maternal exposure via the airways (CuNP, FeNP) or the oral route (NiNP, ZnONP). Decreased pup survival is a well-known effect from water soluble nickel compounds and may therefore be associated to the chemical composition rather than the particulate form. Changes in the offspring organs after birth have been observed in some studies. In offspring from pregnant females exposed by inhalation to TiO_2NP, adverse effects on the cardiac tissue and cardiac functioning have been observed. Further, there is evidence that the brain may be sensitive to maternal NP exposure, as both AgNP, carbon black and AlNP report effects in brain or signs of neurotoxicity after exposure. In the case of AlNP it is to be noted that exposure to the soluble Al-ion is associated to neurodegenerative changes of the brain and thus the neurotoxicity may not specifically be associated to the nanoform. For carbon black, changes in specific cell populations in the central nervous system (CNS) have consistently been observed following maternal airway exposure.

However, as for the data on fertility the non-guideline testing showed a scattered picture of the developmental potential of the various nanomaterials. Thus, the different types of test design, lack of OECD TG testing following inhalation exposure and large variation in nano-characterization, again hampers confirmative conclusions regarding developmental toxicity of individual nanomaterials.

Uncertainties, limitations and data gaps

As indicated above, the majority of the data identified originates from non-OECD TG studies. For some of these studies, there are few animals per group, use of only one dose level or only a single administration of test material. This may introduce uncertainty as to their predictive value. Although a comparable test design may be present for two studies, differences in examination techniques, animal strain, and qualities of the nanomaterial and its characterisation, hampers comparison between studies, even if the same species and exposure route have been applied. This makes overall conclusions for the nanomaterials uncertain and difficult. Of note, if inflammation is a determinant in toxicity, how does particle exposure influence individuals who already suffer from low-grade inflammation (e.g. asthma and obesity).

Overall, studies using inhalation exposure are few in numbers. This is critical, as inhalation exposure may be critical for toxicity of nanomaterials in comparison to oral exposure. Inhalation is the primary route of exposure in the occupational setting and inhalation can be considered a more direct exposure route for the dispersed nanoparticles. Further, uptake of NPs may differ between the oral and the airway routes of exposure and the route of exposure may influence the translocation of particles from one biological compartment to another, and therefore ultimately affect their toxicity. Finally, NPs deposited in the alveoles may be removed relatively slowly and constitute a continuous source of exposure, whereas passage time is much faster in the gastro-intestinal tract.

Importantly, among the retrieved studies there is an almost complete lack of studies on female fertility.

Follow-up/ suggestions/ recommendations

Based on the learnings from this project some general proposals and considerations for future research and testing can be provided:

- Application of a thorough/ more standardized characterisation of the nanomaterial and the nanomaterial exposure to include the most important determinants of toxicity.

- Study the effects of nanoparticles in parallel with larger particles, to gain knowledge of

differences in toxicity relating to size (or other relevant physico-chemical parameters, such as particle form).

- Increase focus on the airway route of exposure.

- Increase focus on female fertility and reproductive parameters.

- Select meaningful periods of exposure. Take into account that particle translocation probably varies considerably during gestation.

- In developmental toxicity, include postnatal functional parameters to a larger degree (offspring fertility, neurofunction- and histology, cardiovascular and immune function).

- Always report gestational and litter parameters

- Follow-up testing of outcomes where previous results raise concern to clarify potential for induction of adverse reproductive or developmental effects.

- Adhere to the principles of OECD TGs to the highest extent possible, even if the full study guideline is not possible to apply. If not included already, include parameters where previous study results raise concern.

- Investigate particle transfer across “barriers” (blood-testes-barrier, placenta), with application of highly sensitive methods of detection of both the bulk material and particles.

- Identify underlying mechanisms of toxicity for grouping of materials. Does the particulate entity as such possess the ability to change foetal development (irrespective of material) or are oxidative stress and inflammation the driving forces?

- Coordination of the testing (e.g. in testing programs) in order to achieve a more systematic approach for the testing

Foreword

Reproductive and developmental toxicity effects are critical adverse effects that can occur upon systemic uptake of a substance. There is a concern that nanomaterials may cause such effects due to their ability to pass cell membranes and biological barriers in the human body.

The scope of this project was to perform a critical review of the current knowledge of the studies on reproductive and developmental effects that have been performed and reported with nanomaterials. The review provides an updated overview of data on reproductive and developmental toxicity of nanomaterials as well as the newest scientific state-of-the-art information.

The major points of interest are:

A complete overview of available data

A presentation and evaluation of the available data

A scientific discussion of the data to provide answers to a series of key questions on the subject

The current report represents the outcome of such a critical review made by DHI A/S and Danish National Research Centre for the Working Environment under the framework contract with ECHA.

1. Description of the project

1.1 Objective of the project

The overall objective of the requested services is to perform literature searches for studies focusing on reproductive and developmental effects of nanomaterials and to review the information available in the public domain.

Reproductive and developmental effects are critical adverse effects that can occur upon systemic uptake of a substance. There is a concern that due to their small size nanomaterials may be able to pass through membranes, enter cells and cause direct (adverse) effects on the target organs. Furthermore, there is a concern that nanomaterials may be able to pass also protective barriers such as the placental barrier, blood-testis barrier or the blood-brain barrier. The number of nanotoxicological studies, including studies examining reproductive and developmental effects, is constantly increasing, but extensive reviews of reproductive and developmental effects of nanomaterials are still missing.

Thus, the current project intends to provide:

A complete overview of available data

A presentation and evaluation of the available data

A scientific discussion of the data with the goal to provide answers to a series of key questions given by ECHA.

The output of this work should elaborate and respond to the following questions:

What studies relevant to reproductive/developmental toxicity are available on nanomaterials?

In vitro, ex vivo and in vivo (animal studies, human case studies or epidemiological data).

What are the test guidelines followed for the studies (if any)? Are the results available in a structured way, e.g. following OECD harmonised templates?

Are there comparable studies in which nanomaterials and bulk sized materials and/or soluble forms of these materials with the same chemical composition are compared, or studies in which different nanoforms of the same nanomaterials are compared? What are the differences in their toxicity profiles? Can any conclusions be drawn?

Is there evidence suggesting that nanomaterials can cross relevant biological barriers (placental barrier, blood-testis barrier, blood-brain barrier (pre- and postnatally)?

Are there differences between different types of nanomaterials (e.g. nanomaterials of different size, shape (e.g. particles versus fibres), surface properties and solubility)?

What factors (e.g. physico-chemical parameters) are known to influence the ability of nanomaterials to reach the developing foetus (in utero) when the mothers/dams/does have been exposed to nanomaterials during pregnancy?

What kind of changes or adverse effects have been observed in the developing foetuses of mothers/dams/does that have been exposed to nanomaterials during pregnancy?

Is there evidence of direct effects (direct interference of the nanomaterial with embryo/foetal tissue function) and/or indirect effects (e.g. induction of the release of mediators in maternal or placental tissue)?

Can any conclusions be drawn? Are there any concerns identified? Are these relevant to humans?

What are the uncertainties of these studies? For example, have the test materials been adequately characterised?

What kind of changes or adverse effects on fertility have been observed in males and females?

Are there differences between different types of nanomaterials (nanomaterials of different size, shape (e.g. particles versus fibres) and solubility)?

Can any conclusions be drawn? Are there any concerns identified? Are these relevant to humans?

What are the uncertainties of these studies? For example, have the test materials been adequately characterised?

2. Definition of the scope of the review and the methodology

The work of the project was planned according to the following four work packages:

Work Package 1, Definition of the scope of the review and the methodology

Work Package 2, Literature search and review

Work Package 3, A transparent analysis of the data

Work Package 4, Final report

The purposes, content and methodology for performing these tasks is described in appendix A, making up the overall scene, the strategy and methodology of the project in order to be able to provide an overview and review of the relevant literature and to answer the questions as raised in section 1.1.

2.1 Definition of the scope of the review and the methodology

The scoping of the study and description of the methodology is described in detail in Appendix A. However, a short summary of the outcome of this work is presented below.

Nanomaterials covered

Nanomaterials within the scope:

- manufactured nanomaterials (MNs) that are commercially available and produced in an industrial scale relevant to REACH regulation

Nanomaterials out of scope:

- advanced use of nanomaterials for medical treatment, diagnostic or analytical purposes are outside of the scope of this report

- nanoparticles in ambient air, combustion derived nanoparticles from engine exhaust and wood burning, asbestos fibres in nanoscale

Database and search terms

Relevant studies to search for:

- studies relevant for assessing reproductive and developmental effects of manufactured nanomaterials in relation to human health with focus on *in vivo* studies using species relevant for human health assessment

Identification of database

Via STN (Scientific and Technical information Network) from Fiz-Karlsruhe:

- **TOXCENTER (Toxicology Center)** is a cluster of bibliographic databases that covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. The records in the file contain bibliographic data, abstracts, indexing terms, chemical names, and CAS Registry Numbers.

- **EMBASE (Excerpta Medica)** is a comprehensive bibliographic database that covers the worldwide literature on biomedical and pharmaceutical fields. It is produced by Elsevier B.V., the world's largest publisher of scientific information.

- **Science Citation Index (SciSearch®)** contains all records published in Science Citation Index ExpandedTM. Records from January 1991 to the present include abstracts, author keywords, and KeyWords Plus®. Authors, bibliographic information cited references, and KeyWords Plus are searchable.

Identification of relevant search terms:

Eight review publications regarding reproductive and developmental toxicity of nanomaterials were studied. Based on the keywords and wording used in these publications, relevant search terms were identified within the following categories:

- Nanomaterial relevant search terms

- Relevant search terms for effects and target organs in relation to developmental and reproductive toxicity

- Test system relevant search terms

To capture relevant nanomaterials the following *nanomaterial relevant search terms* were used:

Nanoparticle#[1]; Nanomaterial#; nanofib?[2]; nanotube#; nanowire; carbon nanotube#; CNT#; MWCNT#; SWCNT#; multiwall; singlewall; graphene; CB; carbon black; Printex90; Printex 90; fullerene#; silver; AgNP; ?NP; gold; nickel; cerium; zinc; silicium; silica; titanium; cadmium; copper; Au; Ni; Ce; Zn; Si; Ti; Cd; Cu*

To capture relevant effects, target organs, exposure periods and mechanisms the following *reproductive and developmental toxicity relevant search* terms were used:

Reproduct?; reprotoxic?; development?; maternal?; paternal?; birth, fetal?; foetal?; fetus; foetus; gestation?, pregnan?; prenatal?; postnatal?, perinatal?; neonatal?; miscarriage; abort?; resorp?; retard?; delayed; newborn#; pup#; birth defect#; abnormal?; congenital?; breast; lactat?; embryo?; terato?; placenta?; ovar?; oocyt?; follic?; uterus, uterine; menstruation; testic?; testis; testes; semen, sperm?; germline; fertil?; infertil?, endocrine?; estrog?; estrus; estrous; anti-estrogen?; antiestrogen?; oestrog?; oestrus; oestrous; anti-oestrogen?; antioestrogen? androgen?; anti-androgen?; antiandrogen?; thyroid?; hormon?; disrupt?; steroid; mechanis?; transfer; distribut?; penetrat?; transport?; translocat?

To capture *relevant target organisms* and *test systems* the following search terms were used

Human#; rat#; mouse; mice; rabbit#; chick? *in vivo*; rodent#, *ex vivo*

Search strategy:

The data search was performed in the "title" field of the database, i.e. the search string should combine the "*nano material relevant terms*" with the "*Reproductive/ developmental toxicity relevant terms*" for example:

[1] #: any letter e.g. plural -s
[2] ?: more letters (any letters)

(*all nano relevant terms used with an OR between them, title search*)

"AND"

(*all reproductive/developmental toxicity relevant terms with an OR between them, title search*)

In order not to gain a lot of hits only relevant to ecotoxicity, or technical development or medical development of nanomaterials, the search is further combined with "*test system relevant terms*" in the search fields of "all fields" in the database i.e.:

"AND"

(*all test system relevant search terms with an "OR" between them, all field search*)

3. Literature search results

Using the databases and the search strategy described in chapter 2 a large number of titles were retrieved (> 8000). Many studies regarding cancer therapy or development of methods were among the hits. The search was therefore further narrowed by applying a number of search terms to the 'all fields' category. This was done to identify the studies regarding nanomaterial investigation in relation to what is relevant for the present assignment, reproduction and developmental toxicity. The additional search terms included were:

nano? graphene, cb, carbon, black, carbon black, (carbon(w)black), printex90, printex, 90, printex 90, (printex(w)90), fullerene# (with an "OR" between)

"AND"

reproduct? reprotoxic? developmental, fertility (with an "OR" between)

This resulted in **a total of 2152 titles/publications**

When evaluating the titles of the references according to the inclusion and exclusion criteria indicated in section 2.3.3.1 **a total of 437 of the 2152 titles were selected** for further assessment of their abstracts. See printout in appendix B showing all the 2152 titles in which the 437 selected titles are marked with green colour and the non-selected titles are marked with red colour.

Due to the surprisingly large number of titles (when compared to the bibliographic analyses by Wang et al. (2018) as described in section 2.2.2) it was decided that the inclusion/exclusion criteria for selection of references based on the abstracts should be further strengthened. In order to focus our resources on the publications considered most relevant for addressing the objective of this project, the suggested criteria in section 2.3.3.2 was elaborated:

Inclusion criteria

Relevant MNs addressed

In vivo test systems targeting human health effects

Relevant exposure route for humans i.e. exposure by oral, dermal, or respiratory route.

Data concerning specific reproductive or developmental endpoints (use of relevant search terms in a relevant context)

Exclusion criteria

Data on irrelevant species (e.g. species for ecotoxicological assessment)

In vivo studies using unrealistic human exposure routes (e.g. s.c., i.p., i.v. injections)

Data not addressing the scope of this project

Abstracts only mentioning in vitro tests

In addition to the principles of these inclusion/exclusion criteria, a preliminary expert assessment was made of the relevance of each study to this project.

Thus, it was decided to focus on the identified *in vivo* studies as such data are considered especially relevant in relation to the questions set out by ECHA in the present assignment (see section 2.2), where investigation of relevant adverse effects to humans are emphasized. *In vivo* data, i.e. data from an intact organism of a relevant species, are much more indicative of reproductive and developmental effects in relation to human exposure compared to data from

in vitro or *ex vivo* studies, where the predictive value is limited due to the artificial way of dosing the cell systems/tissues directly. Systemic absorption of nanomaterials is a key issue for the development of adverse effects in relation to reproductive toxicity and developmental toxicity. As absorption of nanomaterials from oral, dermal and inhalation exposure are generally found to be rather limited and as these routes of exposure are most relevant for humans, it was further decided to focus on *in vivo* data using the oral, dermal and respiratory routes of exposure. Hence, data generated using intravenous, intraperitoneal, or subcutaneous injection was considered less relevant, as in such studies artificial and excessive high systemic exposure levels can be generated. These latter routes furthermore bypass the formation of coronas (proteins and lipids adhering to the surface of the particles) specific for the oral and airways, that may be specific to the port of entry and can influence on the ability of particles to cross biological barriers. To answer the question related to evidence suggesting that nanomaterials can cross relevant biological barriers, data on *ex vivo* placental transfer was, however, included in the assessment.

Results

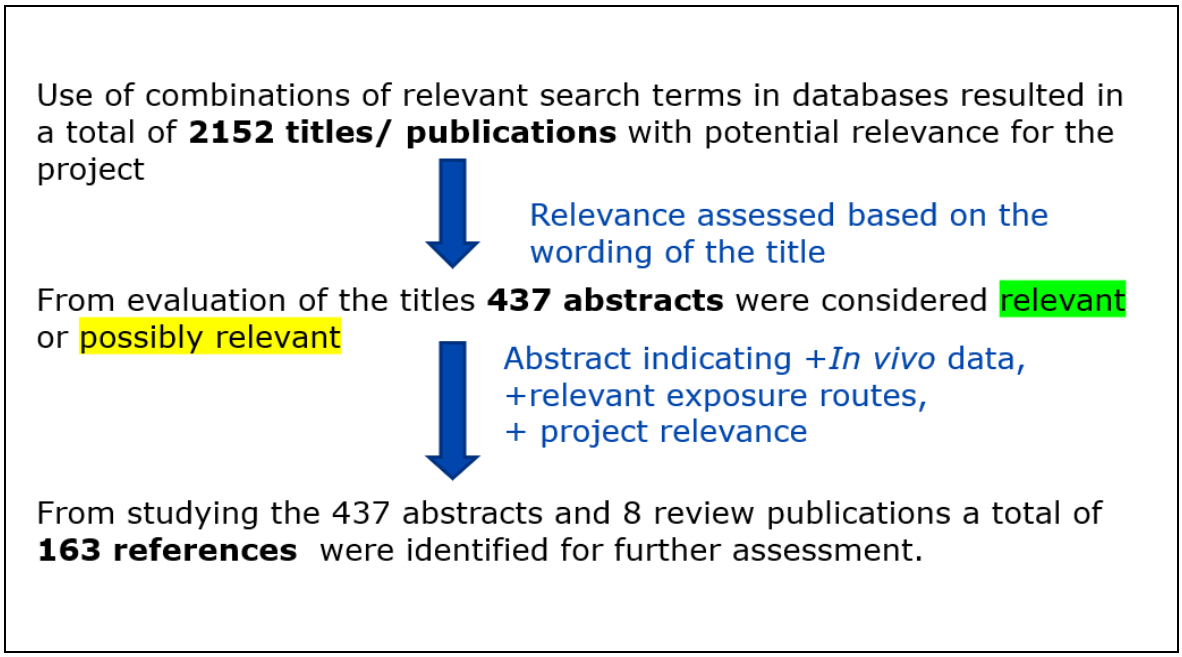
Using these criteria, the abstracts were scored with relevant, possibly relevant, or not relevant.

From the 437 evaluated abstracts a total of 139 references were selected for further examination as **98 references** were scored green and **41 references** scored yellow.

From the assessment of the eight review publications described and discussed in section 2.2 further **24 references** were selected as relevant based on the description in the reviews and the description in the abstract of the references.

Thus, in total **163 references** should be further assessed.

Figure 3-1: Overall outcome of the literature search



4. Analysis of the data

During examination of the 163 full text references, further references were included based on expert assessment so that a total of 177 references were included in the analysis.

Of these 177 references several references were, however, excluded as some references did not pass the exclusion/inclusion criteria after all (e.g. not relevant animal species or not relevant exposure route). Also, some references were reviews that included studies already found by the search, but in some cases contributed to further references to be included. It should be noted that 8 of the 163 references were excluded due to serious doubts as to the scientific quality and validity of the studies (see appendix B2, titanium dioxide).

To give a structured overview of the references, they were split into groups according to the chemical composition of the nanomaterial. The grouping into nanomaterials was applied as indicated in Table 4-1:

Table 4-1: Grouping of references

Nanomaterial	No. of references for full text examination	No. of references considered relevant for further assessment
Titanium dioxide, Appendix B2	46	28
Silver, Appendix B3	34	26
Zinc oxide, Appendix B4	15	10
Silicon dioxide, Appendix B5	13	8
Carbon nanotubes + graphene, Appendix B6	16	7
Carbon black, Appendix B7	21	12
Other nanomaterials*, Appendix B8	32	20
Total	177	111

**nanomaterials with only few (≤ 5) references each, covering the following: Aluminium, Cadmium, Cerium, Cobalt, Copper, Gold, Iron, Lead, Manganese, Nickel, Platinum, Polystyrene, Selenium*

Because of the large amount of references to be reviewed, it was not possible to make an in-depth evaluation of each of the references. Instead a screening for the most relevant information from each of the 111 publications was made and data was extracted and reported in the following template, separately for each nanomaterial:

Template

Legend: *Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Nanomaterial XX					
Reference	**Test material, nano-charactersation**	**Species/ strain. No /group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1 - 11)** **K-score (1 - 4)** **R-score (0, +, ++)** **Comments**
Lee et al. 2018	**Cc**: **Pu**: **Ps**: **Sh**: **Cr**: **Sa**: **Sc**: **Ch**: **Ag**: **Em**: **Ws**:		**Route/ adm:** **Duration/period:** **Exposure levels:**		**N:** **K:** **R:**
Key findings: Fertility					
Key findings: Development					
Key findings: Kinetics					
Wang et al. 2015	**Cc**: **Pu**: - **Ps**: **Sh**:		**Route/ adm:** **Duration/period:**		**N:** **K:** **R:**

Based on the method described by Card & Magnuson (2010) an overall assessment of the nano characterisation and test design and reporting are given as an N(nano)-score and a K-(Klimisch) score.

For nano-characterisation (**N-score**), publications were screened for the following parameters and scored with 1 point for each of the included parameters (i.e. a max of N=11 points):

1. agglomeration and/or aggregation
2. chemical composition
3. crystal structure/crystallinity
4. particle size/size distribution
5. Purity
6. Shape
7. surface area
8. surface charge
9. surface chemistry (including composition and reactivity)
10. whether any characterisation was conducted in the relevant experimental media
11. water solubility

The ten first parameters are parameters suggested by Card & Magnuson (2010). The parameter "water solubility" was further included as this parameter is considered a relevant parameter when assessing the toxicity and kinetics of the nanomaterial.

For the quality regarding study design and reliability of the testing a Klimisch score (1-4) was applied as a **K-score**:

K score 1: reliable without restrictions
K score 2: reliable with restrictions
K score 3: unreliable
K score 4: not assignable due to insufficient experimental details

Further, an **R-score** regarding relevance of information for this project is provided:

R++: information highly relevant (key reference for the assessment)
R+: relevant information (supporting or indicative references)
R0: not relevant for further consideration of relevance

Data on each of the nanomaterials is reported in the above table format in Appendices B2-B8. Below each table, evaluation and overview of the most important findings for the specific nanomaterial are given using the following subheadings:

Data availability

Nano-characterisation

Kinetics

Fertility

Developmental toxicity

Overall evaluation

Data gaps

In the following sections these individual overviews are inserted in order to give an overview of the available data and findings.

4.1 Overview of the data

4.1.1 Sorting of all references described in appendices (B2—B8)

In table 4-2 below, the *in vivo* study entries for each material in appendices B2-B8 are divided into type of data (fertility (F), development (D), kinetics (K)), animal species and exposure route.

Table 4-2. Number of entries reported in tables in appendices B2-B8 sorted in relation to type of data, animal species and exposure route

	Type of data	Rats, oral	Mice, oral	Rats, inh./arte.	Mice inh./arte.	Total	
Titanium dioxide Appendix B2	*F*	4	2	-	-/1	7	28 (29%)
	D	5	2	8/-	3/-	18	
	K	2	-	-	1/-	3	
Silver Appendix B3	*F*	6	-	-	-	6	21 (21%)
	D	5	3	-	1	9	
	K	5	1	-	-	6	
Zinc oxide Appendix B4	*F*	1	4	-	-	5	10 (10%)
	D	3	2	-	-	5	
	K	-	-	-	-	0	

	Type of data	Rats, oral	Mice, oral	Rats, inh./arte.	Mice inh./arte.	Total	
Silicon dioxide Appendix B5	*F*	2	1	-	-	3	5 (5%)
	D	1	-	-	-	1	
	K	1	-	-	-	1	
Carbon nanotubes + graphene Appendix B6	*F*	1	-	-	-/1	2	6 (6%)
	D	1	-	-	-/3	4	
	K	-	-	-	-	0	
Carbon black Appendix B7	*F*	-	-	-	-/2	2	13 (14%)
	D	-	-	-	3/8	11	
	K	-	-	-	-	0	
13 other nanomaterials Appendix B8	*F*	5	3	-	-	8	14 (15%)
	D	-	1	-	2/2	5	
	K	-	-	1/-	-	1	
Total Appendix B2-B8		42 (44%)	19 (20%)	9 (9%)	10/17 (10%/18%)	97 (100%)	97 (100%)

F: entry mainly covering fertility, i.e. grouped under fertility data in appendix B2-B8
D: entry mainly covering development, i.e. grouped under developmental data in appendix B2-B8
K: entry mainly covering kinetic data, i.e. grouped under kinetic data in appendix B2-B8
arte: alternative respiratory tract exposure

It should be noted that the number of entries indicated in table 4-2 do not equal the number of the 111 references for further assessment indicated in table 4-1. In several instances one experiment resulted in several publications that in some instances are included as one entry in the appendix tables. Also, several *ex-vivo* studies are not covered by the entries in table 4-2. A 1:1 comparison between the number of references and the number of entries can therefore not be made.

Below, overall analysis of the findings from each of the nanomaterials/groups of nanomaterials are given (evaluation sections from appendices B2-B8).

4.1.2 Data on nano titanium dioxide (appendix B2)

Data availability

From the literature search 46 publications on titanium dioxide (TiO_2) NPs were identified for further examination in full text. Of these, 10 references were not further assessed, as they were excluded based on the inclusion/exclusion criteria as indicated in appendix B.1. Also, eight references from one specific group of researchers were excluded as serious doubts about the scientific validity of their work have been raised (further described in Appendix B2).

Of the remaining 28 publications, the most relevant and informative data could be extracted from the following publications (scored as R++ or R++/+):

Table 4-3: Nano titanium dioxide data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Shahin & Mohammed 2017 (N:4, K:2. R+/++)	Warheit et al. 2015 (N:9, K:1, R++) Lee et al. 2019 (N:8, K:1, R++)	Geraets et al. 2014 (N:6, K1/2,R++) Lee et al. 2019 (N:8, K:1, R++)
Rats, inhalation	-	Nurkiewicz and Stapleton (2013-2019) (N:8, K:2, R++)	-
Mice, oral	Song et al. 2017 (N:9, K:2, R++)	Philbrook et al. 2011 (N:8, K2, R++)	-
Mice, inhalation/ resp. tract	Lauvås et al. 2019 (N8, K:2, R:++)	Hougaard et al. 2010 (N:8, K2, R+/++) Kyjovska et al. 2013 (N:8, K2, R+/++)	Hougaard et al. 2010 (N:8, K2, R+/++)

Nano-characterisation

Of the publications included in the table above, the N-scores for nano-characterisation of the test item were in the range of 2-9 with an average score of 6.2. Four references did not provide information on the crystal structure. Nine references included testing of the rutile crystal structure and nine references included testing of the anatase crystal structure. Eight references tested a mixture of anatase and rutile TiO_2NPs.

No information on water solubility was given in the publications. In the REACH registration of titanium dioxide (CAS 13463-67-7) covering both the anatase and the rutile forms, it is indicated that nanosized titanium oxide does not dissolve to any relevant extent under regular environmental conditions and test data indicates a water solubility < 6 µg/L (ECHA, January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/15560).

Kinetics

Geraets et al. 2014 tested five different commercial qualities of TiO_2NP, covering both the anatase and the rutile form (particle sizes in the range of 6-20 nm), and found that after i.v. exposure to adult rats only very low levels of elemental titanium could be detected in the organs of rats. No such distribution could be detected after oral exposure to 8.4 - 59.9 mg/ kg bw.

In an OECD Test Guideline (TG) 414 test where pregnant female rats were gavaged with 0, 100, 300 and 1000 mg/kg bw/day of TiO_2NP (anatase/rutile: 80/20) with a particle size of 21 nm, increased levels of elemental titanium were found at the highest dose level in placenta (0.6 mg Ti/kg at the highest dose level vs 0.2 mg Ti/kg in control) (Lee et al. 2019) Levels of Ti in the foetus was not investigated.

Hougaard et al. (2010) did not find distribution of elemental Titanium (Ti) above the detection limit (0.2-5 mg Ti/kg) to milk from lactating mice or to liver from young pups following 1hr daily inhalation of TiO_2NP at 40 mg/m^3 during gestation days (GD) 8-18 of pregnancy.

Fertility

Shahin and Mohammed (2017) exposed adult male Wistar rats by daily oral gavage to 50 mg/kg bw/day of TiO_2NP (anatase form with a particle size of 25 nm) for either 7 days, 14 days, or 21 days. The exposure, in a duration-related manner, caused significant adverse responses in relation to testis and prostate weight; sex hormone levels; biomarkers indicating impaired spermatogenesis; biomarkers for lipid peroxidation and inflammation in testicular tissues; and on sperm parameters**.**

Song et al. (2017) examined testes and sperm quality in male mice after exposure to 0, 10, 50, or 100 mg/kg body bw/day TiO_2NP (anatase form with a particle size of 5-10 nm) by oral gavage for 28 days. Exposure did not affect the weight of the testicles and epididymis at any dose level. Sperm malformation and sperm cell micronucleus rate showed dose related and significant differences at the two highest dose levels. Exposure caused reduction in germ cell number and led to spherospermia, interstitial glands, malalignment, and vacuolization in spermatogenic cells at the two highest dose levels. Superoxide dismutase (SOD) activity significantly decreased at the highest dose level and the malondialdehyde significantly increased at the two highest dose levels, both of which are markers indicating cell damage in testis.

After intratracheal instillation of TiO_2NP (rutile, 20.6 nm) once weekly during seven weeks to adult male mice at a dose level of 63 µg/animal/dosing, no effects on weight of testis or epididymis, daily sperm production or plasma testosterone levels were found by Lauvås et al. (2019).

Developmental toxicity

Warheit et al. (2015) conducted a study following the OECD TG 414 using three non-nanoforms and three nanoforms of TiO_2 (both anatase and rutile forms with a particle size of 42-47 nm of the nanoforms). In all studies female rats were exposed by oral gavage to 0, 100, 300, or 1000 mg/kg bw/day of the test substance. No maternal toxicity or developmental adverse effects were noted in any of the studies.

A similar lack of findings was noted by Lee et al. 2019, that conducted an OECD TG 414 study in which female rats were exposed by oral gavage to 0, 100, 300 and 1000 mg/kg bw/day of TiO_2NP (anatase /rutile form: 80/20, particle size 21 nm).

Bowdridge et al. (2019) and Abukabda et al. (2019) exposed female rats to 12 mg/m3 (6h/day) of TiO_2NP (anatase/rutile: 80/20 with of particle size of 21 nm) by inhalation during gestation. Exposure resulted in increased placental weights and an impaired vascular reactivity in placenta considered as a sign of placenta dysfunction.

Stapleton and co-workers (2013-2019) exposed pregnant rats to TiO_2NP (anatase/rutile (80/20) with a particle size of 21 nm) by inhalation at approx. 10 mg/m^3 for up to 6 hr/day for different periods during gestation and found cardiovascular effects in offspring such as epigenetic and transcriptomic changes in cardiac tissue, reduced vascular reactivity in aorta, and reduction of maximal mitochondrial respiration in aorta tissue.

In Philbrook et al. 2011, female mice were exposed by a single oral gavage to 0, 100 or 1000 mg/kg bw of TiO_2NPs (rutile form, particle size of 50 nm) on day 9 of gestation. At the two highest dose levels TiO_2NPs negatively affected normal progeny development as assessed by a statistically significant increase in the number of foetuses with external morphological defects (5.5% at mid-dose and 2.5% at high dose compared to 0% in control) and at the highest dose level by a significantly greater percentage of non-viable foetuses (7.6% nonviable compared to 1.7% in controls). There was no significant difference between litter sizes, foetal resorptions, or mean foetal weight or length compared to the control group. Also, there was no increase in

the number of skeletal defects in foetuses and no histopathological changes in placentas, foetal livers and foetal kidneys.

Hougaard et al. (2010) exposed female mice by inhalation to approx. 40 mg/m^3 of TiO2NP (particle number concentration 1.7x 10^6 n/cm^3, rutile form, particle size 20.6 nm), 1h/day during GD 8-18. Slight neurobehavioral alterations were observed in the offspring. In the same offspring, Kyjovska et al. (2013) found that the maternal particulate exposure did not affect daily sperm production in the F1 male offspring, although TiO_2 tended to reduce sperm counts/g testicular tissue.

Overall evaluation

The current data indicate that oral exposure to high dose levels of TiO_2NP (e.g. 1000 mg/kg bw/day to pregnant rats) may lead to a small systemic uptake and distribution (measured as elemental Ti) into maternal organs including the placenta, however, at very low levels. Also, after inhalation during the gestation period in mice no increased Ti levels was found in milk or in livers from the pups.

Sparse amounts of data are available regarding effects on reproduction/fertility.
Repeated oral dosing of male rats to 50 mg/kg bw/day of TiO_2NP resulted in decreased prostate and testis weight and further disrupted the hormone profile by significantly decreased serum testosterone level and increased serum estradiol, Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) levels. Normal sperm counts decreased from 88% (control) to 68% after 21 days of exposure. In male mice repeated oral exposure has lead to increased level of sperm malformation and histopathological changes in the germinal tissue at dose levels of 50 and 100 mg/kg bw/day.
In male mice intratracheal instillation of TiO_2NP did not cause any effects on testes, epididymis, sperm count or plasma testosterone levels

Prenatal developmental testing according to OECD TG 414 has been performed using oral exposure of rats to both the anatase and the rutile crystalline form of TiO_2NP without any adverse reproductive/developmental outcome even at dose levels of 1000 mg/kg bw/day.
In pregnant mice a single oral exposure of 0, 100 or 1000 mg/kg bw of TiO_2NP negatively affected normal progeny development at both dose levels (however, in an inverse dose related manner) and resulted in a significantly greater percentage of non-viable foetuses at the highest dose level.
In female rats inhalation of 12 mg/m^3 TiO_2NP during gestation was found to increase placental weights and impair the vascular reactivity in placenta. Also, adverse cardiovascular effects in the offspring has been found after inhalation of approx. 10 mg/m^3 during gestation in rats.

Data gaps

Kinetics:
No data was found in the literature search examining uptake from inhalation of TiO_2NP and the following distribution into gonads, placenta or into organs of the foetus (other than the liver).

Fertility:
Some indicative findings especially on the male reproduction system suggest concern for effects on fertility, however, for example no one-generation guideline study on TiO_2NP is available neither in relation to oral nor inhalation exposure.

Development:
In relation to prenatal developmental toxicity it should be noted that TiO_2NP in various qualities have been covered by OECD TG 414 testing but only using oral exposure in rats. Therefore, data regarding inhalation and other species is lacking in order to make confirmative conclusions on this endpoint. Thus, several studies where pregnant rats were exposed to TiO_2NP by inhalation indicate concern for cardiovascular effects in the offspring.

4.1.3 Data on nano silver

Data availability

Based on screening of the abstracts 34 publications were identified and further examined in full text for silver nano particles (AgNPs).

Of these 34 publications three publications were review articles, while five studies were not considered relevant based on the screening criteria or limited data available (abstracts only).

Of the remaining 26 publications the most relevant and informative data could be extracted from 13 publications which were given the score R++. These covered one oral OECD TG 422 study in rats, four oral studies in male rats examining semen quality and testicular toxicity and three studies and one oral study in rats and mice, respectively, examining developmental toxicity. One study investigated developmental toxicity in mice, following maternal inhalation exposure. Further four studies were found that specifically addressed the kinetics of AgNPs; three were performed in rats and one in the *ex vivo* placenta perfusion model.

Table 4-4: Nano silver data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Elsharkawy et al., 2019 (N:2, K:2, R:++) Hong et al. 2014 (N:3, K:1, R:++) Lafuente et al., 2016 (N:5, K:2, R:++) Lee et al., 2013 (N:4, K:2, R:++) Mathias et al., 2014 (N:3, K:2, R:++) Sleiman et al. 2013 (N:3, K:2, R:+/++)	Yu et al. 2014 (N:3, K:2, R:++) Charehsaz et al., 2016 (N:4, K:2, R:++) Hong et al. 2014 (N:3, K:1, R:++)	Kim et al. 2010 (N:4, K:1, R:++) Kim et al. 2008 (N:4, K:1, R:++) Melnik et al. 2012 (N:4, K:2, R:++) Charehsaz et al., 2016 (N:4, K:2, R:++) Lee et al., 2013 (N:4, K:2, R:++)
Mice, oral	-	Amiri et al. 2011 (N:6, K:2, R:++)	
Mice, inhalation/ resp. tract	-	Campagnolo et al. 2017 (N:6, K:2, R:++)	Campagnolo et al. 2017 (N:6, K:2, R:++)
Other			Vidmar et al. 2018 (N:4, K:2, R:++)

Nano-characterisation

Of the 13 publications considered most relevant for the present project (included in the table above), the N-scores for nano-characterisation of the test item were in the range of 2-6 with an average score of 4. One study had an N-score of 2 only. Solubility was only addressed in one of the assessed articles (kinetics study) where the water solubility of the polyethylene glycol or sodium carboxylate coated AgNP is described as soluble in water (Vidmar et al. 2019). Campagnolo et al. (2017) observes that particles diminish in size from the original size of 20 nm, indicative of dissolution. No REACH registration for silver nanoparticles was found; however, n the REACH registration for silver (Ag, CAS 7440-22-4), the water solubility is given as insoluble (< 0.1 mg/L) (ECHA, January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16155).

Kinetics

A dose-dependent increase in tissue Ag levels was observed in rats after exposure to AgNPs (56 ± 1.46 nm) at 30, 125 and 500 mg/kg bw/day for 28 and 90-days, respectively (Kim et al. (2010) and (2008)). Higher levels of Ag were observed in testis compared to liver, kidney and lungs at 30 and 125 mg/kg bw/day and in brain and blood at all dose levels after 90 days of exposure (Kim et al., 2010). Increased Ag levels in testis, ovaries and brain were observed in rats exposed to 10 and 25 nm AgNPs for 28 days, with very low clearance rate from testes and brain (Lee et al., 2013).

The transfer of AgNPs across the placenta and via milk during lactation was examined in rats exposed intra-gastrically on GD 20 or on lactating day 14-16. The rats were exposed to 1.69-2.2 mg/kg bw AgNPs of 34.9 ± 14.8 nm in diameter labelled with 110mAg radioactive isotope. Transfer of AgNPs across the placenta was found, however the average level of AgNPs accumulated in the foetus of was low (0.085-0.147% of the administered dose). In lactating females, the total accumulation of labeled NPs into the milk exceeded 1.94 ± 0.29% of the administered dose over a 48h period (Melnik et al., 2012). Charehsaz et al. (2016) exposed pregnant rats on GD 7-20 to 20 nm Ag particles at 0, 0.2, 2, 20 mg/kg bw/day, or 20 mg of Ag/kg/day of AgNO3. Ag was found in offspring, indicative of transport across the placenta. Significantly higher Ag levels were found in offspring kidneys at all dose levels. Following inhalation exposure, AgNPs were detected in the placenta, with a total mass concentration of AgNPs of 0.005 ± 0.001 mg/kg. Total silver amounted to 0.082 ± 0.006 mg/kg. A low number of particles was present in foetuses, including the head region. Total silver in foetuses was 0.012 ± 0.003 mg/kg, part of which probably included AgNPs smaller than 13 nm (Campagnolo et al. 2017). The transfer of AgNPs across the placenta was also investigated in the *ex vivo* human placenta model. Perfusions were performed with AgNPs synthesized to mimic commercial NPs. The AgNPs were coated with polyethylene glycol or sodium carboxylate. Ionic Ag was detected in the foetal circulation in low but not negligible amounts after 6 hours of perfusion (Vidmar et al 2018).

Fertility

Elsharkawy et al. (2019) exposed adult male rats to 0, 5.36 or 13.4 mg Ag/kg bw/day twice a week for 6 months as AgNPs (particle size 8.93-33.4 nm). Significant decrease in sperm viability as well as histopathological changes were observed at both exposure levels. Also, significant decrease in testosterone level and a significant increase in LH level were detected, however no effects on morphology was detected. Effects on spermatogenesis after prepubertal exposure to very low dose levels (15 µg/kg bw/day) was also found in Wistar rats exposed to AgNPs (86 nm) on post natal day 23-53/8 (Mathias et al., 2014; Sleiman et al., 2013). Lafuente et al. (2016) exposed male SD rats for a duration of 90 days to 0, 50, 100 and 200 mg/kg bw/day to Polyvinyl pyrrolidone (PVP)-coated AgNPs and found effects of sperm morphology at 50 and 100 mg/kg bw/day, but not at 200 mg/kg bw/day. Lafuente et al. (2016) did not find effects on sperm count and sperm motility and viability.

In two other studies, no effects on testes weight and histopathological parameters were found in rats exposed to 20 and 25 nm AgNPs up to a dose level of 500 mg/kg bw/day 28 day (Lee et al., 2013). Also, Hong et al. (2014) in an OECD 422 study with oral gavage of male and female SD rats to 0, 62.5, 125, 250 mg/kg bw/day to AgNPs (8.8 nm) found no effects on reproductive parameters, following exposure for a total of 42 days. These studies did, however, not investigate sperm parameters or sperm morphology.

Developmental toxicity

In the majority of the studies examining developmental toxicity following oral exposure, no effects on foetal survival, growth and morphology were reported in the studies (Hong et al., 2014; Amiri et al., 2011; Yu et al 2014; Charehsaz et al., 2016).

In the OECD TG 422 study performed by Hong et al. (2014), no effects on development of the offspring or on the on the exposed females were observed. Similarly, no effects were found in a prenatal developmental toxicity study in which rats were exposed from GD6 to 19 to 0, 100, 300, and 1000 mg/kg bw/day of AgNPs with a particle size of 6.45 ± 2.55 nm (Yu et al., 2014).

In mice exposed prenatally to synthesized non-commercial AgNPs (10 nm, 30 nm) and ionic silver ($AgNO_3$) at a dose level of 0.26 mg/kg/day from GD 0 until delivery, cognitive and behavioural abnormalities, mitochondrial dysfunction and upregulation of the genes relevant to the innate immune system in the brain were detected accompanied by high concentration of silver present in the brain of male pups. The same effects were not seen in female offspring (Amiri et al. 2011).

Signs of increased oxidative stress in the brain of offspring were found by Fatemi et al. (2013) in rats prenatally exposed to AgNPs during gestation.

Campagnolo et al. (2017) exposed female mice by inhalation during the first two weeks of gestation and observed increased rate of resorptions and levels of inflammatory mediators in the placenta, and decreased oestradiol levels in maternal plasma.

Overall evaluation

In rats, exposure to AgNPs leads to measurable levels of Ag in testis, ovaries and other organs, and the clearance may be rather low in testis. Following exposure to AgNP during pregnancy, AgNPs may at low levels cross the placenta and lead to AgNP exposure of the foetuses. One study indicated that particles partly dissolved during the period of exposure (2 weeks), which may decrease particle size and increase translocation, either as dissolved Ag or as very small particles. Further evidence of penetration across the placenta is available from the human placenta *ex vivo* model. No studies of kinetics were found in mice. Also, no kinetic data was found in relation to inhalation exposure.

Toxicity in testes and germinal tissue and reduced sperm quality as well as changes in sex hormone levels have been found in male rats subjected to repeated exposure to AgNPs. However, no effects on fertility were observed in a combined repeated dose/reproductive toxicity study (OECD TG 422) with oral exposure of rats to AgNPs up to a dose level of 1000 mg/kg bw/day including male and female rats. Also, in this study no developmental effects were noted. This is in alliance with other developmental studies, were most indicate no effects on foetal survival, growth and morphology. There are some findings that indicate that maternal exposure to AgNPs may affect brain development and function and oxidative stress in the outcome.

Data gaps

Kinetics:
No data on uptake from inhalation of AgNPs and the following distribution into gonads, placenta or foetus was found from the literature search. Further, no data is available regarding reproductive and developmental toxicity from inhalation exposure to AgNP.

Fertility:
One OECD TG 422 study is available, where no effects were found, however there are available data regarding adverse effects on testes and spermatogenesis, which implies that more data is needed to clarify the effects.

Development:
No standard prenatal developmental toxicity testing (OECD TG 414) has been performed with AgNPs. Although one OECD TG 422 study was performed, foetal exposure and distribution to the foetal brain as well as indications of foetal neurotoxicity indicate a need for data that could be provided by an extended one-generation study with the inclusion of neurobehavioural and neuropathological endpoints.

4.1.4 Data on nano zinc oxide

Data availability

Based on screening of the abstracts, 15 publications on zinc oxide nano particles (ZnONPs) were identified and further examined in full text.

Of these 15 publications, one publication was a review article, while four studies were not considered relevant based on the screening criteria.

Of the remaining 10 publications most relevant and informative data could be extracted from six publications (scored with R++ or R+/++). These covered two oral studies with male mice examining semen quality and testicular toxicity and one developmental toxicity, one oral study in mice and one in rats (conducted according to the OECD TG 414 study protocol, this study was covered by two publications).

Table 4-5: Nano zinc oxide data with highest R-score

	Fertility data	Developmental toxicity data
Rats, oral	-	Hong et al. 2014 (a+b); (N:6, K:1, R:++)
Mice, oral	Radhi et al. 2019 (N:4, K:2, R:++) Talebi et al. 2013 (N:2, K:2, R:+/++) Tang et al. 2019 (N:3, K:2, R:+/++)	Teng et al. 2019 (N:6, K:1, R:++)

Nano-characterisation

Of the 10 publications included in the table above the N-scores for nano-characterisation of the test item were in the range of 2-6 with an average score of 3.9. It may be noted that three studies had an N-score of 2 only. Solubility has been determined in one publication where a dissolution of 6.2-8.2% was measured in gastric fluid (Teng et al. 2019). In the REACH - registration water solubility in the range of 1.1 - 47 mg/L is given for various nano-qualities of ZnO (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16139).

Kinetics

Teng et al. (2019) found increased Zn content in placenta and foetuses of mice when dams were orally exposed during GD7-GD17 to ZnONP with a particle size of 13 nm at an exposure level at about 280 mg/kg bw/day. This was not seen in dams exposed to 57 nm and 1900 nm ZnONPs. Zn content was measured after digestion of the organs in nitric acid, so no data on accumulation of particles can be concluded.

Hong et al. (2014 a+b), however, did not find increased Zn levels in foetuses from rats exposed to ZnONP (particle size 20 nm) during GD5-GD19 to 0, 100, 200, and 400 mg ZnONP/kg bw/day.

Fertility

Radhi et al. (2019) exposed male mice to 0,100 and 200 mg ZnONP/kg bw/day (particle size of 50 nm) for 7 or 14 days. In all exposed groups significantly reduced testes, epididymal, seminal vesicle and prostate weights were observed. The percent of abnormal sperm cells was also increased at both dose levels.

Talebi et al. (2013) exposed male mice to ZnONP for 35 days at 0, 5, 50 and 300 mg/kg bw/day (particle size not indicated). Significant impairment of sperm number and motility and increased percentage of abnormal sperm were noted in mice exposed to 50 and 300 mg/kg bw/day. Also, at the two highest dose levels histopathological changes was observed in testicular tissue. Similar results were found by Tang et al. (2019) following exposure of male mice to 50, 150 and 450 mg ZnONPs/kg bw/day for 30 days. In addition, Tang et al. (2019) observed a dose related decrease in serum testosterone levels and a downregulation of the *StAR* gene (involved in testosterone synthesis) in testes.

Developmental toxicity

At exposure of pregnant mice to ZnONP sized 13 and 57 nm at a dose level of 7.2 mg ZnONPs mg/dam (about 280 mg/kg bw/ day), pathological lesions was observed in the placenta (swelling of trophoblast giant cells and accumulation of neutrophils. ZnONPs (13 nm) further caused decreased placental weight (g/foetus) and foetal developmental toxicity recorded as decreased viability, foetal weight and crown-rump and tail length. The organogenesis was more vulnerable than the peri-implantation period. None of the effects were seen after exposure particles with a diameter of 1900 nm (Teng et al. 2019).

In pregnant rats exposed on GD5-GD19 to 0, 100, 200, and 400 mg ZnONP/kg bw/day (20 nm), significant increases in the number of foetuses with visceral variations was observed at 400 mg/kg bw/day. Reduced maternal food consumption and decreased liver weight and increased adrenal gland weight was observed at the two highest dose levels (Hong et al., 2014a+b). This study was conducted according to OECD 414 and in compliance with GLP (Good Laboratory Practice).

Overall evaluation

Toxicity in testes and germinal tissue and reduced sperm quality has been found in two studies where male mice were subject to repeated exposure to ZnONP.

In pregnant mice exposure of ZnONP may result in increased zinc level in level in placenta and foetuses after exposure to 13 nm ZnONP but not for 57 nm particles. No increased Zn levels has been found in foetuses from rats to 20 nm ZnONPs.

A prenatal developmental toxicity in rats found visceral variations in pups only at maternal toxic doses, while in mice developmental toxicity and reduced number of live pups were seen at levels with no obvious maternal toxicity.

Data gaps

Kinetics:
No data on uptake from inhalation of ZnONP and the following distribution into gonads, placenta or foetus was found from the literature search.

Fertility:
Although data indicate concern for testicular toxicity no reproductive toxicity studies are available. Furthermore, there are no studies addressing potential placental toxicity.

Development:
Although an oral OECD TG 414 study has been conducted in rats showing no concern, conclusive data is missing as data from oral exposure in mice indicates some concern for developmental toxicity.

No data is available for the inhalational exposure route.

4.1.5 Data on nano silicon oxide

Data availability

Based on screening of abstracts, 13 publications were identified for further examination in full text. Of the 13 references two of the references were only available as conference abstracts and two other references were reviews. One publication was not considered relevant due to lack of nano-characterisation.

Of the remaining 8 publications most relevant and informative data could be extracted from five publications (scored with R++). These covered two oral studies in rats which were conducted according to OECD TG 416 (two-generation study by Wolterbeek et al. (2015)) and OECD TG 414 (prenatal developmental toxicity study by Hoffmann et al. (2015)). One study on reproductive toxicity in mice (Ren et al., 2016). Further, two studies on kinetics are included, one in rats (Lee et al., 2014) and one in the *ex-vivo* human placenta model (Poulsen et al., 2015).

Table 4-6: Nano silicon oxide data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Wolterbeek et al. 2015 (N: 7, K: 1, R: ++)	Wolterbeek et al. 2015 (N: 7, K: 1, R: ++) Hofmann et al. 2015 (N: 7, K: 1, R: ++)	Lee et al. (2014) (N: 4, K: 2, R: ++)
Mice, resp. tract	Ren et al. 2016 + Zhang et al. 2016 (N: 4, K: 2, R: ++)		
Human placenta			Poulsen et al. 2015 (N: 6, K: 2, R: ++)

Nano-characterisation

Of the 8 publications for detailed examination, the N-scores for nano-characterisation of the test item were in the range of 3-7 (average 5.7). No data is given on any of the publications regarding water solubility of silicon oxide nano particles (SiO_2NP). In the REACH registration of nano silicon dioxide the water solubility of all non surface-treated SAS (Synthetic amorphous silica) products (silica gel, colloidal, precipitated and pyrogenic SAS) is indicated to be in the range of 100 mg/L or higher (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/15556/4/9).

Kinetics

Lee et al. (2014) found that oral administration of SiO_2NP (particle sizes of 15 nm or 89 nm)

was predominantly distributed to the kidneys, liver, lungs, and the spleen in rats exposed to 500 and 1000 mg/kg bw of SiO_2NP. The SiO_2NPs were found to retain their particulate form, although more decomposition was observed in kidneys especially for 15 nm particles. No increase in content of silicium was observed in testes and ovaries, indicating no or very low distribution to these organs. No data on the kinetics of SiO_2NP from inhalation or exposure to the respiratory tract was found in the data search.

Poulsen et al. (2015) used the human placenta *ex vivo* model and found penetration of SiO_2NP to the foetal circulation, of 4.2 ± 4.9% and 4.6 ± 2.4% for 25 and 50 nm NPs after 6 hours of perfusion with a concentration of 100 mg SiO_2NP /L in the maternal circulation compartment.

Fertility

Ren et al. (2016) and Zhang et al. (2016) (the same study reported twice) found increased malformation of sperms and decreased sperm motility and concentration in the epididymis in mice after intratracheal instillation of SiO_2NP (57.7 nm) at a dose level of 2 mg/kg bw/instillation every third day for a period of 45 days. SiO_2NP exposure was associated with induction of oxidative stress in the testis and led to apoptosis and necroptosis of the spermatogenic cells.

Wolterbeek et al. (2015) conducted an OECD TG 416 two-generation study in which rats were orally dosed to SiO_2NP (primary particle size 10-25 nm and a surface area of 230 m^2/g, and mainly as agglomerates in the test solution) at dose levels of 0, 100, 300, or 1000 mg/kg bw/day. No effects were found for any reproductive or developmental toxicity parameters in this study.

Developmental toxicity

Hofmann et al. (2015) conducted an OECD TG 414 prenatal developmental study in which rats were orally dosed to SiO_2NP (same test item/batch as in Wolterbeek et al. (2015)). No effects were found for any developmental parameters in this study.

Overall evaluation

The current data indicates that orally administered SiO_2NP does not reach the testes or the ovaries in rats. An *ex vivo* study with a human placenta indicates that SiO_2NP may have the potential for a low degree of translocation across placenta, at least at the late stage of pregnancy.

In mice exposure to monodispersed SiO_2NP by tracheal instillation, histopathological findings in testes and adverse effects on semen quality indicate that SiO_2NP may interfere with male fertility.

In contrast, oral exposure to even high dose levels of agglomerated SiO_2NPs (at dose levels up to 1000 mg/kg bw/day) did not result in adverse effects on fertility or foetal development when tested in rats according to OECD TGs 416 and 414.

Data gaps

Kinetics:
No data on uptake from inhalation of SiO_2NP and the following distribution into gonads, placenta or foetus was found in the literature search

Fertility:
There is no data available in relation to inhalation exposure. Intratracheal administration in mice indicates concern on toxicity on testes and spermatogenesis.

Development:
There is no data available in relation to inhalation exposure.

4.1.6 Data on carbon nanotubes and graphene

Data availability

Based on screening of the abstracts, 16 publications were identified and further examined in full text. One publication considered graphene, the remaining MWCNT. The majority of the studies are investigations relevant for airway exposure to carbon nanotubes, and there are three studies on oral exposure.

Of the 16 publications three publications are review articles, while five studies were not considered relevant based on the screening criteria or limited information available.

The data was therefore extracted from the remaining five studies. These covered four studies in mice, three studies with intratracheal administration (Skovmand et al., 2018; Johansson et al., 2017; Hougaard et al., 2013) and one oral study (Vasyukova et al., 2015). The final study administered MWCNTs by the oral route in rats (Lim et al., 2011a+b). The main focus of the studies is reproductive toxicity in males and females and developmental toxicity. Besides gestational parameters, male testes and sperm parameters and behaviour were addressed in one study. No guideline studies were found.

Table 4-7: Carbon nanotubes and graphene data with highest R-score

	Fertility data	Developmental toxicity data
Mice, oral	Vasyukova et al., 2015 (N:4, K:2, R:++)	Vasyukova et al., 2015 (N:4, K:2, R:++)
Mice, respiratory tract	Skovmand et al., 2018 (N:6, K:2, R:++) Johansson et al., 2017 (N:5, K:2, R:++) Hougaard et al., 2013 (N:5, K:2, R:++)	Johansson et al., 2017 (N:5, K:2, R:++) Hougaard et al., 2013 (N:5, K:2, R:++)
Rat, oral		Lim et al., 2011a+b (N:4, K:2, R:++)

Nano-characterisation

Of the five publications included in the table above, the N-scores for nano-characterisation of the test item were in the range of 4-6 with an average score of 4.8. Solubility was not addressed in any of the studies. Some of the studies refer to more in-depth characterization data in other publications, but these were not considered in the scoring. Two REACH registrations for MWCNT are available (Graphite and MWCNT). In the REACH registrations, the water solubility is given as < 2 mg/L at 20 °C and a pH of 7.5 - 9.2 for MWCNT and 0 mg/L (insoluble) for graphite. (ECHA January 2020, MWCNT https://echa.europa.eu/registration-dossier/-/registered-dossier/13454#SubNav4; ECHA January 2020, Graphite: https://echa.europa.eu/registration-dossier/-/registered-dossier/16080).

Kinetics

There was no data on particle kinetics.

Fertility

Males: Skovmand et al., (2018) exposed mature male NMRI mice to four different types of carbonaceous nanomaterials including graphene oxide (18 µg/mouse/i.t. for 7 weeks). The study is also described in section 4.1.7 on carbon black. The mice were exposed weekly for 7 weeks, and testes were examined for effects in sperm counts and motility, as well as for daily sperm production and sperm integrity. Despite the sustained pulmonary inflammatory response, semen parameters were unaffected in the male NMRI mice. Vasyukova et al., 2015 performed an oral study in male C57B/6× DBA2 mice, exposed by oral gavage to 0.3, 3, and 30 mg MWCNT/kg/day on 30 consecutive days. No effects on the testes or any of the sperm parameters investigated were observed. No changes in hormone levels (FSH and LH) were seen. The treated males were bred with untreated females C57B/6 × DBA2 mice. A dose-dependent decrease of fertilizing capacity of 15-40% was registered at all dose levels.

Females: In the study of lung exposure to MWCNT, Hougaard et al. (2013) exposed mature, female C57BL/6J mice to 67 µg MWCNT by intratracheal instillation one day prior to mating. A short delay in the delivery of the first litter (5 days) was observed for exposed females. In a follow-up study, naïve female C57BL/6J mice were intratracheally exposed once to 67 µg MWCNT. Compared to normal estrous cycling determined prior to exposure, exposure to MWCNT significantly prolonged the estrous cycle (by approximately 2 days, i.e., from 5.3 days before exposure to 7.2 days for exposed cycles). However, the estrous cycle immediately after the exposed cycle was significantly shortened ($p < 0.001$). Another group of females was intratracheally exposed to 2, 18 or 67 µg MWCNT on the day before cohabitation with unexposed males. No consistent effects were seen on time to delivery of a litter (Johansson et al. (2017).

Developmental toxicity

In Lim et al (2011a+b), Sprague-Dawley rats were exposed to 40, 200 or 1000 mg/kg MWCNT/kg bw by oral gavage from GD6 to GD9. No effects on foetal growth, viability, or morphological development were observed. A decrease in maternal thymus weight was found at 1000 mg. The no-observed adverse effect level of MWCNTs was therefore considered to be 200 mg/kg/day for exposed dams, and 1000 mg/kg/day or more for embryonic development (Lim et al., 2011a+b).

In the study of intratracheal lung exposure to MWCNT, Hougaard et al. (2013) exposed mature, female C57BL/6J mice to 67 µg MWCNT by intratracheal instillation one day prior to mating. Litter parameters, behaviour and daily sperm production were similar in control and exposed offspring. No consistent effects were seen on litter parameters, such as litter size, sex ratio, implantations and implantation loss following exposure of female mice by intratracheal administration to 2, 18 or 67 µg MWCNT on the day before start of cohabitation with unexposed males (Johansson et al. (2017). Fujitani et al. (2012) exposed pregnant ICR mice on day 9 of the gestation to 3, 4 and 5 mg/kg body weight. Foetuses were examined for external and skeletal anomalies on day 18 of gestation. The incidences of foetal malformations in the groups given 4 or 5 mg/kg body weight were statistically higher compared to controls. No or very low-level malformations were seen after instillation of 3 mg/kg bw/day. (However, this study is considered of lower relevance as very poor characterisation of the MWCNT was given)

Overall evaluation

Weekly airway exposure of adult male mice to four different types of carbonaceous nanomaterials including graphene oxide did not alter semen parameters, but 30 days of oral exposure to MWCNTs decreased fertilizing capacity of males. Female exposure to MWCNT on the day prior to cohabitation with an unexposed male increased time-to-delivery of a first litter in one study, but not in another study. Intratracheal exposure to MWCNT did interfere with estrous cycling, increasing the length of the exposed cycle but decreasing the length of the

following cycle. Overall, these findings indicate that exposure to MWCNTs may interfere with adult fertility, but no firm conclusions can be drawn on the basis of the present studies.

No findings regarding effects on development were observed in two studies using instillation of MWCNT to female mice on the day prior to cohabitation with naïve males (~2.5 mg/kg). However, one study (with very poor characterization of the MWCNT) found indications of developmental effects in mice after one intratracheal instillation during gestation (at and above 4 mg/kg).

Data gaps

Kinetics:
No data on the distribution of carbon nanotubes into gonads, placenta or foetus was found from the literature search.

Fertility:
Only sparse and very scattered published data with the testing of only mice is available concerning fertility effects of carbon nanotubes and graphene exposure. Standard OECD testing determining fertility of carbon nanotubes and graphene using relevant exposure routes (oral or inhalation exposure) is therefore needed.

Development:
Limited data of the developmental effects of carbon nanotubes and graphene is available. Thus, standard OECD testing determining prenatal developmental toxicity of carbon nanotubes and graphene using relevant exposure routes (oral or inhalation exposure).

4.1.7 Data on carbon black

Data availability

Based on screening of the abstracts, 21 publications were identified and further examined in full text. All studies are investigations relevant for inhalational exposure to carbon black. The majority of the studies use intranasal or intratracheal administration of carbon black nanoparticles (Printex90).

Of these 21 publications two publications are review articles, while three studies were not considered relevant based on the screening criteria or only abstract available.

Of the remaining publications most relevant and informative data could be extracted from 10 studies. Some studies are covered by more than one reference (see table below). These covered three inhalation studies, three intranasal instillation studies and four studies with intratracheal administration. All studies are performed in mice. The main focus of the studies is developmental toxicity, with focus on offspring brain development and male reproductive function. The remaining studies investigated the effects on male reproductive function following exposure to carbon black in adulthood. No guideline studies were found.

Table 4-8: Carbon black data with highest R-score

	Fertility data	Developmental toxicity data
Mice, respiratory tract	Skovmand et al., 2018 (N:5, K:2, R:++) Yoshida et al., 2009 (N:4, K:2, R:++)	Kyjovska et al., 2013 (N:4, K:2, R:++) Onoda et al., 2017b (N:5, K:2, R:++) Onoda et al., 2014 (N:6, K:2, R:++) Umezawa et al., 2018 (N:5, K:2, R:++) Skovmand et al., 2019 (N:5, K:2, R:++) Yoshida et al. (2010) (N:3, K2, R++) Jackson et a. (2011, 2012a+b) (N:6, K:2, R:++)

There is one full REACH registration (1 000 000 - 10 000 000 tonnes per annum) of carbon black available. Carbon black is not classified in the REACH dossier. The toxicological data referred to in the dossier for reproduction and developmental effects is the publication by Jackson et al. (2012a) (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16056).

Nano-characterisation

Of the 10 studies included in the table above, the N-scores for nano-characterisation of the test item were in the range of 3-6 with an average score of 4.4. Only one study had an N-score of only 3. Solubility in water was described in two publications as low (Kyjovska et al. 2013) and insoluble (Onoda et al., 2014). The solubility in the REACH dossier is given as below 1 mg/L, which was the detection limit (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16056). Several available characteristics on carbon black is, however, summed up in Jackson et al. (2012a+b; 2011).

Kinetics

No data on kinetics.

Fertility

Skovmand et al., (2018) exposed mature male NMRI mice by intratracheal instillation to four different types of carbonaceous nanomaterials, including two types of carbon black particles (Printex90 and Flammrus 101) as well as graphene oxide and diesel exhaust particles. The mice were exposed once a week for seven weeks, and testes were examined for effects in sperm concentration and motility as well as daily sperm production and sperm integrity. Despite the sustained pulmonary inflammatory response, an eight-week exposure to graphene oxide, Flammruss 101, Printex 90 and the diesel particle SRM1650b in the present study did not appear to affect semen parameters, daily sperm production or testosterone concentration in male NMRI mice.

Yoshida et al., (2009) found a decrease in the daily sperm production and testosterone levels of male ICR mice after exposure to carbon black, 0.1 mg/mouse by intratracheal administration once a week for 10 weeks. Three different sizes were tested (14, 56, 95 nm CB) and further one group received 14 nm CB, where the particle number concentration is the same as that of 56-nm. Furthermore, vacuolation of the seminiferous tubules was observed in 14-nm CB, 56-nm CB, and 95-nm CB groups. The effects of nanoparticles on the male reproductive system seemed to depend on particle mass rather than on particle number (Yoshida et al., 2009). It should be noted that the vehicle used contains 0.05% tween 80, which has lipophilic and hydrophilic properties and may enhance permeability through cellular membranes because of their effects on tight junctions.

Developmental toxicity

Developmental toxicity was studied for effects on three organ systems in the offspring, i.e. the male reproductive system, the central nervous system and the immune system.

Three studies investigated the effects of maternal exposure to carbon black on male reproductive function in the offspring:

In Kyjovska et al. (2013), the fertility of the in utero exposed offspring were investigated in C57BL/6J mice mated with CBA/J mice. The C57BL/6J mice were exposed *in utero* on gestation days 7, 10, 15 and 18 via maternal exposure by intratracheal instillation of 67 µg CB (Printex90)/day. The time it took breeding couples of a prenatally CB exposed F1 C57BL/6J male and a naïve CBA/J female to deliver a first F2 litter was slightly extended compared to F1 control C57BL/6J males cohabiting with naïve CBA/J females, although not statistically significant and no correlation between sperm content/daily sperm production and time-to-first F2 litter was found.
The same group performed a study in NMRI mice, exposed to Printex90 particles by whole body inhalation on GD 4 to 18. The dams were exposed to 4.6 and 37 mg/m^3 for 45 min per day. No changes in gestation length, number and loss of implantations, offspring weights, litter size and sex ratio for exposed females and offspring compared to control females and offspring were seen. Also, no significant changes were observed in body and reproductive organ weights, epididymal sperm parameters, daily sperm production, plasma testosterone or fertility of the male offspring examined though four generations (F1-F4) (Skovmand et al. 2019).

Signs of toxicity in testes and reduced DSP was found by Yoshida et al., (2010), after prenatal exposure to 14-nm carbon nanoparticles was administered intratracheally on days 7 and 14 of gestation. Contrary to this, Skovmand et al. (2019) did not find any effect in the investigated sperm parameters, which were sperm motility, daily sperm production and sperm chromatin structure. The study by Skovmand et al (2019) was an inhalation study, which is considered more relevant for human extrapolation.

Several studies have found effects on the level of glial fibrillary acidic protein (GFAP) expression in the cerebral cortex after CB exposure.

Umezawa et al., (2018) found dose dependent increase in expression of glial fibrillary acidic protein (GFAP) in astrocytes around blood vessels in the cerebral cortex and hippocampus, indicative of reactive astrogliosis, and enlarged lysosomal granules were observed in brain perivascular macrophages in 5 week old offspring after prenatal exposure in NMRI mice. The dams were exposed by inhalation to 0, 4.6 or 37 mg/m^3 carbon black Printex 90 on GD 4 to 18 (45 min/day). The authors also observed altered offspring behavior in the open field test, and decreased number of parvalbumin-positive interneurons were decreased in the motor and prefrontal cortices at weaning (this was only investigated at the highest dose level (Umezawa et al., 2018). Very similar findings regarding expression of GPAP were observed in Onoda et al. (2017b), when pregnant ICR mice were exposed intranasally to carbon black Printex 90 at 2.9, 15, or 73 µg/kg on GD days 5 and 9. Brains were again collected from male offspring at 6 weeks of age (one pup per litter for each outcome was used). An increase in GFAP expression in the cerebral cortex was detected together with increased aquaporin-4 expression in the brain parenchyma region around blood vessels and altered expression levels in the cerebral cortex of mRNAs associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production. These changes are similar to what is observed with aging (Onoda et al 2017b). This was also found in a similar study performed previously by Onoda et al. (2014).

Altered open field test behaviour after CB exposure was also found in Jackson et al. (2011) after maternal intratracheal instillation on GD 7, 10, 15 and 18 to a cumulative dose of Printex 90/animal. In this study maternal inhalation exposure to Printex90 of 42 µg/m^3 on GD 8-18 also induced liver DNA damage in the mothers and the in utero exposed offspring (Jackson et al 2012a).

Two studies found effects on the offspring immune system after maternal exposure to a total of 190 µg/kg bw Printex90 by intranasal instillation on GD 5 and 9 (El-Sayed et al., 2015; Shimizu et al., 2014).

Overall evaluation

Fertility following exposure of adult animals

Intratracheal instillation of carbon black (0.1mg/mice) weekly for 10 weeks induced testicular toxicity, with decreased daily sperm production and testosterone levels of male ICR mice, but not in NMRI mice exposed to the same dose for 7 weeks. No other effects on fertility was observed. Apart from mouse strain, there are several other differences between the two studies, the most important is probably the vehicle composition, as the ICR mouse study consisted of saline with 0.05% tween 80 compared to nanopure water in the NRMI study. Hence tween possesses both lipophilic and hydrophilic properties and is therefore able to partition between lipid and protein structures. Tween is also known to enhance permeability by altering tight junctions and cellular membranes (Skovmand et al., (2018). It is, however, not known, whether this is the underlying reason for the observed difference between the studies.

Gestational exposure by the maternal airways did not seem to affect gestational and litter parameters. Findings in two studies indicated that maternal exposure could interfere with offspring development of the immune system. Equivocal results regarding the effects of maternal CB exposure and effects on sperm parameters in male offspring were observed.

Exposure of pregnant mice to carbon black via the airways results in changes in protein expression in the brain of the offspring (Onoda et al., 2014 and 2017b; Umezawa 2018). These changes were in form of increased expression of the protein GFAP. GFAP increases naturally in the brain with age, but the changes induced in one of the studies were similar to levels normally observed in much older animals (Onoda et al. 2017b). Summing up, this kind of change have been observed in several studies, in two different mouse strains as well as in intranasal instillation exposure. Related changes have been observed in two additional studies, not described in detail here (Onoda et al 2017a+c). Other CNS changes included altered mRNA expression levels in the cerebral cortex associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production. Finally, changes in parvalbumin positive interneurons bear high resemblance to observations in established animal models of maternal inflammation. Hence, the heavily reduced expression of PV+ in the cortex are furthermore indicative of a schizophrenia-like phenotype (Umezawa et al., 2018). However, more data is needed to elucidate the effects of the altered expression levels.

Data gaps

Kinetics:
No data on the distribution of carbon black into gonads, placenta or foetus was found from the literature search.

Fertility:
Only data on inhalational exposure in mice is available concerning fertility effects. No standard OECD testing of carbon black using relevant exposure routes (oral in particular) is available.

Development:
Limited data of the developmental effects of carbon black is available. Thus, standard OECD testing determining prenatal developmental toxicity of carbon black using relevant exposure routes (oral or inhalation exposure) are missing.
The histopathological changes observed in offspring of carbon black exposed mothers raise concern about the long-term functional consequences hereof, e.g. due to increased neurodegeneration.

4.1.8 Data on other nanomaterials

The 32 references found for the other 13 nanomaterials, of which 19 references are further evaluated in appendix B8, are not discussed further in this section. Instead, the main findings from the studies in appendix B8 are included in section 4.2 if they are considered to contribute to answering the key questions on kinetics, fertility and development asked in section 1.1.

A short overview of the type of data is given below showing data availability, type of data, animal species, exposure route and N-, K-, R- scoring for the references:

Table 4-9: Data availability on other nanomaterials

	Kinetics	Fertility	Development
Aluminium	Zhang et al. 2018 (N:5; K:2; R:++) Mice nasal drip exposure	-	Zhang et al. 2018 (N:5; K:2; R:++) Mice nasal drip exposure
Cadmium oxide	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.
Cerium oxide	Geraets et al. 2012 (N:8; K:1-2; R:++) Rats inh.	Qin et al. 2019 (N:5;K:2; R:++) Mice oral	-
Cobalt (tricobalt tetraoxide)	-	Hussien & Mohamed 2018 (N:3; K:2/3; R:+) Mice oral	-
Copper	Adamcakova-Dodd et al. 2015 (N:5; K:2; R:++) Mice inh.	Kalirawana et al. 2018 (N:3; K:2; R:++) Rats oral	Adamcakova-Dodd et al. 2015 (N:5; K:2; R:++) Mice inh.
Gold	Myllynen et al. 2008 (N:4; K:2; R:++) ex vivo human placenta Gupta et al. 2018 (N:4; K:2; R:++) Rat oral	Gupta et al. 2018 (N:4; K:2; R:++) Rat oral	-
Iron	Park et al. 2017 (N:4; K:2; R:++) Mice intratracheal instillation	-	Park et al. 2017 (N:4; K:2; R:++) Mice intratracheal instillation
Mangan oxide	-	Negahdary et al. 2015 (N:3; K:2/3; R:0/+) Rats oral	-
Nickel	-	Kong et al. 2014 + 2016 + 2019 (N:7; K:1; R:++) Rats oral (OECD TG 415) Hu et al. 2019 (N:7; K:2; R:+) Mice oral	-

Platinum	-	-	Park et al. 2010 (N:2-3; K:2-3; R:+) Mice oral
Polystyrene	Wick et al. 2010 (N:7; K:2; R:+) ex vivo human placenta Grafmüller et al. 2015 (N:6; K:2; R:+) ex vivo human placenta	-	-
Selenium	-	Liu et al. 2017 (N:2; K:2; R:+) Rats oral	-

4.2 Overall evaluation of the collected data

Below, the key questions asked in section 1.1 are indicated (***bold italic letters***) and afterwards answers based on the findings in this project is given.

4.2.1 Data availability

What studies relevant to reproductive/developmental toxicity are available on nanomaterials?

Based on a structured literature search in selected relevant databases from STN 2152 publications were identified using search terms relevant for identifying publications studying manufactured nanomaterials relative to reproductive and developmental toxicity. The retrieved publications were screened for relevance by reading of the titles of the publications, with emphasis on identification of *in vivo* studies. This reduced the number to 437 publications which were further evaluated based on abstracts and additional exclusion and inclusion criteria (e.g. related to relevance of exposure routes for the human situation) and expert assessment. As a result, 177 relevant references were identified for full text examination. Data from studies described in 111 of these references was further examined and described in tabulated form in seven nanomaterial specific appendices. The remaining 63 references were excluded for various reasons (e.g. not meeting inclusion criteria after all, reviews, or poor quality).
Nanomaterials tested
Half of all the identified *in vivo* data is related to titanium dioxide (29%) and silver (21%), while 35% of the data covers the zinc oxide (10%), silicon dioxide (5%), carbon nanotubes and graphene (6%), and carbon black (14%).

In vitro, ex vivo and in vivo (animal studies, human case studies or epidemiological data)?

The process aimed to identify the most relevant data for assessment of developmental and reproductive toxicity of nanomaterials relative to the human situation, therefore the focus was on *in vivo* studies. *In vitro* and also to some extent *ex vivo* studies are therefore only included to a very limited degree in this report. No human case studies or epidemiological data in relation to reproductive and developmental toxicity of manufactured nanomaterials was found.

Table 4-2 gives an overview of the identified studies, sorted according to the nanomaterial studied, type of outcome (kinetics, fertility, developmental toxicity), animal species, and exposure route. From this it is seen that all *in vivo* animal testing was conducted in rats and mice.

Exposure route
Sixty four of the indicated *in vivo* data pertain to oral exposure (rats 44% and mice 20%) while 36% of the studies used airway exposure, with 18% pertaining to inhalation exposure (rats 9% and mice 9%) and the remaining 18% used alternative respiratory tract administration, such as intratracheal instillation (rats 0% and mice 18%).

Testing of industrial chemicals for developmental and reproductive toxicity most often apply the oral route of exposure to maximise systemic exposure. Inhalation is, however, the primary route of exposure in the occupational setting and also bears some relevance for exposure to consumer products. Inhalation can be considered a more direct exposure route for the dispersed nanoparticles, as airborne nanoparticles may deposit directly on the inner surface of the airways. In contrast, oral exposure by gavage and in food and airway exposure by instillation implicate that nanoparticles may have potentially interacted with and undergone changes in the dosing medium (food or vehicle). Further, uptake of NPs may differ between the oral and the airway routes of exposure. The route of exposure may influence the translocation of particles from one biological compartment to another, and therefore ultimately affect their toxicity. This is because the port of entry influences the composition of the corona, i.e. the proteins adhering to the surface of the particles, and the corona may have dramatic influence on the ability of particles to cross biological barriers (Pietroiusti et al. 2013).Finally, NPs deposited in the alveoles may be removed relatively slowly and constitute a continuous source of exposure, whereas passage time is much faster in the gastro-intestinal tract.

Also, data indicate that nanoparticles deposited in the lung, even at very low exposure levels, can cause local reactions in lung tissue inducing release of inflammatory mediators that might subsequently have consequences for the functioning of other organ systems, thereby introducing secondary effects. Overall, that data pertaining to the airway route of exposure is of outmost importance when assessing the toxicity of nanomaterials – also in relation to reproductive and developmental toxicity.

What are the test guidelines followed for the studies (if any)? Are the results available in a structured way, e.g. following OECD harmonised templates?

Only few studies followed the OECD harmonized test guidelines. For the following nanomaterials testing according to OECD test guidelines have been performed:

TiO_2NP: OECD TG 414, oral exposure in rats
ZnONP: OECD TG 414 oral exposure in rats
SiO_2NP: OECD TG 414 and OECD TG 416, both oral exposure in rats
AgNP: OECD TG 422 oral exposure in rats
NiNP: OECD TG 415 oral exposure in rats

It should be noted that no data is available for OECD TG testing by inhalation. However, for FeNP a modified OECD TG 421 study was carried out in mice, albeit using intratracheal instillation as the means of exposure.

For the studies that did not adhere to OECD guideline testing, many can be considered hypothesis generating rather conclusive studies, as often only a single to few target organs or bioindicators of effects (e.g. mechanistic or functional parameters, for example gene expression in gonadal tissue or vascular reactivity in placenta) are studied rather than commonly accepted end-points for fertility or development. These may indeed be relevant, but there is less experience in their interpretation relative to reproductive and developmental toxicity.

In relation to testing for adverse effects on fertility following exposure in adulthood, a great amount of non-guideline studies has been conducted. Interestingly, in several such studies (e.g. of TiO_2NP, ZnONP SiO_2NP, AgNP, NiNP, CuNP, AgNP, Mn_2O_3NP, and SeNP) testicular

tissue and sperm parameters were observed to be detrimentally affected by NP exposure. However, in OECD TG 422 studies of AgNP and OECD TG 416 testing of SiO_2NP, no adverse changes were observed for fertility parameters or histology of the reproductive organs. Here it should however be noted that several of the studies reporting adverse effects of e.g. AgNP do so for sperm parameters (motility, normal sperm, viability), but these were not investigated in the OECD TG 422 study. When NiNP was tested according to OECD TG 415, no effects on mating success and pregnancy rate were observed, although NiNP in this test also (as in non-guideline studies) was found to induce toxicity to the testes and lower sperm quality.

For the other nanomaterials indicating toxicity to testes and sperm quality in non-guideline studies, follow-up with testing of mating and reproductive success is lacking.

In relation to developmental toxicity, several studies exposed pregnant rats or mice for varying periods during gestation (either by single or repeated exposure) and examined the offspring for non-guideline endpoints (e.g. effects on the cardio-vascular system, specific cell populations in the brain, gene expression in selected organs etc.). Several of these studies applied only a single or two dose levels and/or used a low number of animals per dose level. They do therefore not systematically cover all types of organs and toxicity parameters as prescribed in the OECD TGs. Additional follow-up testing may often be needed for firm conclusions to be drawn on the presence or absence of developmental effects.

Are there comparable studies in which nanomaterials and bulk sized materials and/or soluble forms of these materials with the same chemical composition are compared, or studies in which different nanoforms of the same nanomaterials are compared? What are the differences in their toxicity profiles? Can any conclusions be drawn?

Testing substances in bulk form alongside of nanoforms are rather seldomly performed in testing of nanomaterials for reproductive and developmental toxicity, and only few studies have been found.

For titanium dioxide OECD TG 414 testing has been conducted with three different pigment-grade qualities (i.e. non-nanoforms) and three nanoforms in parallel at the same dose levels (Warheit et al. 2015). None of the particle types interfered with gestation, foetal or litter parameters, and thereforeno differences in response was noted between the nano- and the non-nanoform.

For zinc oxide, a non-nanoform (particle size of 1900 nm) was tested in parallel with two qualities of nanoparticles (13 or 57 nm) in an oral study. Pregnant mice were exposed by oral gavage (280 mg/kg bw/day) during two different periods of gestation (Teng et al. 2019). The two nanoforms caused pathological lesions in placenta, and the 13 nm particle had negative effects on foetal growth. For this particle size, increased zinc levels were observed in the placenta and in foetuses. No adverse effects nor transplacental transfer was seen for the bulk form.

For silver particles a non-nanoform (particle size 323 nm) has been tested in parallel with three nanoforms (particle sizes of 22, 41 and 71 nm, respectively) using repeated oral administration at 1 mg Ag/kg bw/day for 14 days in male and female mice (Park et al. 2010). No Ag was detected in any tissue after administration of the two larger Ag particles, but Ag was detected in testes following exposure to the 22 and 42 nm AgNPs. When Charehsaz et al. (2016) exposed pregnant rats to 20 nm Ag particles or AgNO3 at 20 mg Ag/kg/day, offspring tissue levels were generally similar or lower if their dams had been exposed to AgNO3 rather than the AgNPs. Only for plasma did $AgNO_3$ offspring present with statistically significantly higher concentration than in the corresponding AgNP group. There might be slight differences in offspring deposition patterns between NP and ionic Ag.

For nickel particles a non-nanoform (particle size of 3.3 µm) were tested together with a nano-form (particle size in the range of 30-100 nm). Particles were administered to rats of both sexes by oral gavage at 45 mg/kg bw/day from 10 weeks before mating and throughout gestation and lactation. The nanoparticles apparently affected the offspring survival rate at birth to a greater extent than the microform. Nanosized particles also seemed to affect hormone levels in male and female rats more than the microsized particles at the same level of exposure (Kong et al. 2014). The negative effect on sperm motility was comparable among the different particle sizes while histopathological examination of testes showed more pronounced adverse effects of the nanoparticles compared to the micro-sized particles in the seminiferous tubules (Hu et al. 2019).

For zinc oxide, silver and nickel a higher degree of either tissue distribution or toxicity has been found for particles in the nano-range compared to particles. As these three metals are water soluble to some degree, an explanation for increased distribution and toxicity may be that the increased surface area of the nanoparticles will promote dissolution of the smaller particles to a larger degree than the larger particles and thereby increase the bioavailability of the solubilized metal ions.

TiO_2 is the only non-soluble material that has been tested for both nano- and micro-size. As no adverse effects was observed for either size-range, the study offers no information regarding differences in toxicity profiles between particles of different sizes. Based on the present selection of studies, no firm conclusions can be drawn with respect to this issue.

4.2.2 Kinetics

Is there evidence suggesting that nanomaterials can cross relevant biological barriers (placental barrier; blood-testis barrier, blood-brain barrier (pre- and postnatally)?

The main data on transport and distribution of nanoparticles available from the present studies applying oral and airway exposure, relates to TiO_2NPs, AgNPs, SiO_2NPs and ZnONPs. Focus in the present project was on distribution to the gonads as well as across the placental barrier. Studies focusing on kinetics in general were therefore not included. It should be kept in mind that for particles to pass the blood-testis and the placental barrier they would have to cross two barriers. First the air-blood lung or the intestinal barrier and then the blood-testes or the placental barrier.

Placental barrier:
An OECD TG 414 test was performed with TiO_2NPs, where female rats were exposed orally by gavage with 0, 100, 300 and 1000 mg/kg bw/day 21 nm TiO_2NPs. The exposure to 1000 mg/kg bw/day increased levels of elemental titanium in the placenta compared to control animals (0.6 mg/kg vs 0.2 mg/kg) (Lee et al. 2019). Hougaard et al. (2010) did not observe distribution of elemental Ti above the detection limit in liver from pups nor in milk from lactating mice after 1hr of daily exposure to TiO_2NP at 40 mg/m^3 during GD8-18 in mice.

Transfer across the placenta was seen in rats exposed intra-gastrically to labelled AgNP at 1.69-2.2 mg/kg bw (35 nm) on GD 20. However, the average level of AgNPs accumulated in the foetus of was very low (0.085-0.147% of the administered dose) (Melnik et al., 2012). Charehsaz et al. (2016) dosed pregnant rats with AgNP or $AgNO_3$ by gavage from gestation day 7 to 20. Offspring tissue contents of Ag were numerically higher in all treated compared to control offspring, but statistically significantly so only in kidneys and at all dose levels. Following treatment with $AgNO_3$, increased concentrations were found in offspring lungs and plasma, indicating some difference in deposition patterns Ag in ionic and particulate form. Following inhalation exposure during the first two weeks of gestation, AgNPs were detected in the placenta and in foetuses (including the head region). Overall levels of Ag were significantly increased in both the placenta and foetuses. In the foetus, detected Ag was almost entirely in ionic form or as NP of less than 13 nm in diameter, whereas in the placenta approximately 6%

of the total Ag were present in particulate form. In comparison, 21% of the total Ag in the maternal lungs were still in particle form. The author concludes, that Ag translocating the lung or the placenta does so as ions or small readily dissolving particles (Campagnolo et al. 2017).

Similarly, Teng et al. (2019) found increased Zn content in the placenta and foetuses of mice, when the dams were orally exposed during to 13 nm ZnONP but not to 57 nm and 1900 nm ZnONPs. Zn content was measured after digestion of the organs in nitric acid, so no data on accumulation of Zn in particle form is provided. Hong et al. (2014 a+b) did not find increased Zn levels in foetuses from rats exposed by gavage to 20 nm ZnONP during GD5-GD19 at levels of up to 400 mg ZnONP/kg bw/day.

The transfer of nanoparticles across the placenta was also investigated in the *ex vivo* human placenta model. When perfusions were performed with AgNPs, ionic Ag was detected in the foetal circulation in low but not negligible amounts after 6 hours of perfusion (Vidmar et al. 2018). Poulsen et al. (2015) also found transfer of SiO2NP particles to the foetal circulation in this model, of 4.2 ± 4.9% and 4.6 ± 2.4% for 25 and 50 nm NPs after 6 hours of perfusion with a concentration of 100 mg SiO_2NP /L in the maternal circulation. Polystyrene beads of different particle sizes (50, 80, 240, 500 nm) were also able to cross from the maternal to the foetal circulation, however, the nanoparticles to a significantly higher extent than the micro-particles (Wick et al. 2010). Grafmüller et al. (2015) found that functionalised (-COOH) polystyrene beds crossed the placenta to a significantly lower amount that non-functionalised poly styrene beads. When transfer of gold nanoparticles (AuNPs) were investigated using particle sizes of 10, 15 or 30 nm, no transfer of across the placenta was found. AuNPs were however found located in placental tissue (Myllynen et al., 2008).

Based on available data, there are indications that nanoparticles are able to cross the placenta and reach the foetal compartment following maternal exposure by the oral route. A single study using inhalation exposure also shows this for Ag. However, the majority of the studies assesses the tissue concentrations of the NP material rather than detection of the particles. Two *in vivo* studies on placental transfer of Ag do however asses the actual particle transfer. In Melnik et al. (2012) >0.2% of the administered dose (labelled AgNPs) was detected in the foetus, and in Campagnolo et al. (2017) detecting AgNPs in the placenta and foetuses, the latter in very low amounts. Transport across the placenta of polystyrene beads was also documented in the *ex vivo* placenta perfusion model (Wick et al., 2010). Several of the studies may however not have been able to detect translocation at low tissue concentrations. Here it should be noted, that even if only small proportions of the administered dose translocate, it may still represent a considerable number of particles. More data is needed, with focus on detection of transfer of the particles across the human placenta is needed, especially following exposure routes of relevance for the human situation.

Gonads:
Transfer of nanoparticles across the blood-testis barrier was investigated in several studies with AgNPs. A dose-dependent increase in tissue Ag levels was observed in rats after oral exposure to AgNPs of 56 nm at 30, 125 and 500 mg/kg bw/day for 28 and 90-days, respectively (Kim et al. (2010 and 2008), and higher levels of Ag were observed in the testes compared to other tissues after 90 days of exposure (Kim et al., 2010). Increased Ag levels in testes, ovaries and brain were also observed in rats exposed to 100 or 500 mg/kg/day of 10 and 25 nm AgNPs for 28 days (Lee et al., 2013). The clearance rate from testes, ovaries and brain was found to be very low. In testes, no clearance was seen even after 4 months recovery in the high dose group of both sizes of AgNPs and at the low dose of 25 nm particles (Lee et al., 2013).

Distribution to the testes was also investigated for SiO_2NP, where distribution was predominantly to the kidneys, liver, lungs, and spleen in rats orally exposed to 500 and 1000 mg/kg bw of SiO_2NP of 20 and 100 nm. No increase in content of silicium was observed in testes and ovaries, indicating no or very low distribution to these organs (Lee et al. 2014). Similarly, Geraets et al. (2014) tested five different commercial qualities of TiO_2NP covering

both the anatase and the rutile forms (particle sizes in the range of 6-20 nm) and found that after i.v. exposure of rats only very low levels of elemental titanium could be detected in testes and ovaries nor the other organs assessed. No distribution could be detected after oral exposure to 8.4 - 59.9 mg/ kg bw (Geraets et al., 2014).

In one study on gold nanoparticles, Gupta et al. (2018), assessed the distribution of gold nanoparticles to testes in male rats exposed to 20 µg 5-20 nm AuNPs/kg/day for 90 day by TEM. Electron micrographs showed aggregates of gold nanoparticles in the interstitial spaces of the testis, including the seminiferous tubules. Large aggregates were also detected near, crossing the outer membrane and inside Leydig cells. The Leydig cells however appeared structurally intact. AuNPs were also detected in Sertoli cell cytoplasm, and membrane bound AuNPs were detected close to developing spermatids as well as in germ cell cytoplasm entrapped in lysosomal bodies (Gupta et al. 2018).

Thus, data suggests that the distribution of NPs to reproductive organs may depend on the type of nanomaterial. One study on gold nanoparticles found particles in interstitial spaces including in the seminiferous tubuli and inside cells. More data is needed to elucidate the distribution to testis and female reproductive organs.

Offspring blood-brain barrier:
Charehsaz et al. (2016) dosed pregnant rats with AgNP or $AgNO_3$ by gavage from gestation day 7 to 20. Offspring brains showed increased contents of Ag in all treated compared to control offspring, but not statistically significantly so. Onoda et al. 2014 exposed pregnant mice to carbon black by intranasal instillation on gestational days 5 and 9 and collected brains from the offspring 6 and 12 weeks after birth. Evaluation of brain tissue by transmission electron microscopy did not lead to identification of structures indicating that carbon black had been transferred to the offspring brains.

Milk:
A few studies are available on the transfer of nanoparticles via milk. Hougaard et al. (2010) did not find distribution of elemental Ti above the detection limit (0.2-5 mg Ti/kg) to milk from lactating mice after 1 hr daily exposure to TiO_2NP at 40 mg/m^3 during GD8-18.

In rats exposed orally on lactation days 14-16 with 35 nm AgNPs, the total accumulation of labeled NPs into the milk was > 1.9% of the administered dose over a 48h period. Charehsaz et al. (2016) dosed pregnant rats with AgNP or $AgNO_3$ by gavage and collected stomachs from the pups on postnatal day 2. Ag level in milk increased with dose, with the highest levels observed in offspring from mothers exposed to $AgNO_3$. Ag level did not differ statistically significantly from controls, but this may be due to milk being collected only from 2-3 pups/group. It is furthermore possible that the continuous removal of milk by the suckling pups prevented Ag from accumulating to a significant degree.

No data on kinetics were found for CB, MWCNT and graphene.

In summary, there are several indications from these studies that nanoparticles are able to cross biological barriers. However, the majority of the data is based on measurements of the tissue concentrations of the elemental material of the nanoparticle rather than the presence of the particulate entities. Data suggests that distribution of the particles depends on the type of nanomaterial and/or size of the nanoparticle. More data is needed to elucidate the distribution to sensitive tissues, with focus on identification of the specific particles. Translocation rates may be very low, but the number of translocated particles may still be very high and e.g. equal the number of cells in the studied organ (Pietroiusti et al. 2013). Importantly, the sensitivity of the methods applied to detect particles might not be high enough to detect transfer of small amounts of particles, as also stated by some authors (e.g. Hougaard et al. 2011).

Are there differences between different types of nanomaterials (e.g. nanomaterials of different size, shape (e.g. particles versus fibres), surface properties and solubility)?

and

What factors (e.g. physico-chemical parameters) are known to influence the ability of nanomaterials to reach the developing foetus (in utero) when the mothers/dams/does have been exposed to nanomaterials during pregnancy?

The two questions are to a great extent overlapping and are therefore covered together.

The data described above indicate that there may be an effect of size on the rate of translocation. Exposure of pregnant mice to ZnONP may increase zinc level in level in the placenta and foetuses, as observed after exposure to 13 nm but not 57 nm ZnONP. *Ex-vivo* perfusions with polystyrene beads of different particle sizes (50, 80, 240, 500 nm) found that the nanoparticles to a significantly higher extent than the micro-particles were able to cross the placental barrier (Wick et al. 2010).

In fact, several factors may determine whether particles translocate across the placenta, and rodent studies using intravenous administration indicate that transplacental passage depends on other particle characteristics than size, e.g. surface coating and charge, dose and duration of exposure, animal model, timing during pregnancy and maternal state of health (Buerki-Thurnherr et al. 2012, Pietroiusti et al. 2013).

Yang et al. (2012) performed a key study, albeit using intravenous exposure. Pregnant mice were exposed to 13 nm gold NPs with different surface modifications. Particles were administered from GD5.5 to 15.5. Three different surface modifications were used to explore the effects of nanomaterial functionalization on maternal-foetal transfer; coating with ferritin for optimal biocompatibility, coating with PEG (polyethylene glycol) to reduce interaction with cells and proteins, and coating with an anionic material (citrate) for negative surface charge. With exposure prior to GD11.5, all three nanoparticles could be detected in foetal tissues in significant amounts. Thereafter levels declined dramatically. This change corresponds with the maturation of the placental barrier function in mice. Overall, particles coated with ferritin and PEG accumulated to a much higher degree than the citrate-modified NP. (Yang et al. 2012). Hence, foetal exposure to NP is highly dependent on the stage of gestational maturation as well as the surface composition of the particles.

That NPs with negative surface coating to a lesser degree translocates the placenta corresponds well with a study in the placenta *ex vivo* model. Here polystyrene beads functionalized with (-COOH) cross the placenta to a significantly lower amount compared to non-functionalised polystyrene beads (Grafmüller et al. 2015). Whether modification of the particle surface (-COOH-modification for negative charge, -NH_4-modification for positive charge, and PEGylation) affects translocation across the placenta have also been studied for other particle types in the *ex vivo* human placenta model for other nanomaterials (Au, TiO_2, Ag). The results indicated that surface modification may exert some (limited) influence on particle uptake by and translocation across the placenta, but interpretation of the results were hampered by high agglomeration of the particles in the perfusion medium for firm conclusions to be drawn (Aengenheister et al., 2018; 2019; Vidmar et al., 2018).

In another line of research, Tian et al. (2013) investigated if placental transfer differs between healthy and diseased conditions. Pregnant mice were exposed to AuNP of three different sizes, 3, 13 or 32 nm by intravenous injection on GD17. In the healthy condition none of the particles translocated the placenta to a significant degree. However, the two smallest particles accumulated to a significantly higher degree, when intrauterine inflammation had been induced by injection of lipopolysaccharide, compared to in healthy control mice, the 3 nm particle to a

much higher degree than the 13 nm particle. The 32 nm particles did not cross the placenta in healthy or in mice with intrauterine inflammation (Tian et al. 2013).

4.2.3 Fertility

What kind of changes or adverse effects on fertility have been observed in males and females?

The majority of the identified studies applying exposure via the gastrointestinal tract or the airways investigate effects on male fertility, whereas female fertility is much less studied. The studies are generally performed on adult mice or rats, or pre-pubertal/pubertal animals. The main adverse effects reported are reduced sperm quality and daily sperm production as well as effects on weight of the gonads. The effects on fertility performance was sparsely investigated.

TiO_2NP:
Three studies are available on TiO_2NP. Two in adult male rats and one in adult male mice. Shahin and Mohammed (2017) exposed adult male Wistar rats by daily oral gavage to 50 mg/kg bw/day of 25 nm TiO_2NP for 7, 14 or 21 days. The study report adverse effects such as reduced testis and prostate weights; effects on sex hormone levels, biomarkers indicating impaired spermatogenesis and on sperm parameters**.** The effects were related to the duration of the exposure, i.e. the longer the exposure the more pronounced the effects. Similarly, Song et al. (2017) report increased sperm malformations and sperm cell micronucleus rate as well as levels of markers indicating cell damage in the testes, following oral exposure to 50 and 100 mg/kg TiO_2NP of 5-10 nm for 28 days. They did, however, not find effects on the weight of the testicles and epididymis at any dose level.

Effects on male reproductive parameters (weight of reproductive organs, daily sperm production and plasma testosterone levels) was however not seen in mice after intratracheal instillation of 63 µg of TiO_2 (rutile, 20.6 nm) once weekly for 7 weeks to adult male mice (Lauvås et al. 2019).

Silver:
For silver, toxicity in testes and germinal tissue and reduced sperm quality as well as changes in sex hormone levels have been observed in male rats subjected repeatedly to AgNPs. Several studies investigated effects on male fertility in rats. A significant decrease in sperm viability and histopathological changes in the testes were observed in adult male rats exposed to AgNPs with a particle size of 8.93-33.4 nm at 5.36 or 13.4 mg Ag/kg bw/day, by oral gavage twice weekly for 6 months. A significant decrease in testosterone level and a significant increase in LH level were also detected (Elsharkawy et al., 2019). Effects on sperm morphology, spermatogenesis after prepubertal oral exposure to very low dose levels (15 µg/kg bw/day) was similarly found in Wistar rats exposed to AgNPs (86 nm) on postnatal days 23-53) (Mathias et al., 2014; Sleiman et al., 2013). In another study, Lafuente et al. (2016), did not find effects on sperm count and sperm motility and viability, but they did report effects on sperm morphology after oral exposure to 50 and 100 mg/kg bw/day of PVP-coated AgNPs for 90 days.

In contrast to these findings, no effects on gonads and fertility parameters were found in an in an OECD 422 study with oral gavage of male and female Sprague Dawley rats with 8.8 nm AgNPs (at 62.5, 125, 250 mg/kg bw/day) following exposure for a total of 42 days (Hong et al. (2014)). Also, no effects on testes weight and histopathology were found in rats exposed to 20 and 25 nm AgNPs up to an oral dose of 500 mg/kg bw/day in a 28-day study by Lee et al. (2013). The latter studies, Lee et al. (2013) and Hong et al (2014), did not assess sperm parameters or sperm morphology. Thus, for AgNP, effects in spermatogenesis seems to occur after repeated oral exposure. The potential effects on fertility needs further assessment.

ZnONP:
Toxicity in testes and germinal tissue and reduced sperm quality has been found in three studies where male mice were subjected to repeated exposure to ZnONP. Male mice were orally exposed to 0, 100 and 200 mg ZnONP/kg bw/day (particle size of 50 nm) for 7 or 14 days. In all exposed groups significantly reduced testes, epididymal, seminal vesicle and prostate weights were observed. The percent of abnormal sperm cells was also increased at both dose levels (Radhi et al. (2019). Similarly, Talebi et al. (2013) exposed male mice to ZnONP for 35 days at oral dose levels 0, 5, 50 and 300 mg/kg bw/day. Significant impairment of sperm number and motility, increased percentage of abnormal sperm and histopathological changes in testis were found in mice exposed to 50 and 300 mg/kg bw/day. Comparable results were found by Tang et al. (2019) following oral exposure of male mice to 50, 150 and 450 mg ZnONPs/kg bw/day for 30 days. In addition, Tang et al. (2019) observed a dose related decrease in serum testosterone levels and a downregulation of the *StAR* gene (involved in testosterone synthesis) in testes.

SiO_2NP:
In mice exposure to monodispersed SiO_2NP by tracheal instillation, histopathological findings in testes and adverse effects on semen quality indicate that SiO_2NP may interfere with male fertility.) Increased malformation of sperm cells and decreased sperm motility and concentration in the epididymis were observed in mice after intratracheal instillation of SiO_2NP (57.7 nm) at a dose level of 2 mg/kg bw/instillation every third day for a period of 45 days. The exposure furthermore resulted in induction of oxidative stress in the testis and led to apoptosis and necroptosis of the spermatogenic cells (Ren et al. (2016) and Zhang et al. (2016)).

In contrast, a two-generation study in rats performed according to OECD TG 416 in which rats (male and female) were orally dosed with 0, 100, 300, or 1000 mg/kg bw/day SiO_2NP (10-25 nm in agglomerates) found no effects were for any reproductive parameters including sperm count and daily sperm production (Wolterbeek et al. 2015).

MWCNT and graphene:
Nanoparticle effects were examined for MWCNT in both male and female mice. In a study on male C57B/6× DBA2 mice, exposed by oral gavage to 0.3, 3, and 30 mg MWCNT/kg/day on 30 consecutive days, no effects on the testis or any of the sperm parameters were observed, however when bred with untreated females a dose-dependent significant decreases of fertilizing capacity of 15-40% was registered, starting already at the lowest dose level (Vasyukova et al., 2015)

In female mice, intratracheal exposure to 67 µg of MWCNT on the day prior to co-habitation with an unexposed male increased time-to-delivery of a first litter in one study (Hougaard et al. (2013). This was, however, not found in a follow-up study, where female mice were intratracheally exposed to 2, 18 or 67 µg of MWCNT on the day before cohabitation with unexposed males (Johansson et al. (2017)). However, when naïve female C57BL/6J mice were exposed to MWCNT significantly prolongation was observed of the oestrous cycle during which MWCNT exposure took place, by approximately 2 days, whereas significant shortening of the estrous cycle immediately after the exposed cycle was observed.

One study by Skovmand et al. (2018) investigated graphene oxide NP in mature male NMRI mice. The mice were exposed to four different types of carbonaceous nanomaterials including graphene oxide (18 µg/mouse/i.t. for 7 weeks) by intratracheal instillation. Sperm concentration and motility as well as daily sperm production and sperm integrity were unaffected by the exposure.

Overall, these findings indicate that exposure to MWCNTs may interfere with adult female fertility when exposed via airways, but no firm conclusions can be drawn on the basis of the present studies.

*Carbon black**:***
In the study described above by Skovmand et al. (2018), weekly intratracheal exposure to four different types of carbonaceous nanomaterials, including two types of carbon black particles (Printex90 and Flammrus 101) for 7 weeks, did affect semen parameters, daily sperm production or testosterone concentration in male NMRI mice in mature male NMRI mice (Skovmand et al., 2018).

A decrease in the daily sperm production and testosterone levels of male ICR mice was however, found by Yoshida et al., (2009) after exposure to 0.1 mg CB/mouse by intratracheal administration once a week for 10 weeks. Three different sizes were tested (14, 56, 95 nm CB) and further one group received 14 nm CB, where the particle number concentration was the same as that of the 56-nm particle (14 N). Also, vacuolation of the seminiferous tubules was observed in the 14-nm CB, 56-nm CB, and 95-nm CB groups. These results suggest that carbon nanoparticle-exposure has adverse effects on the mouse male reproductive function which may depend on particle mass rather than particle number (Yoshida et al., 2009). No other effects on fertility was observed. Apart from mouse strain, there are several other differences between the two studies, the most important is probably the vehicle composition, as the ICR mouse study used vehicle consisting of saline with 0.05% tween 80 compared to nanopure water only in the NRMI study. As tween possesses both lipophilic and hydrophilic properties is might therefore be able to partition between lipid and protein structures and has been shown to enhance permeability by altering tight junctions and cellular membranes (Skovmand et al., (2018). It is however not known, whether this is the underlying reason for the observed difference between the two studies.

Other nanomaterials:
For other nanoparticles, scattered data is available for cadmium oxide, cerium oxide, cobalt, copper, gold, manganese oxide, nickel and selenium. Effects on male fertility such as sperm damage, reduced daily sperm production, reduced testis weight and motility were reported in studies of cerium oxide, cobalt, cobber, manganese oxide, and nickel (Qin et al., 2019; Hussien & Mohamed 2018; Kalirawana et al. 2018; Negahdary et al. 2015). Effects on female fertility was observed for cadmium oxide, with reduced incidence of pregnancy after cadmium oxide exposure in nanoform. For some of these nanomaterials, it must be noted that there are already know effects on reproduction on the bulk material. Hence, cadmium, cobalt and nickel, have harmonized classification regarding reproductive toxicity. It is therefore likely that the effects seen from nano particle exposure is related to the toxicity of the elemental material in ion form rather that nanoparticle exposure.

Are there differences between different types of nanomaterials (nanomaterials of different size, shape (e.g. particles versus fibres) and solubility)?

Conflicting data is available on the effects on male reproductive parameters after exposure to several nanomaterials. There are indications of effects from several studies on all the investigated nanomaterials. However, available OECD TG studies could not find effects on fertility parameters. Sperm parameters was not investigated in the OECD GT 422 studies available in rats. For some of the nanomaterials, the effects may be due to effects of the material, rather than the particle size. The large variation in exposure time and duration, characterization of NPs as well as studied endpoints (e.g. histology, daily sperm production, sperm parameters) make it very difficult to compare and conclude on the results. More data is therefore needed to elucidate the effects on fertility and reproductive parameters of nanoparticle exposure to the different particles. The matter may be further complicated by differential sensitivity between the rodent strains relative to the effects of particles on male reproductive parameters. As an example, a recent study of the effects of inhalation of welding fume with a high content of nanosized particles found that the studied reproductive outcomes seemed more prone to disruption in Sprague Dawley compared to Brown Norway rats (Skovmand et al. 2020).

Can any conclusions be drawn? Are there any concerns identified? Are these relevant for humans?

From the above summary of the effects on fertility, it can be noted that the observation of effects on several endpoints give raise to concern related to effects of NPs on fertility, in particular for male fertility (testis, sperm production, motility and viability). Female fertility have only been studied to a very limited extent. These effects represent endpoints of relevance for humans as long as species specific mechanisms of the test animal have not been identified and documented for the test species. As the results are not consistent, further data, performed according to OECD standards are needed, preferably including assessment of sperm parameters.

What are the uncertainties of these studies? For example, have the test materials been adequately characterised?

Some of the non-OECD TG guideline studies use few animals/groups, only one dose level or administration of a single dose only. This might introduce uncertainty as to the predictive value of these testing schedules.

For intratracheal instillation studies uncertainty pertain as to the direct translation to inhalation exposure and corresponding inhalation exposure level. Although such estimation may give an indication of relevant lung burden for the nanomaterial there is still both qualitative and quantitative uncertainties in this type of testing. Instillation studies may however be used to rank NPs according to toxicity and serve as proof-of-principle studies that can then later be tested with inhalation exposure (Masakazu et al. (2018)).

The data regarding toxicity to fertility for silicon oxide also indicate that the extent of agglomeration/aggregation of the nanomaterial in the test vehicle may play a role in relation to the toxicological response and that this may bring uncertainty into the test.

Regarding characterization of the study material, the studies considered most relevant (R: ++ or R+/++) for having assessed effects on fertility have obtained the following N-scores for nano-characterisation:

TiO_2NP: three studies with N-scores in the range of 4-9 (average N: 7.0)
AgNP: five studies with N-scores in the range of 2-5 (average N: 3.4)
ZnONP: three studies with a N-score of 2-4 (average N: 3.0)
SiO_2NP: two studies with a N-score of 4 and 7 (average N: 5.5)
MWCNT: four studies with N-scores in the range of 4-6 (average N: 5.0)
Carbon black: two studies with N-scores of 4 and 5 (average N: 4.5)

When these scores are compared to full characterization with a score of 11, in general a low characterization rate of the studies exist. The studies used for assessment of nanoparticulate titanium oxide, silicon oxide and MWCNT are considered to be the most acceptable, whereas data from studies with an N-score of four or less may be more uncertain with regard to the nanomaterial as several "unknowns" pertain to the characterization. The low level of characterization further makes it difficult to compare and conclude on the results even within the specific nanomaterials. It should be noted that in some studies, the particles are characterized in previous publications, which was not considered in the present report. For some NPs, such as carbon black, many studies use the same manufactured type of particle (Printex90), which makes comparison more reliable but at the same time hampers extrapolation to other types of black carbon.

4.2.4 Development

What kind of changes or adverse effects have been observed in the developing foetuses of mothers/ dams/ that have been exposed to nanomaterials during pregnancy?

TiO_2NP:
For nano titanium dioxide both absence of developmental effects as well as adverse effects such as *impaired placental functioning, reduced pup viability and adverse cardiac effect in offspring* have been reported:
For nano titanium dioxide, four OECD TG 414 studies have been conducted, dosing rats orally with both the anatase and rutile crystalline forms. In none of these studies any indication of developmental toxicity was observed, even at the highest exposure level of 100 mg/kg bw/day (Warheit et al. 2015 and Lee et al. 2019).

However, studies in rats using inhalation exposure at a dose level of 10 mg/m^3 of anatase/rutile (80/20) TiO_2NP with a particle size of 21 nm have found cardiac effects in the offspring such as reduced vascular reactivity in the aorta reduction of maximal mitochondrial respiration in aorta tissue. Also, epigenetic and transcriptomic changes were observed in cardiac tissue (Stapleton et al. 2013-2019). The same quality of nanomaterial resulted in in increased placental weights and impaired vascular reactivity in the placenta as a sign of dysfunction in the placenta in rats exposed by inhalation during gestation (Bowdridge et al. 2019 and Abukabda et al. 2019). Hougaard et al. (2010) exposed female mice by inhalation to approx. 40 mg/m^3 TiO_2NP, 1h/day during GD8-18 and observed slight behavioral changes.

In mice a significantly greater percentage of non-viable foetuses were found in female mice orally dosed once on GD 9 with 1000 mg/kg bw/day of rutile TiO_2NP (Philbrook et al. 2011).

On the basis of these studies, it is not possible to conclude whether the difference in findings between rats or mice owes to differential sensitivity in the two species or different qualities of the tested nanomaterial, nor whether induction of developmental effects of TiO_2NP depends on the route of exposure as the outcomes tested (foetal development vs. change in offspring organ function after birth) for oral and inhalation exposure only overlap to a limited extent.

AgNP:
For nanosized silver particles no effects on development of the offspring was observed in an OECD 422 study using oral exposure at dose levels of up to 250 mg/kg bw/day of AgNPs with a diameter of 8.8 nm (Hong et al. 2014). Similarly, no effects were found in a prenatal developmental toxicity study in which rats were exposed on GD6-19 up to a dose level of 1000 mg/kg bw/day of AgNPs with an average particle size of 6.5 nm (Yu et al., 2014). However, some concern for developmental toxicity was described by Fatemi et al. (2013) due to observation of increased oxidative stress in the brain of offspring from female rats orally exposed from GD9 to the end of gestation to 25 mg/kg bw/day of AgNP (particle diameter of 20 nm). Only one inhalation study was identified. Campagnolo et al. (2017) exposed female mice by inhalation during the first two weeks of gestation and observed increased rate of resorptions and levels of inflammatory mediators in the placenta, and decreased oestradiol levels in maternal plasma.

Thus, some concern remains to developmental effect by the oral route, while potential toxicity following inhalation exposure have been explored to a very limited extent.

ZnONP:
In pregnant rats orally exposed on GD5-GD19 to 0, 100, 200, and 400 mg ZnONP/kg bw/day (20 nm) in a study performed according to OECD TG 414, significant increase in the number of foetuses with visceral variations was observed at 400 mg/kg bw/day. Reduced maternal food consumption, decreased liver weight and increased adrenal gland weight were observed at the two highest dose levels (Hong et al., 2014a+b).

For nanosized zinc oxide particles, oral exposure of pregnant mice to 13 nm particles at a dose level of 7.2 mg ZnONPs mg/dam (about 280 mg/kg bw/day) resulted in pathological lesions in the placenta, and decreased placental relative to foetal weight, foetal viability, foetal weight, crown-rump and tail length (Teng et al. 2019).

Even though an oral OECD TG 414 study has been conducted in rats, conclusive data (from oral exposure and not a least from inhalation exposure) are lacking for an evaluation of the potential for induction of developmental toxicity of ZnNP.

SiO_2NP:
For nanosized silicon dioxide particles, Wolterbeek et al. (2015) conducted an OECD TG 416 two-generation study in which rats were orally dosed to SiO_2NP (primary particle size 10-25 nm, surface area of 230 m^2/g) at dose levels of 0, 100, 300, or 1000 mg/kg bw/day. No effects were found for any reproductive or developmental toxicity parameters in this study. Further, Hofmann et al. (2015) conducted an OECD TG 414 prenatal developmental study in which rats were orally dosed to SiO_2NP (same test item and batch as in Wolterbeek et al. (2015)). No effects were found for any developmental parameter. Together, these findings indicate low - if any - concern for developmental toxicity of SiO_2NPs following oral exposure, while no evaluation can be made in relation to inhalation exposure for which no data is available.

Carbon nanotubes + graphene:
Only data on the developmental toxicity of MWCNTs was identified. In Lim et al. (2011a+b), Sprague-Dawley rats were exposed to 40, 200 or 1000 mg/kg MWCNT/kg bw by oral gavage from GD6 to GD9. No effect on foetal growth, viability, or morphological development were observed.

In a study using intratracheal exposure to MWCNT, mature female C57BL/6J mice were exposed to 67 µg of MWCNTs one day prior to cohabitation with a mature unexposed male. Litter parameters, behavior and daily sperm production were similar in control and exposed offspring (Hougaard et al. (2013)). Also, no consistent effect was seen on litter parameters following exposure of female mice by intratracheal administration to 2, 18 or 67 µg MWCNT on the day before start of cohabitation with unexposed males (Johansson et al. (2017).
Fujitana et al. (2012) in a study using intratracheal exposure of mice to MWCNT on GD 9 found statistically increased incidences of foetal malformations at exposure levels of 4 or 5 mg/kg bw, however, no characterisation of the test item was given.

Although no concern has been found in these studies limited data is available for an overall evaluation.

It has to be noted that testing according to the OECD TGs standards regarding developmental and reproductive toxicity for oral and not at least for inhalation exposure is missing for SWCNT, MWCNT and graphene.

Carbon black:
For carbon black, developmental toxicity was studied for gestation and litter effects as well as for effects on three organ systems in the offspring, i.e. the male reproductive, central nervous system and immune systems.

Gestational exposure by the maternal airways did not seem to affect gestational and litter parameters in several studies (malformations not studied) (Kyjovska et al. 2013; Skovmand et al. 2019; Yoshida et al. 2010). Findings in two studies indicated that maternal exposure could interfere with offspring development of the immune system (El-Sayed et al., 2015; Shimizu et al., 2014). Equivocal results regarding the effects of maternal CB exposure and effects on sperm parameters in male offspring were observed (no findings in Skovmand et al. 2019, toxicity in Yoshida et al. 2010).

Exposure of pregnant mice to carbon black via the airways resulted in histological changes in the brain of the offspring (Onoda et al., 2014 and 2017b; Umezawa 2018). These changes were in the form of increased expression of the protein glial fibrillary acidic protein (GFAP). GFAP increases naturally in the brain with age, but the changes induced in one of the studies compared with levels normally observed in much older animals (Onoda et al. 2017b). This kind of change have been observed in several studies, in two different mouse strains and following exposure by inhalation at levels below the Danish occupational exposure limit as well as by intranasal instillation. Related changes have been observed in two additional studies, not described in detail here (Onoda et al 2017a+c). Other CNS changes included decrease in the number of parvalbumin-positive interneurons in the motor and prefrontal cortices and altered mRNA expression levels associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production in the cerebral cortex.

The findings do not indicate that maternal airway exposure poses a risk to pregnancy but the histopathological changes in the offspring central nervous system does raise concern of the long-term functional consequences hereof.

Thus, limited and scattered data of the developmental effects of carbon black is available. Testing according to the OECD TGs standards regarding developmental and reproductive toxicity, including examination for neurobehavioral changes, following oral and not least inhalation exposure is missing for carbon black.

Other nanomaterials:
Nickel nanoparticles (NiNPs) in an OECD TG 415 study negatively impacted birth survival rates at all oral dose levels of 5, 15, 45 mg/kg bw/day and to a higher degree for nanoparticles (90-100nm) compared to microparticles (3.3 µm) (Kong et al. 2014).

Aluminium nanoparticles (AlNPs) induced behavioural changes, induced increased oxidative stress and decreased neurotransmitter activity in the cerebral cortex of offspring from female mice exposed by nasal drip at a dose level of 50 mg/kg bw/day from 14 days before mating to the day of giving birth (Zhang et al. 2018)

Cadmium oxide nanoparticles (CdONP) delayed neonatal growth in pups from female mice exposure during GD 5-17 by inhalation exposure to 230 µg/m3 (Blum et al. 2012+2014).

Copper nanoparticle (CuNPs) reduced survival rate of 7 weeks old pups delivered from female mice exposed during GD 3-19 by inhalation at 3.5 mg/m^3. Further expression of several Th1/Th2 and other genes related to the immune response in offspring spleens were significantly up- or down-regulated, indicating strong immunomodulatory effects (Adamcakova-Dodd et al. 2015).

Iron nanoparticles (FeNP) increased mortality and induced significant haematological and biochemical changes in offspring (especially in females) delivered from female and male mice exposed to mice at 4 mg/kg by intratracheal instillation according to a modified OECD TG 421 test design (Park et al. 2017).

Is there evidence of direct effects (direct interference of the nanomaterial with embryo/foetal tissue function) and/or indirect effects (e.g. induction of the release of mediators in maternal or placental tissue)?

It is very difficult to answer this question on the basis of the current data of inhalation and oral exposure, where in-depth evaluation of single references investigating various hypothesis for mechanistic action. Thus, no confirmative mechanisms have been concluded in any of the references, but induction of oxidative stress in the target tissues is often indicated as the plausible cause of a toxic response.

Particulates may confer their oxidative and inflammatory action directly in the tissues or may even trigger specific receptors. In the alveoli, deposited particles may generate inflammation and oxidative stress (Braakhuis et al. 2014), and inflammatory mediators may be released to the blood stream to reach reproductive organs. Inflammatory mediators are biologically active molecules that may trigger a range of responses in the tissues, such as vascular events and endocrine disruption, which may affect fertility and foetal development in an indirect manner (Kim et al. 2015; Lan and Yang 2012; Meyer 2014). Even if particles do not enter the blood stream, the effects may therefore not be confined to the presence of or immediate proximity of particles at the port of entry or organ of accumulation. Both the direct effects of particles and the indirect effects of inflammation may therefore have potential to interfere adversely with fertility and foetal development, with potential adverse consequences. Several studies have aimed to investigate if NPs induce pregnancy complications through oxidative stress and inflammation, e.g. by administration of antioxidants alongside the exposure to particles in experimental animal studies. In many cases, co-administration of antioxidants alleviated the adverse effects to some degree, compared to animals exposed to the particles without antioxidants (Yamashita et al. 2011; Qi et al. 2014; Onoda et al. 2017; Huang et al. 2014).

However, it is generally recognized that it is methodologically very challenging to separate direct and indirect effects of nanomaterials. Dugershaw et al. (in preparation) is presently reviewing evidence for indirectly induction of developmental toxicity following maternal exposure to nanomaterials during gestation. Of importance, the described pathways should not to be considered mutually exclusive. Hence, intrauterine inflammation has been shown to increase transfer of nanoparticles from the maternal to the foetal compartment (Tian et 2013). However, in addition to induction of oxidative stress and inflammation, several specific mechanisms are proposed to be of importance, including activation of toll-like receptors in the placenta, interference with endocrine signalling and uteroplacental developmental and function and extracellular vesicle signalling. The mechanisms driving reproductive and developmental toxicity of NPs are however still poorly understood.

Can any conclusions be drawn? Are there any concerns identified? Are these relevant for humans?

From answering of the first question in this section it can be noted that different toxicological endpoints give raise to concern relative to:

TiO_2NP: effects on placenta and cardiovascular system in the offspring (rats, inhalation)
AgNP: developmental neurotoxicity (rats, oral)
ZnONP: effects on placenta, foetal growth and offspring viability (mice, oral)
SiO_2NP: no specific concerns based on the present material
MWCNT: no specific concern based on the present material
Carbon black: developmental neurotoxicity (mice, inhalation and resp. tract exposure)
AlNP: developmental neurotoxicity (mice, resp. tract exposure)
CuNP: decreased offspring viability (mice, inhalation)
FeNP: decreased offspring viability (mice, resp. tract exposure)
NiNP: decreased offspring viability (rats, oral)

All of these endpoints represent human relevant endpoints as long as specific mechanisms for these effects in the test species have not been identified and documented. For Ni this is in agreement with already identified developmental toxicity for the material as such, i.e. Ni is classified for developmental toxicity. For the remaining particle types, it is interesting that the main concerns do not relate to fetotoxicity, but rather to functional alterations in the offspring after birth (developmental neuro- and cardiovascular toxicity). Albeit there are test guidelines for developmental neurotoxicity (OECD TG 426), it is not often used in regulatory testing. For effects on the developing cardiovascular system, there is no accepted OECD TG. It has previously been concluded that for inhalation exposure to NPs, the main concern is probably functional alterations in the offspring rather than fetotoxicity (Hougaard et al. 2015). Whether this is a general issue for synthetized NMs is however difficult to delineate due to the

differences in the outcomes studied for each NM (present results, Ema et. al. 2016), but the issue deserves attention.

What are the uncertainties of these studies? For example, have the test materials been adequately characterised?

For studies not adhering to OECD TGs, few animals per group, use of only one dose level or single dose levels may introduce uncertainty as to the predictive value of the testing.

For intratracheal instillation studies uncertainty pertain to the translation of findings relative to inhalation exposure and estimation of a corresponding exposure level for inhalation. Although such estimation may give an indication of relevant lung burden for the nanomaterial there is still both qualitative and quantitative uncertainties in this type of testing, as instillation exposure imply a high dose rate.

Data on silicon oxide (regarding fertility) also indicate that the extent of agglomeration/ aggregation of the nanomaterial in the test vehicle may play a rule in relation to the toxicological response and thus this may bring uncertainty into the test. Thus, methods for test sample preparation and dispersion must be described and documented in order to limit such uncertainties.

Although a comparable test design is given for two tests, differences in examination techniques, use of different qualities of the nanomaterial and/or different level of characterization hamper comparison of apparently identical studies 1:1 even if the same test species and exposure route has been used for a nanomaterial. This makes overall conclusions for each of the nanomaterials uncertain and difficult.

Regarding characterization the studies considered most relevant (R: ++ or R+/++) for assessing developmental toxicity have obtained the following N-scores for nano-characterisation:

TiO_2NP: four studies with N-scores in the range of 3-6 (average N: 4.0)
AgNP: four studies with N-scores in the range of 3-6 (average N: 4.0)
ZnONP: two studies both with a N-score of 6 (average N: 6.0)
SiO_2NP: two studies both with a N-score of 7 (average N: 7.0)
MWCNT: four studies with N-scores in the range of 4-5 (average N: 4.5)
Carbon black: seven studies with N-scores in the range of 3-6 (average N: 4.8)
AlNP: one study, N:5
CdONP: one study N:4
CuNP: one study, N:5
FeNP: one study, N:4
NiNP: one study N:7

When these scores are compared to the maximum score of 11, the studies used for assessment of nano zinc oxide and silicon oxide are considered the most acceptable, whereas data from studies with an N-score at 4 or less may be considered less representative as several "unknowns" pertain to the characterization.

However, it has to be acknowledged that for several of the parameters used in the Card & Magnuson scoring, knowledge is lacking as to how these parameters may affect toxicity, and not all these parameters in the Card & Magnuson system can be considered as equally relevant for the toxicity of the nanomaterial. Thus, in "Appendix R7-1 for nanomaterials applicable to ECHA´s Endpoint specific guidance" toxicological relevant parameters for a REACH registration of a nanomaterial is indicated to be: chemical composition, particle size, shape and aspect ratio and surface chemistry (ECHA 2017).

Also, data on water solubility is considered relevant as water soluble nanomaterials, especially for dermal and oral exposure, may increase bioavailability of the chemical(s) of which the nanoparticle is composed. Findings in the one inhalation study of silver indicate that this also may be the case for airway exposure. On the other hand, high persistency/low water solubility may increase the potential for local tissue reactions in the lungs after inhalation, due to the very slow clearance of nanosized particles.

The specific surface area may be a further important parameter as increased specific surface area may promote dissolution and bioavailability of the nanomaterial. Also, increased specific surface area may increase the potential for local tissue reactions in the lungs due to higher degree of surface contact to lung tissue.

5. Conclusions

Data availability:
Based on a structured literature search in selected databases from STN (Scientific and Technical information Network), 2152 publications were identified using search terms relevant for identifying publications with manufactured nanomaterials in combination with a range of search terms relevant for description of reproductive and developmental toxicity. From this, 111 studies covering mainly *in vivo* testing of manufactured nanomaterials were extracted and analysed. These *in vivo* studies used either oral or airway exposure (inhalation or intratracheal instillation) in rats or mice as the test species.

Testing of titanium dioxide and silver in nanoform was most frequent and together contributed with 48% of the studies. Nanosized particles of zinc oxide and silicon oxide and carbon-based nanomaterials together contributed with 34% of the studies, while 13 different nanomaterials were tested in the remaining 18% of the studies.

Six OECD TG studies were found (three OECD TG 414 with TiO_2NP, ZnONP, SiO_2NP, respectively; one OECD TG 422 with AgNP; one OECD TG 416 with SiO2NP and one OECD TG 415 with NiNP). These studies were all conducted in rats, exposed with oral gavage.
For the studies that did not comply with OECD guideline testing, many can be considered proof-of-concept studies and hypothesis generating rather than concluding studies, as often only a single to few target organs or bioindicators of effects were studied (e.g. mechanistic or functional parameters, for example gene expression in gonadal tissue or vascular reactivity in placenta) rather than commonly accepted end-points for adverse effects on fertility or development. These may indeed be relevant, but there is less experience in their interpretation relative to reproductive and developmental toxicity.

Studies testing of materials in nanoform in parallel with bulk substance to uncover differences in toxicity between nano- and micro-sized particles were few in numbers among the studies included in this report.

With respect to characterisation of the particles, very different levels were provided in the study reports. In fact, only the minority of the references provided data on all the key parameters: chemical composition, particle size, shape/aspect ratio and surface chemistry as indicated as toxicological relevant parameters in the guidance for REACH registration of nanomaterials.

Kinetics:
Findings in the retrieved literature indicate that nanoparticles are able to cross biological barriers, including the blood-testes barrier and the placenta. The majority of the data on the subject is however based on measurements of the tissue concentration of the elemental material of the nanomaterial, rather that assessment of the actual presence of particles in relevant tissue. Thus, the found increases in tissue contents of elemental nickel, silver and zinc may to some extent owe to dissolution of the particles and subsequent distribution of the ions rather than distribution in particulate form.

Overall, the available data suggests that distribution of the particles may depend on the level of exposure, type of nanomaterial, the size of the particles, and that also surface modification and agglomeration should be taken into account. For placental passage, the stage of gestation is also an important factor. Here it should be considered that particles already in the body at the time of impregnation might easily access the developing embryo, even if there is no placental transfer. More data is needed to elucidate the distribution to sensitive tissues. Further, data focusing on detection of nanoparticles in tissue is warranted.

Fertility:
Conflicting data is available on the effects on male fertility after exposure to nanomaterials. There are indications of effects on sperm parameters, such as sperm count, daily sperm

production and sperm morphology and motility in several non-guideline studies for most of the investigated nanomaterials. There are however inconsistencies between studies. For some particle types, available OECD TG studies did not report adverse effects on male reproductive nor fertility parameters. Sperm parameters was however not investigated in the OECD GT 422 studies available in rats.

For some of the nanomaterials, the effects on testes and sperm parameters could probably be explained by the chemical composition of the particles rather than exposure to the particulate entities, as the reproductive toxicity are well-established for e.g. cadmium and cobalt. In general, there were large variations among the studies relative to timing and duration of exposure as well as characterization of the materials. Another issue relates to vehicle composition of the vehicle as a potential factor affecting toxicity. Overall, only very fragmented pictures emerge from the study results, hampering firm conclusions. More systematic studies of nanomaterials relative to reproductive toxicity are therefore needed to elucidate the effects on male fertility and reproductive parameters of nanoparticles of different sizes and composition.

A very important conclusion based on the retrieved studies is the almost complete lack of studies on female fertility.

Development:
In general OECD TG testing with oral exposure to rats indicate to limited concern for developmental toxicity of TiO_2NP, ZnONP, SiO_2NP and AgNP. These studies do however mainly investigate toxicity related to fetotoxicity and growth, with only few functional parameters being assessed in the offspring.

Based on review of also non-guideline studies, some endpoints of concern have come up related to developmental effects.

For nano titanium dioxide and nano zinc oxide, effects on the placenta were reported after inhalation and oral exposure, respectively. Maternal exposure to CuNP, FeNP and NiNP and ZnONP was associated with decreased survival and/or growth and viability of the offspring pups after maternal exposure via the airways (CuNP, FeNP) or the oral route (NiNP, ZnONP). Decreased pup survival is a well-known effect from water soluble nickel compounds and may therefore be associated to the chemical composition rather than the particulate form. Changes in the offspring organs after birth have been observed in some studies. In offspring from pregnant females exposed by inhalation to nano titanium dioxide, adverse effects on the cardiovascular system have been observed. Further, there are evidence that the brain may be sensitive to maternal NP exposure, as both AgNP, carbon black and AlNP report effects in brain or signs of neurotoxicity after exposure. In the case of AlNP, it has to be noted that exposure to the soluble Al-ion is associated to neurodegenerative changes of the brain and thus the neurotoxicity may not specifically be associated to the nanoform. For carbon black, changes in specific cell populations in the CNS have consistently been observed following maternal airway exposure.

However, as for the data on fertility the non-guideline testing proved a scattered picture of the developmental potential of the various nanomaterials. Thus, the different types of test design, lack of OECD TG testing following inhalation exposure and the sometimes low level of nano-characterization, again hampers confirmative conclusions regarding developmental toxicity of individual nanomaterials.

Uncertainties and limitations data gaps:
As indicated above, the majority of the data identified originates from non-OECD TG studies. For some of these studies, there are few animals per group, use of only one dose level or only a single administration of test material. This may introduce uncertainty as to their predictive value. Although a comparable test design is present for two tests, differences in examination techniques, animal strain, and qualities of the nanomaterial and its characterization hampers

comparison of studies even if the same species and exposure route have been applied. This makes overall conclusions for the nanomaterials uncertain and difficult. Of note, if inflammation is a determinant in toxicity, how does particle exposure influence individuals that already suffer from low-grade inflammation (e.g. asthma and obesity).

Overall, studies using inhalation exposure are few in numbers. This is critical, as inhalation exposure may be critical for toxicity of nanomaterials in comparison to oral exposure. And, importantly, among the retrieved studies there is an almost complete lack of studies on female fertility

Follow-up/ suggestions/ recommendations:
Based on the learnings from this project some general proposals and considerations for future research and testing can be provided:

- Application of a thorough/ more standardized characterisation of the nanomaterial and the nanomaterial exposure to include the most important determinants of toxicity.

- Study the effects of nanoparticles in parallel with larger particles, to gain knowledge of differences in toxicity relating to size (or other relevant physico-chemical parameters, such as particle form).

- Increase focus on the airway route of exposure.

- Increase focus on female fertility and reproductive parameters.

- Select meaningful periods of exposure. Take into account that particle translocation probably varies considerably during gestation.

- In developmental toxicity, include postnatal functional parameters to a larger degree (offspring fertility, neurofunction- and histology, cardiovascular and immune function).

- Always report gestational and litter parameters

- Follow-up testing of outcomes where previous results raise concern to clarify potential for induction of adverse reproductive or developmental effects.

- Adhere to the principles of OECD TGs to the highest extent possible, even if the full study guideline is not possible to apply. If not included already, include parameters where previous study results raise concern.

- Investigate particle transfer across “barriers” (blood-testes-barrier, placenta), with application of highly sensitive methods of detection of both the bulk material and particles.

- Identify underlying mechanisms of toxicity for grouping of materials. Does the particulate entity as such possess the ability to change foetal development (irrespective of material) or are oxidative stress and inflammation the driving forces?

- Coordination of the testing (e.g. in testing programs) in order to achieve a more systematic approach for the testing

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Appendix A: Definition of the scope of the review and the methodology

A.1 Scope

A.1.1 Relevant nanomaterials

According to the EU-adopted recommendation for a definition of a nanomaterial, a "Nanomaterial" means (2011/696/EU):

A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

In this project, however, it is the aim to focus the search and evaluation of data on types of manufactured nanomaterials (MNs) that are commercially available and produced in an industrial scale relevant to REACH regulation. This means that inclusion of data in relation to more advanced use of nanomaterials for medical treatment, diagnostic or analytical purposes is considered less relevant and borderline for the scope of this report.

Also, knowledge and data from incidental generated nanomaterial such as nanoparticles in ambient air (including combustion derived nanoparticles from engine exhaust and wood burning), and also naturally occurring asbestos fibres in nanoscale are not covered in this project.

A.1.2 Relevant studies/data

This project focused on data especially relevant for assessing reproductive and developmental effects in relation to human health. This means that data generated from species used for environmental assessment, e.g. earth worms, larvae and fish, are not included unless the data specifically indicate that the findings are relevant to human health assessment.

Also, in trying to answer the questions in section 1.1 it is considered necessary to identify the key literature and to focus on the original literature with description of testing and generation of the data in order to evaluate the quality, reliability and relevance of data, rather than to identify and refer to data from secondary sources.

A.2 Level of knowledge based on current reviews

In order to get a basis for the literature search to be performed in WP2 it was decided to build on the existing knowledge as given in the most recent scientific reviews on nanomaterials and reproductive and developmental effects.

Examination of these scientific reviews will provide this project with an initial understanding of the scientific state-of-the-art of the topic and enable a more focussed and systematic literature search. This will give us an indication of which type of experimental data is available, which nanomaterials have mainly been tested, to which extent the tested nanomaterials have been adequately characterised, and which key aspects are considered important in the field of nanomaterial and reproductive/developmental effects, etc. Also, reference lists from the

reviews will provide the project with a lot of relevant references for further review and evaluation.

Thus, the current reviews will enable scoping of the project and may give indications as to which extent a series of relevant questions can be answered:

What studies relevant to reproductive/developmental effects are available on nanomaterials?

Which type of information is available (in vitro/ex vivo/in vivo)?

Are the test guidelines followed for the studies?

Which types of nanomaterials have mostly been tested and how well are they characterised?

Are there comparable studies in which nanomaterials and bulk sized materials and/or soluble forms of these materials with the same chemical composition are compared, or studies in which different nanoforms of the same nanomaterials are compared?

What are the key issues raised about nanomaterials and reproductive/developmental effects?

A.2.1 Recent reviews

The reviews were selected based on of the project members' many years of work and expertise with this specific area supplemented with a targeted web-search for additional reviews. Although several reviews were identified (see below), the list of reviews should not be considered the result of a systematic search or a complete overview of reviews. The intention of the collection of recent reviews was to obtain a starting point for the systematic literature search (WP 2) rather than to give a detailed overview of the complete list of all reviews. The following reviews were selected:

Wang R et al. (2018). Potential adverse effects of nanoparticles on the reproductive system. International Journal of Nanomedicine 2018:13 8487–8506

Brohi RM et al. (2017). Toxicity of Nanoparticles on the Reproductive System in Animal Models: A Review. Front. Pharmacol. 8:606.

Ema M (2017) A review of reproductive and developmental toxicity of silver nanoparticles in laboratory animals. Reprod Toxicol. Jan; 67:149-164

Hougaard KS et al. (2017). Developmental toxicity of engineered nanomaterials. Chapter 19 in: Reproductive and developmental toxicology (2nd edition), 333-357. https://doi.org/10.1016/B978-0-12-804239-7.00019-6

Ema M et al. (2016). Reproductive and developmental toxicity of carbon-based nanomaterials: A literature review. Nanotoxicology, 10:4, 391-412

Hougaard KS et al. (2015). A perspective on the developmental toxicity of inhaled nanoparticles. Reproductive Toxicology 56 (2015) 118–140

Larson JK et al. (2014). Engineered Nanomaterials: An Emerging Class of Novel Endocrine Disruptors. BIOLOGY OF REPRODUCTION 91(1):20, 1–8

Iavicoli et al (2013). The Effects of Nanomaterials as Endocrine Disruptors. Int. J. Mol. Sci. 2013, 14, 16732-16801

A.2.2 Findings from the reviews

In Table A-1 below an overview of the information from the reviews is given in relation to:

the number of references included

the nanomaterials tested

type of studies (in vitro/in vivo)

species tested

route of administration used

period of exposure

information given in either abstract or conclusion of the review

keywords (identified from either the title, abstract, conclusions, or headings of sections/ tables in the review)

comments.

Also, all the references from each review were transferred into Endnote and sorted for duplicates to obtain a collection of potentially relevant references for this project.

Table A-1: Description of reviews

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Wang R et al. (2018) (95 refs)	SWCNT MWCNT Graphene Zinc oxide Silver Gold CB Iron Iron oxide CeTe QD Nickel Silicium PbS TiO2 PeGbPLA-polymer	*In vitro* Primarily sperm cells	Rats, mice	Oral, s.c.; i.v., resp. instillation	Single exposure and various periods during gestation Up to 90 days repeated exposure.	NPs can pass through the blood–testis barrier, placental barrier, and epithelial barrier, which protect reproductive tissues, and then accumulate in reproductive organs. NP accumulation damages organs (testis, epididymis, ovary, and uterus) by destroying Sertoli cells, Leydig cells, and germ cells, causing reproductive organ dysfunction that adversely affects sperm quality, quantity, morphology, and motility, or reduces the number of mature oocytes and disrupts primary and secondary follicular development. In addition, NPs can disrupt the levels of secreted hormones, causing changes in sexual behaviour. However, the current review primarily examines toxicological phenomena. The molecular mechanisms involved in NP toxicity to the reproductive system are not fully understood, but possible mechanisms include oxidative stress, apoptosis, inflammation, and genotoxicity. Previous studies have shown that NPs can increase inflammation, oxidative stress, and apoptosis and induce ROS, causing damage at the molecular and genetic levels which results in cytotoxicity.	Reproductive system, germ cell, sperm, fertility, testis, ovary, uterus, oocytes, follicular development, ROS, oxidative stress, blood-testes, translocation, hormone levels, mechanisms	17 *in vivo* tests 12 *in vitro* test systematically tabulated

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Brohi et al. 2017 (179 refs)	Ag Gold SWCNT MWCNT CB MnO2 CdO Iron oxide CdTe QD TiO2 SiO2	Few *in vitro* data	Focus on *in vivo* data, mainly in mice and rats	i.p., i.v., s.c., Parenteral, oral, inh, instillation	Few details	Studies on the toxicity of NPs in the reproductive system of animals are increasing, but effectively the field is still in its preliminary stages. While there is evidence to suggest the entry of some NPs into both male and female reproductive organs, both directly in adult animals and in utero, the studies were carried out with widely varying doses and administration routes, making direct comparisons and definitive conclusions difficult. While Si-based NPs appear to have few, if any, toxic effects, Ag and TiO2-based NPs may be more dangerous, with an impact on cells in the seminiferous tubules, immune and inflammatory reactions, and sperm motility and morphology. Transplacental transfer of many types of NPs, including Au, TiO2, SiO2, C, and QDs, is established in animal models, and there is evidence suggesting that in many cases this results in the transfer of NPs to the vulnerable foetus, with varying toxic effects on, for example the foetal brain, nerve development and future fertility.	Reproduction, reproductive system, uterus, ovaries, testis, sperm cells, transplacental transfer. Oxidative stress. Nanotoxic. Distribution in organs	Many references but few details regarding study design and exposure details.

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Ema et al (2017) (84 refs)	Ag	Few data	33 *in vivo* studies. Mice, rats, rabbits	i.p., i.v., s.c., oral, resp. instillation	Single exposure and various periods during gestation Up to 90 days repeated exposure.	A number of studies have reported the possible effects of AgNPs on reproduction and development; however, data are still very limited, and the studies were performed using a wide range of dose levels (15 µg/kg–1000 mg/kg) and sizes (average size of 6.45–323 nm) of AgNPs. In developmental toxicity studies, maternal toxicity was also induced. Developmental effects should be examined at submaternal toxic doses to clarify whether embryonic and foetal alterations, including morphological and functional alterations, death, and growth restriction, are the result of direct effects on the embryo/foetus, indirect (maternally-mediated) effects, or a combination of the two. Further studies on the reproductive and developmental toxicity of AgNPs require the use of state-of-the-art methodologies, and administration route and doses relevant to human exposure. Additional studies are also warranted to elucidate the mechanism of reproductive and developmental toxicity following low level exposure to AgNPs	Reproductive/ developmental toxicity, nanoparticles, blood-testes barrier transfer, testicular/sperm toxicity, developmental neurotoxicity	Focus on *in vivo* data, data on 33 *in vivo* studies, systematically reported in tables with details.

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Hougaard et al. (2017) (approx 140 refs)	Graphite SWCNT fullerenes MWCNT CB Gold Silver TiO2 Polystyrene QDs	Few *in vitro* data	Rats, mice	Inh, oral, i.v., resp. instillation, s.c.	Single exposure and various periods during gestation	Data published so far indicate that MNs may pass the placenta. The question is to which degree transfer takes place, and how placental transfer depends on physicochemical properties of the nanoparticles. Maternal exposure to engineered nanoparticles may potentially affect foetal development directly as well as via indirect pathways. Toxicity might also occur due to toxic compounds associated with the particles them-selves. A true challenge is that nanoparticles might not need to cross the placenta or even enter the maternal bloodstream to affect foetal development. Severe limitations apply to the study designs and reporting of effects.	Development, reproductive toxicity, pregnancy, central nervous system, reproductive system, immune system, translocation, mechanism of action	Relative few studies using rats compared to mice. Limitations of study design and reporting of data.
Ema et al. 2016 (118 references)	Graphite SWCNT fullerenes MWCNT CB	Few *in vitro* data	Mice, rats, chicken, Zebra fish Focus on *in vivo* studies	i.v.; i.p.; oral, inh, resp. instillation. dispersed in water	Single exposure and various periods during gestation	Overall, the available data provide initial information on the potential reproductive and developmental toxicity of carbon-based MNs. However, confirmatory studies using well-characterized MNs, state-of-the-art study protocol and appropriate route of exposure, are required to clarify the findings and provide information suitable for risk assessment.	Embryolethal, teratogenic, testicular toxicity, brain morphology, abortion Mechanisms (ROS, oxidative stress), placenta. Physicochemical properties Study design	Systematic study descriptions of 30 studies using rats/mice. Details concerning substance ID and characterization given as far as possible. Limitations of study design and characterisation of the MN.

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Hougaard et al. (2015) (193 refs)	TiO2 CB CdO MWCNT Gold Silver SiO2 ZnO Fullerenes SWCNT CdTe/CdS QD	*In vitro*, (mainly on placental transfer)	Rat (few studies), mice (most studies)	Inh, instillation, i.v., s.c., i.p., oral	Single exposure and various periods during gestation	Although the available database on NP describes several organ systems in the offspring to be potentially sensitive to maternal inhalation of particles, large uncertainties exist about the implications of such exposures for embryofoetal development as well as for possible long-term health effects later in life. The emerging picture suggests that embryofoetal exposure to NP after exposure via relevant routes (inhalation and oral) may be limited. However, exposure of the conceptus has been shown to occur in experimental studies, and translocation from the maternal lungs to the foetus has yet to be studied. Overall, experimental studies indicate that adverse health effects of such exposures cannot be excluded, but at present the potential hazard has not been characterized. A testing strategy on developmental and reproductive toxicity is needed, however, several gaps remain to be filled before a testing strategy can be established.	Developmental toxicity, reproductive toxicity, instillation, inhalation, pregnancy, foetal effects, translocation, placenta, immune system, nervous system, germline tissue, testing strategy,	37 studies described in tables with details
Larson et al. (2014) 103 refs	Gold TiO2 Cobalt-Chromium Silver ZnO CB QD	*In vitro* tests	Mice, rats	Oral, resp instillation, i.p.	Single exposure and various periods during gestation Up to 90 days repeated exposure	Studies have shown that MNs may mediate adverse endocrine-disrupting effects on several endpoints of mammalian reproductive physiology (e.g., steroidogenesis, spermatogenesis, pregnancy). However, a series of gaps in knowledge and research needs are identified.	Steroidgenesis, endocrine disruptors, reproduction	Focus on endocrine disruption. Very few details regarding the specific tests. Often lack of correlation between *in vitro* and *in vivo* effects of MNs.

Reference (no of refs)	MNs	*In vitro*	*In vivo*, Species	Route of exposure	Exposure Duration	Key findings from abstract or conclusion	Key words (extracted from abstract/ conclusion/ headings)	Comments
Iavicoli et al (2013 (179 refs)	Silver Gold Aluminium Al2O3 Fullerene CB CdS QD CdSe QD CdTe QD CeO2 CNTs MWCNT Iron oxide CrCl3 MnO MoO3 SiO2 ZnS	In vitro Semen, ovarian cells, oocytes follicles, MCF-7 cells	Mice, rats	Inh, s.c., oral, i.v., resp. instillation,	Single exposure and various periods during gestation Up to 90 days repeated exposure	Data currently available indicate that several types of NPs can adversely affect the endocrine system, and in particular the male and female reproductive system. In fact, the results of the studies presented in this review suggest that NPs are able to disrupt the endocrine system by exerting cytotoxic effects and damaging the constituent cells of endocrine organs. At the same time there is a serious lack of information on the potential nanoparticle hazard to human health, particularly on their possible toxic effects on the endocrine system. However, current data support the notion that different types of nanoparticles are capable of altering the normal and physiological activity of the endocrine system. However, a critical evaluation of these findings suggests the need to interpret these results with caution since information on potential endocrine interactions and the toxicity of nanoparticles is quite limited.	Nanoparticle, endocrine disruption, reproductive health/system, estrogenic, thyroid function, neuroendocrine system	A total of 48 in vitro studies and 61 in vivo studies are tabulated with details.

MNs tested

The reviews most often include test data on:

Silver, 8 reviews

Gold, 6 reviews

Carbon based nanomaterials (CB, SWCNTs, MWCNTSs, fullerenes, graphene), 6 reviews

Titanium dioxide, 5 reviews

Cd-containing quantum dots, 5 reviews

Silicium oxide, 4 reviews

Zinc, zinc oxide, 4 reviews

***In vitro/in vivo* data**

The majority of the reviews focused on data from *in vivo* studies, except the review by Iavocoli et al. (2013) that also discussed endocrine effects and focused on *in vitro* studies.

Most *in vivo* studies were conducted with mice. Significantly fewer studies were conducted in rats and only very few studies in rabbits.

Exposure routes

Most of the *in vivo* studies were performed using the oral route of exposure. However, some studies also used exposure by either intravenous, peritoneal or subcutaneous injections, by respiratory tract instillation (intrathracheal instillation) or by inhalation.

Exposure duration

Most of the *in vivo* studies were performed using single or multiple exposure during the gestation period, however, also data from repeated dose studies (up to 90 days studies) were reported.

Key findings from abstract or conclusion

Regarding the distribution in the body the reviews indicate that MNs may enter into both male and female reproductive organs and may pass the placenta barrier (Wang et al. 2018, Brohi et al. 2017. Hougaard et al. 2017). The question is to which extent transfer takes place, and how for example placental transfer depends on physicochemical properties of the nanoparticles. No papers have as yet been published where inhaled particles are identified in the foetus.

The most recent review by Wang et al (2018) summarizes the overall potential for adverse effects of MNs on reproduction and development. They concluded that exposure and accumulation of MNs may damage organs (testis, epididymis, ovary, and uterus) by destroying Sertoli cells, Leydig cells, and germ cells, causing reproductive organ dysfunction that can adversely affect sperm quality, quantity, morphology, and motility or reduce the number of mature oocytes and disrupt primary and secondary follicular development. In addition, MNs can disrupt the levels of secreted hormones, causing changes in sexual behaviour. Further, it is indicated (as by several other reviews) that the molecular mechanisms involved in MN toxicity to the reproductive system are not fully understood, but studies indicate that possible mechanisms include oxidative stress, apoptosis, inflammation, and genotoxicity. Further, Larson et al. (2014) and Iavicoli et al. (2013) found data showing that different types of

nanoparticles are capable of altering the normal and physiological activity of the endocrine system, hence nanoparticles were proposed to act as endocrine disruptors.

In general, it is expressed that testing of the toxicity of MNs with respect to reproductive and developmental toxicity is still in its preliminary stages, and the use of very different techniques and study designs makes it very difficult to draw conclusions and comparisons between the studies .A wide range of dose levels and administration routes have been used, which makes it difficult to distinguish between primary developmental effects and effects secondary to maternal toxicity and to assess whether the effects are applicable to a relevant human exposure route. Furthermore, the review concludes that for carbon particles, functional endpoints in the offspring are mainly assessed for carbon black, whereas gestational and foetal outcomes are mainly assessed for the other types of nanoparticles.

Hougaard et al (2017) found that much research in the field gives the impression of hypothesis generation.Most studies, for example, did not include maternal and traditional gestational measures (e.g., maternal weight gain, litter size, birth weights) even if these are easy to record. Furthermore, it is often difficult to extract the number of pregnant dams included in each exposure group and if more than one pup per litter was used for investigation of effects, increasing the potential for litter effects. Therefore, systematic studies complying to state-of-art guidelines are strongly needed as well as strategies for and advice about designing the studies.

Keywords

A series of relevant keywords (potential search terms) was obtained from the terminology used in the reviews:

nanoparticle, nanomaterial, nanoeffects, nanotoxic, physicochemical properties

reproduction, reproductive system, reproductive toxicity, reproductive health

development, developmental toxicity, foetal effects, embryolethal, teratogenic, abortion, developmental neurotoxicity

fertility, pregnancy, embryo, foetus

germ cell, sperm, testis, blood-testes, sperm cells, testicular toxicity, sperm toxicity, germline tissue

ovary, ovaries, uterus, oocytes, follicular development, transplacental transfer, placenta, placenta transfer

steroidogenesis, endocrine disruptors, endocrine disruption, estrogenic, thyroid function, neuroendocrine system, hormone levels

translocation, distribution, central nervous system, immune system, brain morphology, nervous system,

ROS, oxidative stress, mechanisms, mechanism of action,

study design, instillation, inhalation, testing strategy

Bibliometric analysis

In addition to the review/overview papers presented above, the result from a recent bibliometric analysis on reproductive and developmental toxicity of nanomaterials was reviewed.

Wang Z et al. (2018) made a bibliometric overview of the amount of publications available in the field of nanomaterials and reproductive and developmental toxicity. Wang Z et al. used the

search terms ("nanomaterial*" or "nanoparticle*" or "nano*" and "reproductive toxicity" or "developmental toxicity" or "embryo toxicity" or "fetal toxicity" or "birth defect*") and combined these in the following text strings: (("nanomaterial*" OR "nanoparticle*" OR "nano*") AND ("reproductive toxicity*" OR "developmental toxicity*" OR "embryotoxicity*" OR "embryo toxicity*" OR "embryo developmental toxicity*" OR "embryo-fetal toxicity*" OR "embryo-fetal developmental toxicity*" OR "fetal toxicity*" OR "birth defect*")).

Data were obtained from the Science Citation Index (SCI)-Expanded database of the Web of Science and the time span was set from 2006 to December 2016.

Based on this search strategy Wang Z et al. (2018) identified 266 journal articles related to nanomaterials and reproductive and developmental toxicity. Based on the illustrations in the Wang Z et al. (2018) publication the following distribution among the various test systems was found:

Zebrafish: 69 publications

Mouse: 55 publications

Rat: 35 publications

Xenopus laevis: 20 publications

Cells: 19 publications

Drosophilia melanogaster: 17 publications

Chicken: 15 publications

Caenorhabditis elegans: 11 publications

Considering the testing in mice, rats, chicken and cells (as most relevant to this project) these test systems covered a max of 124 publications (as some overlap may occur if publications included several test systems).

Further the publications covered the following nanomaterials:

Silver: 54 publications

TiO2: 45 publications

ZnO: 39 publications

CNTs: 33 publications

QDs: 25 publications

Gold: 21 publications

Graphene: 18 publications

Iron: 15 publications

SiO2: 15 publications

CuO: 12 publications

In other words, if there was no overlap between the references there would be approximately 280 references covering reproductive and developmental testing of these MNs in relation to both environment and human health.

"Only" 124 publications were extracted by Wang Z et al. (2018) covering testing of MNs concerning reproductive and developmental toxicity in relation to human health. On the one

hand, this would indicate a big overlap of the used references in the reviews (in total approximately 1200 references were given from the eight reviews in table 1). On the other hand, the search strategy used by Wang Z et al. (2018) may not have been able to find all references considered relevant in the reviews.

As a result, we decided that the search strategy for this project must be broader than the one used by Wang Z et al. (2018) in order to cover more than the 124 publications as found.

A.3 Search strategy

A.3.1 Relevant databases for the search

Relevant literature will be searched in different databases licensed by DHI via the globally used information service STN®[3]. The following three clusters/databases are evaluated relevant for original peer-reviewed scientific papers within the purpose of the project:

TOXCENTER (Toxicology Center) is a cluster of bibliographic databases that covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. The records in the file contain bibliographic data, abstracts, indexing terms, chemical names, and CAS Registry Numbers.

TOXCENTER is composed of the following file segments:

- ANEUPL (Aneuploidy File)
- BIOSIS® (BIOSIS Previews/RN®) is a bibliographic database covering worldwide research on all biological and biomedical topics. Records contain bibliographic data, indexing information, and abstracts for most references. (1969 to the present).
- CAplus℠ (Chemical Abstracts Plus) is the most current and comprehensive chemistry bibliographic database available from Chemical Abstracts Service (CAS). CAplus covers international journals, patents, patent families, technical disclosures, technical reports, books, conference proceedings, dissertations, electronic-only journals, and web preprints from all areas of chemistry, biochemistry, chemical engineering, and related sciences from 1907 to the present, as well as over 35,000 records for patents and journal articles dated before 1907.
- CIS Abstracts (Congressional Information Service Abstracts).
- CRISP (Toxicology Research Projects).
- DART (Development and Reproductive Toxicology File).
- EMIC (Environmental Mutagen Information Center File).
- EPIDEM (Epidemiology Information System,).
- ETIC (Environmental Teratology Information Center File).
- FEDRIP (Federal Research in Progress).
- HAPAB (Health Aspects of Pesticides Abstract Bulletin).
- HMTC (Hazardous Materials Technical Center File).
- IPA (International Pharmaceutical Abstracts) from Thomson Scientific is a bibliographic file containing international coverage of pharmacy and health-related literature in information, the practice of pharmacy, pharmaceutical education, and the legal aspects of pharmacy and drugs (1970 to the present).
- MEDLINE (MEDlars onLINE)[4] is a bibliographic database produced by the U.S. National Library of Medicine (NLM). The database covers worldwide biomedical literature, the

[3] http://www.stn-international.de/index.php?id=123

[4] Medline is part of PubMed – see http://wayback.archive-it.org/org-350/20180312141605/https://www.nlm.nih.gov/pubs/factsheets/dif_med_pub.html

citations of which appear in Index Medicus, Index to Dental Literature, the HealthSTAR database, and International Nursing Index. Over 99% of MEDLINE's citations are references to journal articles (1950 to the present).
- PESTAB (Pesticides Abstracts).
- PPBIB (Poisonous Plants Bibliography).
- RISKLINE (Swedish National Chemicals Inspecorate).
- TSCATS (Toxic Substances Control Act Test Submissions).

EMBASE (Excerpta Medica) is a comprehensive bibliographic database that covers the worldwide literature on biomedical and pharmaceutical fields. It is produced by Elsevier B.V., the world's largest publisher of scientific information.

Science Citation Index (SciSearch®) contains all records published in Science Citation Index Expanded™. Records from January 1991 to the present include abstracts, author keywords, and KeyWords Plus®. Authors, bibliographic information cited references, and KeyWords Plus are searchable
EMBASE (Excerpta Medica) is a comprehensive bibliographic database that covers the worldwide literature on biomedical and pharmaceutical fields. It is produced by Elsevier B.V., the world's largest publisher of scientific information.

Science Citation Index (SciSearch®) contains all records published in Science Citation Index ExpandedTM. Records from January 1991 to the present include abstracts, author keywords, and KeyWords Plus®. Authors, bibliographic information cited references, and KeyWords Plus are searchable.

A.3.2 Keywords for assessment and use in search strategy

The overall strategy for the literature search in work package 2 is to find all relevant data using search term and search strings that capture the following

A relevant nanomaterial (according to 2.1.1)

A relevant effect and/or exposure period (according to section 2.1.2)

A relevant test system/species (according to section 2.1.2)

Based on the keywords obtained from Table 2.1 and further consultation among the project group members the following search terms as indicated below were selected:

Nanomaterial relevant terms

To capture relevant nanomaterials the following search terms will be used:

Nanoparticle#[5]; Nanomaterial#; nanofib?[6]; nanotube#; nanowire; carbon nanotube#; CNT#; MWCNT#; SWCNT#; multiwall; singlewall; graphene; CB; carbon black; Printex90; Printex 90; fullerene#; silver; AgNP; ?NP; gold; nickel; cerium; zinc; silicium; silica; titanium; cadmium; copper; Au; Ni; Ce; Zn; Si; Ti; Cd; Cu*

[5] #: any letter e.g. plural -s
[6] ?: more letters (any letters)

Reproductive/developmental toxicity relevant terms

To capture relevant effects, target organs, exposure periods and mechanisms the following search terms will be used:

> Reproduct?; reprotoxic?; development?; maternal?; paternal?; birth, fetal?; foetal?; foetus; foetus; gestation?, pregnan?; prenatal?; postnatal?, perinatal?; neonatal?; miscarriage; abort?; resorp?; retard?; delayed; newborn#; pup#; birth defect#; abnormal?; congenital?; breast; lactat?; embryo?; terato?; placenta?; ovar?; oocyt?; follic?; uterus, uterine; menstruation; testic?; testis; testes; semen, sperm?; germline; fertil?; infertil?, endocrine?; estrog?; estrus; estrous; anti-estrogen?; antiestrogen?; oestrog?; oestrus; oestrous; anti-oestrogen?; antioestrogen? androgen?; anti-androgen?; antiandrogen?; thyroid?; hormon?; disrupt?; steroid; mechanis?; transfer; distribut?; penetrat?; transport?; translocat?

Test system relevant terms

To capture relevant target organisms/test systems the following search terms will be used:

> Human#; rat#; mouse; mice; rabbit#; chick? in vivo; rodent#, ex vivo

Search strategy

The aim of the literature search is to find available studies where specific MNs have been examined for reproductive and developmental toxicity relevant to humans. As observed when looking at the recent review literature, most relevant and important data were found from *in vivo* testing as the *in vitro* data at the current state in general were considered much more uncertain for interpretation and relevance to humans. Thus, the focus of our search will be to identify publications with *in vivo* testing of MNs. Further, it is the strategy to use a wide series of search terms as indicated above with an OR in-between them. This will enable us to capture a high number of potentially relevant references even if we concentrate the search to the title of the publications. In other words, if a title does not contain any of these search term, it is not likely that the publication will be relevant to the purpose of our project.

Thus, the first level of screening (level 1 screening) is an assessment of the relevance of the title of the publication and the search will be performed in the "title" field of the database, i.e. the search string should combine the "*nano material relevant terms*" with the "*Reproductive/ developmental toxicity relevant terms*" for example:

(*all nano relevant terms used with an OR between them, title search*)

AND

(*all reproductive/developmental toxicity relevant terms with an OR between them, title search*)

In order not to gain a lot of hits only relevant to ecotoxicity, or technical development or medical development of nanomaterials, the search is further combined with "*test system relevant terms*" in the search fields of "all fields" in the database, for example:

AND

(*all test system relevant search terms with an OR between them, all field search*)

A.3.3 Inclusion/exclusion criteria

The inclusion/exclusion criteria described below and the selection strategy of screening level 1-3 will be applied to the references retrieved from the literature search while key references from the reviews from Table A-1 will go directly to screening level 3. Relevant key references from the reviews are considered to be references that are presented/highlighted in tables in the reviews as the most relevant references.

A.3.3.1 Screening level 1: Assessment of titles

Level 1 for selection of relevant publications will focus on the title itself from the hits obtained from the search strategy above.

The inclusion and exclusion criteria for level 1 screening are:

Inclusion criteria

At this level the found literature is evaluated based on the title of the reference. It is evaluated whether the title indicates a content that fits into the scope of this project. This includes:

Indication that specific MNs are addressed

Indication of in vivo test systems targeting human health

Indication of data concerning specific reproductive or developmental endpoints (in the title use of relevant search terms in a relevant context)

If the title based on expert assessment is considered relevant or potentially relevant, the reference will be selected to move on to a second screening where the abstract is evaluated.

Exclusion criteria

If title refers to effects/testing in species typically used for ecotoxicological assessment (fish larvae, earth worms etc.)

If title refers to the use/development of nanomaterials for medical purposes

If title refers to analytical methods for determination of the nanomaterial

If title refers to development of new products or use area for the nanomaterial

If title refers to mechanistic data (e.g. ROS generation or similar) rather than data with specific reproductive or developmental endpoints

Besides these examples, it is evaluated whether the title indicates a content that is considered outside the scope of this project (expert evaluation).

Documentation

The list of references retrieved from the search will be provided in a separate appendix. All references selected for second screening will be highlighted in green.

A.3.3.2 Screening level 2: Assessment of abstracts

At this level the abstract from the references selected in level 1 will be further studied and evaluated, as the further information in the abstract will give information as to the relevance to this project.

Inclusion criteria

Specific MNs addressed

In vivo test systems considered especially relevant to this project, targeting human health

Data concerning specific reproductive or developmental endpoints (use of relevant search terms in a relevant context)

Abstracts only mentioning in vitro tests and mechanistic data only included if specific relevance to human health is indicated

Exclusion criteria

Data on irrelevant species (e.g. species for ecotoxicological assessment)

Data not addressing the scope of this project

Abstracts only mentioning in vitro tests and mechanistic data not included if specific relevance to human health is not indicated

In addition to the principles of these inclusion/exclusion criteria, a preliminary expert assessment will be made for the relevance of each study to this project as each abstract will be scored with relevant, possibly relevant or not relevant.

Documentation

The list of abstracts retrieved from the search will be provided in a separate appendix. All abstracts will be given a score as indicated above by highlighting the abstracts as relevant, possibly relevant or not relevant.

The references given relevant or possibly relevant will be collected in electronic versions and the refences will be gathered in the Endnote system.

This documentation is the output of work package 2.

A.3.3.3 Level 3 assessment: Analysis of the publications

At this level the original references will be downloaded for further examination.

At level 3 assessment, the references passing screening level 1 and 2 will be included as well as relevant key references from the reviews in Table 2.1.

Some of the references have been obtained by a score of only possibly relevant. As the selection was based only on abstracts, a pre-screening of the relevance of the study for this project will be performed according to the level 2 inclusion/exclusion criteria before further analysis of the reference. If the study is considered not relevant after pre-screening, it is excluded from the project at this point and marked with R0 (not relevant). The remaining studies will undergo the examination described below.

Based on the method described by Card & Magnuson (2010) an overall assessment of the nano characterisation and the test design and reporting are given as an N(nano)-score and a K-(Klimisch) score.

For nano-characterisation the following parameters will be looked for and scored with 1 point for each of the parameters (i.e. a max of N=11 points):

1. agglomeration and/or aggregation
2. chemical composition
3. crystal structure/crystallinity
4. particle size/size distribution
5. Purity
6. Shape
7. surface area
8. surface charge
9. surface chemistry (including composition and reactivity)
10. whether any characterisation was conducted in the relevant experimental media
11. Water solubility

The ten first parameters are parameters suggested by Card & Magnuson (2010). The parameter "water solubility" was further included as this parameter is considered a relevant parameter when assessing the toxicity and kinetics of the nanomaterial.

For the quality regarding study design and reliability of the testing a Klimisch score (1-4) was applied as a K-score:

K score 1: reliable without restrictions
K score 2: reliable with restrictions
K score 3: unreliable
K score 4: not assignable due to insufficient experimental details

Further, an R-score regarding information relevant for this project is given.

R++: information highly relevant (key reference for assessment)
R+: relevant information (supporting or indicative references)
R0: not relevant for further consideration of relevance

Screening, evaluation and extraction of data

Using the above approach, the references were screened and scored and the relevant findings from the references were extracted and reported in the following template:

Template

Legend: *Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Nanomaterial XX					
Reference	**Test material, nano-characterisation**	**Species/ strain. No /group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1 - 11)** **K-score (1 - 4)** **R-score (0, +, ++)** **Comments**
Lee et al. 2018	**Cc**: **Pu:** **Ps**: **Sh:** **Cr**: **Sa**: **Sc**: **Ch**: **Ag:** **Em**: **Ws:**		**Route/ adm:** **Duration/period:** **Exposure levels:**		**N:** **K:** **R:**
Key findings: Fertility					
Key findings: Development					
Key findings: Kinetics					
Wang et al. 2015	**Cc**: **Pu:** - **Ps**: **Sh:**		**Route/ adm:** **Duration/period:**		**N:** **K:** **R:**

Appendix B: Reporting and evaluation of data from literature

B.1 Template and methodology for the evaluation

B.2 Titanium dioxide

B.3 Silver

B.4 Zinc oxide

B.5 Silicon dioxide

B.6 Carbon nanotubes + graphene

B.7 Carbon black

B.8 Other nanomaterials (Aluminum, Cadmium, Cerium, Cobalt, Copper, Gold, Iron, Lead, Mangan oxide, Nickel, Platinum, Polystyrene and Selenium)

B.1 Template and methodology for evaluation of the found literature

As indicated in section 3 the following inclusion/exclusion criteria will be used when examining the found literature in full text:

Inclusion criteria

- relevant MNs addressed (see section 2.1.1)
- *in vivo* test systems targeting human health using relevant
- relevant exposure route for humans i.e. exposure by oral, dermal, or respiratory route.
- Data concerning specific reproductive or developmental endpoints (use of relevant search terms in a relevant context)

Exclusion criteria

- Data on irrelevant species (e.g. species for ecotoxicological assessment)
- *In vivo* using unrealistic human exposure routes (e.g. s.c., i.p., i.v. injections)
- Data not addressing the scope of this project
- Abstracts only mentioning *in vitro* tests
- In addition to the principles of these inclusion/exclusion criteria, a preliminary expert assessment will be made for the relevance of each study to this project.

Due to the large amount of references passing the criteria above it is not possible to make a detailed in-depth analysis of each reference. However, based on a screening of the full text references data will be extracted, reported and evaluated in the following template:

Template

Legend: *Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Nanomaterial XX					
Reference	**Test material, nano-characterisation**	**Species/ strain. No /group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1 - 11)** **K-score (1 - 4)** **R-score (0, +, ++)** **Comments**
Lee et al. 2018	**Cc:** **Pu:** **Ps:** **Sh:** **Cr:** **Sa:** **Sc:** **Ch:** **Ag:** **Em:** **Ws:**		**Route/ adm:** **Duration/period:** **Exposure levels:**		**N:** **K:** **R:**
Key findings: Fertility					
Key findings: Development					
Key findings: Kinetics					
Wang et al. 2015	**Cc:** **Pu:** - **Ps:** **Sh:**		**Route/ adm:** **Duration/period:**		**N:** **K:** **R:**

Based on the method described by Card & Magnuson (2010) an overall assessment of the nano characterisation and the test design and reporting will be given as an N(nano)-score and a K-(Klimisch) score.

For nano-characterisation the following parameters will be looked for and scored with 1 point for each of the parameters (i.e. a max of N=11 points):

1. agglomeration and/or aggregation
2. chemical composition
3. crystal structure/crystallinity
4. particle size/size distribution
5. Purity
6. Shape
7. surface area
8. surface charge
9. surface chemistry (including composition and reactivity)
10. whether any characterisation was conducted in the relevant experimental media
11. Water solubility

The ten first parameters are parameters suggested by Card & Magnuson (2010). The parameter "water solubility" was further included as this parameter is considered a relevant parameter when assessing the toxicity and kinetics of the nanomaterial.

For the quality regarding study design and reliability of the testing a Klimisch score (1-4) was applied as a K-score:

K score 1: reliable without restrictions
K score 2: reliable with restrictions
K score 3: unreliable
K score 4: not assignable due to insufficient experimental details

Further, an R-score regarding information relevant for this project is given.

R++: information highly relevant (key reference for assessment)
R+: relevant information (supporting or indicative references)
R: 0 not relevant for further consideration of relevance

B.2 Titanium dioxide (TiO2NP)

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Titanium dioxide (TiO2NP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance (see Appendix B1) N-score (1-11) K-score (1-4) R-score (R0, R+, R++)** **Comments**
Fertility, rats					
Hussein et al. 2019	**Cc**: TiO_2 **Pu:** - **Ps**: <50 nm **Sh:** spherical **Cr**: - **Sa**: > 60 m^2/g **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Sprague-Dawley male rats 10 males/group	**Route/ adm:** Oral, gavage **Duration/period:** Daily exposure for 30 days **Exposure levels:** 0, 300 mg/kg bw/day (+ exposure in combination with antioxidants (morin and rutin))	Examination of hormone levels Semen quality examinations Antioxidant status in testicular tissues Histopathological examination of testes	**N:** 4 **K:** 2/3 **R:** + Only one dose level with TiO_2 alone Crystallinity not indicated
Key findings:	TiO_2 NPs significantly reduced the levels of sex hormones: testosterone (from 4.4 ng/ml to 0,50				At oral dose level

Fertility	ng/ml), Follicle stimulating hormone (FSH; from 0.76 ng/ml to 0,19 ng/ml), and Luteinizing hormone (LH; from 1.9 ng/ml to 1.2 ng/ml), reduced sperm motility (from 92% to 47%), viability (from 88% to 43%), and sperm cell count (decrease of 28%). and increased sperm abnormalities (from 16% to 41%), in addition to damaging the testicular histological architecture. TiO_2 NPs furthermore resulted in the downregulation of 17β-HSD and the upregulation of proapoptotic gene (Bax) transcripts in the testicular tissues. It was concluded that TiO_2 NPs may cause oxidative stress that negatively impacts the production of sperm.				of 300 mg/kg bw/day significant adverse effects regarding sperm quality, histopathology, sex hormone levels and antioxidant status were observed
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Shahin & Mohammed 2017	**Cc**: TiO_2 **Pu:** 99.7% **Ps**: 25 nm **Sh:** **Cr**: anatase **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: -	Adult male Wistar rats 12 rats/group	**Route/ adm:** Oral/ gavage **Duration/period:** Daily for either 7 days, 14 days or 21 days **Exposure levels:** 0 and 50 mg/kg bw/day (+ groups co-exposed with the antioxidant morin)	Prostate and testes organ weights and histopathology Sperm examinations Biochemcial investigations of inflammation	**N: 4** **K: 2** **R: +/++** It may be noted that a dose-response relationship (i.e. exposure-duration relationship) was found for all the examined parameters.
Key findings: Fertility	The organ weight of the prostate and testes were significantly decreased in an exposure-duration manner. Prostate from 0.48 g to 0.29 g and testes from 2.54 g to 1.92 g after 3 weeks of exposure. TiO_2 exposure also disrupted the sex hormone profile in an exposure-duration manner, as manifested by signficantly decreased levels of serum testosteronel and increased serum levels of estradiol, LH and FSH.				Oral exposure at 50 mg/kg/day in rats from 1 to 3 weeks caused exposure-duration related effects in relation to: testis

	Testicular γ-GT and ACP activities (indicators of impaired spermatogenesis) were elevated in an exposure-duration manner. Enhanced lipid peroxidation in both prostatic and testicular tissues also occurred in a expopsure-duration manner. TiO_2 provoked prostatic and testicular inflammation, as manifested by significantly elevated TNF-α levels in prostatic and testicular tissues. Normal sperm counts decreased from 88% (control) to 68% after 3 weeks of exposure and to a lesser extent at the lower exposure durations.				and, prostate weight; sex hormone levels; biomarkers indicating impaired spermatogenesis; biomarkers for lipid peroxidation and inflammation in testicular tissues, and on sperm parameters**.**
Key findings: Development	-				
Key findings: Kinetics	-				
Sharafutdinova et al. 2018	**Cc**: TiO_2 **Pu:** - **Ps**: 40-60 nm **Sh:** **Cr**: rutile **Sa**: 40-60 m^2/g **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**:-	Male Wistar rats 10 male rats/group	**Route/ adm:** Oral gavage **Duration/period:** Single exposure **Exposure levels:** 0, 50 mg/kg bw	One group of animals was killed and examined 14 days post exposure and one group 30 days post exposure. Histopathological examination of testes.	**N: 4** **K: 2/3** **R: +**
Key findings: Fertility	**Testis** Substantial degenerative changes in the spermatogenic epithelium were revealed: thinning, disorganization of layers, and detachment of sperm cells from the basement membrane. Immunohistochemical analysis revealed reduced proliferative activity and differentiation potential of epithelial cells, which was confirmed by changes in the expression of Ki-67 and c-kit markers.				

Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Tassinari et al. 2014	**Cc**: TiO_2 **Pu:** 98% + detection of metallic impurities **Ps**: 20-60 nm **Sh:** spherules and irregular shape **Cr**: anatase **Sa**: 45-55 m^2/g **Sc**: - **Ch**: - **Ag:** large agglomerates < 1600 nm **Em**: - / **Ws**: -	Sprague-Dawley rats 7 rats/sex/group	**Route/ adm:** Oral gavage **Duration/period:** Daily for 5 days **Exposure levels:** 0, 1, 2 mg/kg bw/day	On day 6 organs and blood samples were collected for histopathological examination and determination of sex hormone levels. Assessment of tissue distribution of elemental Ti.	**N: 7** **K: 2** **R: +**
Key findings: Fertility	In males, testosterone levels were significantly increased at 2 mg/kg bw/day and triiodothyronine levels were dose-related, significantly decreased at both dose levels. In females, testosterone levels were significantly decreased at 2 mg/kg bw/day. In the ovary, significant increased incidences of apoptosis in granulosa cells in both dose groups. No histopathological changes found in testes at any dose level.				Repeated exposure caused effects on sex hormone levels in both male and female rats and histopathological changes in ovaries at very low exposure.
Key findings: Development	**-**				
Key findings: Kinetics	Elemental Ti-content significantly increased in ovarian tissue at all dose levels in a dose-related manner.				TiOs particles distributed to ovary tissue

Fertility, mice					
Song et al. 2017	**Cc**: TiO_2 **Pu:** 99.8% **Ps**: 5-10 nm **Sh:** indicated as nearly rhabditiform **Cr**: anatase **Sa**: 120 m^2/g **Sc**: - **Ch**: - 20.7 mV - +116 mV **Ag:** as agglomerates **Em**: 363 – 619 nm I test vehicle **Ws:-**	Male ICR mice 15 mice/group	**Route/ adm:** Oral gavage **Duration/period:** Daily for 28 days **Exposure levels:** 0, 10, 50, or 100 mg/kg body bw/ day	Sperm quality, morphological changes in testes, and oxidative damage indexes were investigated	**N:** 9 **K:** 2 **R: ++**
Key findings: Fertility	**Testis** Exposure did not affect the weight of the testicles and epididymis of male mice at any dose level. **Sperm quality** Sperm malformation and sperm cell micronucleus rate showed dose related and significant differences compared to controls at the two highest dose levels. Exposure caused reduction in germ cell number and led to spherospermia, interstitial glands, malalignment, and vacuolization in spermatogenic cells at the two highest dose levels. **Oxidative stress** Superoxide dismutase (SOD) activity significantly decreased at the highest dose level and the malondialdehyde significantly increased at the two highest dose levels both markers of cell damage in testis.				Exposure at 50 and 100 mg/kg bw/day associated with significant impairment of sperm quality and pathological damage of testicular tissue.
Key findings: Development	-				
Key findings: Kinetics	**-**				

Lauvås et al. 2019	**Cc**: TiO_2 (UV-titan L181) **Pu:** 70.8 wt%- **Ps**: average 20.6 nm **Sh:** elongated and needle-shaped particles **Cr**: rutile **Sa**: 107.7 m^2/g- **Sc**: coated with polyalcohols, modified with Zr, Si, Al **Ch**: - **Ag:** mainly as agglomerates/aggregates **Em**: - **Ws**: -	Male C57BL/6J mice 15-16 mice/ group	**Route/ adm:** intratracheal instillation **Duration/period:** Once weekly for seven weeks **Exposure levels:** 63 µg/animal/ week Total dose: 441 µg/animal (representing the estimated lung deposition at the Danish occupational exposure limit of 6 mg Ti/m3)	Bronchoalveolar lavage Testes Sperm counts Testosterone analysis Behaviour.	**N: 8** **K: 2** **R: ++**
Key findings: Fertility	BALF cell composition showed neutrophil granulocyte influx as indication of pulmonary inflammation in animals exposed to TiO2NP. No effects on weight of testes or the epididymis, sperm counts or plasma testosterone levels.				
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Karimipour et al. 2018	**Cc**: TiO_2 **Pu:** **Ps**: 10-25 nm **Sh:** **Cr**: 99% anatase **Sa**: - **Sc**: -	Female NMRI mice 10 female mice/group	**Route/ adm:** Oral gavage **Duration/period:** Daily for 5 weeks	The female rats were mated after 5 weeks of exposure and fertility parameters assessed. Histopathological examination of ovaries.	**N:** 3 **K: 2/3** **R: +** Low K and N score and only one dose level

	Ch: - **Ag:** - **Em**: **Ws**:		**Exposure levels:** 0, 100 mg/kg bw/day	Serum sex hormone levels	
Key findings: Fertility	**Ovaries** TiO_2 NP administration induced histological alterations in the ovary, including degeneration and reduction of ovarian follicles, ovarian cyst formation and disturbance of follicular development. **Fertility** Compared to controls, animals in the TiO2NP group showed significant reduction of pregnancy rates and numbers giving birth.TiO2NP caused significant reduction in oocyte number, fertilization rate, and pre-implantation embryo development ($p<0.001$). Furthermore, malondyaldehyde and estrogen hormone levels were significantly ($p<0.01$) increased in mice receiving TiO2NP.				Histopathological changes in ovaries and reduced fertility
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Development, rats					
Bowbridge et al. 2019	**Cc:** TiO_2 (Evonik Aeroxide) **Pu:** - **Ps**: 21 nm **Sh**: spherical like **Cr:** anatase/rutile 80/20 **Sa:** 48 m^2/g **Sc:** - **Ch:** -56.6 mV **Ag:** 188 nm **Em: -** **Ws: -**	Female Sprague Dawley rats 8 females/ group	**Route/ adm:** Inhalation **Duration/period:** Daily exposures on GD10-GD15 **Exposure levels:** 0, 12 mg/m^3, 6h/day corresponding to a cumulative dose of 525 µg/animal after six days of exposure	Uterine vascular function Placental efficiency Pup and placental weights Hormone levels	**N: 7** **K: 2** **R: +**

Key findings: Fertility	**-**				
Key findings: Development	Placental weights were increased in exposed (0.99 g) versus control rats (0.70 g), whereas pup weights (4.01 g vs 4.15 g) and placental efficiency (fetus weight/placental weight: 4.5 vs. 6.4) were decreased in exposed rats. Further, exposure augmented uterine artery vasoconstrictor responses. Estrogen level was decreased at GD 20 in exposed (11 pg/ml) versus control rats (67 pg/ml).				Gestational exposure caused increase in estrogen levels, increase in placenta weight and decrease in pup weight.
Key findings: Kinetics	**-**				
Lee et al. 2019	**Cc**: TiO_2 **Pu:** 100% **Ps**: 21 nm **Sh:** spherical **Cr**: anatase /rutile: 80/20 **Sa**: - **Sc**: hydroxyl groups on surface **Ch**: - 35 mV **Ag:** aggregates of 342 nm **Em**: - **Ws**: -	Female Sprague-Dawley rats 12 females/group + 4 females/group for tissue distribution	**Route/ adm:** Oral gavage **Duration/period:** Daily GD5-GD19 **Exposure levels:** 0, 100, 300 and 1000 mg/kg bw/day	According to OECD 414 and GLP Distribution of elemental Ti to maternal brain and liver and placenta.	**N:** 8 **K:** 1 **R:** ++
Key findings: Fertility	**-**				
Key findings: Development	In the maternal and embryo-fetal examinations, there were no marked toxicities in terms of general clinical signs, body weight, food consumption, organ weights, macroscopic findings, cesarean section parameters and fetal morphological examinations.				No effects observed on any fertility and developmental parameters

Key findings: Kinetics	In the distribution analysis, titanium contents were increased in the maternal liver, maternal brain and placenta after exposure to high doses of TiO_2 NP. Increased levels of elemental titanium in placenta (0.6 vs 0.2 mg/kg) at the highest dose level compared to control.				Distribution of titanium into placenta
Abukabda et al. 2019	**Cc**: TiO_2 (Evonik, Aeroxide) **Pu:** - **Ps**: 21 nm **Sh:** particles **Cr**: 80% anatase, 20% rutile **Sa**: 48 m^2/g - **Sc**: - **Ch**: - 56.6 mV **Ag:** agglomerates of 188 nm **Em**: - **Ws**: -	Female Sprague-Dawley rats 8-14/ group	**Route/ adm:** Inhalation **Duration/period:** Daily on GD11-GD16, 6 h/exposure **Exposure levels:** 0, 12 mg/m^3	On GD 20, placentas, umbilical artery and vein were isolated and subjected to ex vivo functional examination.	**N: 7** **K:1/2** **R: +**
Key findings: Fertility	-				
Key findings: Development	Increased placental vascular resistance and impaired umbilical vascular reactivity, which indicate that inhalation of TiO_2 during gestation impairs fetoplacental vascular reactivity.				Alterations in placenta functioning observed. The authors indicate that the implication of this needs to be further studied.
Key findings: Kinetics	**-**				
Engler-Chiurazzi et	**Cc**: TiO2 **Pu:** -	Female Sprague-Dawley rats	**Route/ adm:** Inhalation	Examination of pups at 5 month of age in	**N: 2** **K: 2**

al. 2016	**Ps**: - **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: 171 nm in exposure chamber **Solubility**:	4 females/group	**Duration/period:** GD 7-20 **Exposure levels:** 10.4 mg/m3 5hr/day 4 days/week or in average 7.8 exposures	a standard battery of locomotion, learning, and anxiety tests. 11 male F1 rats/ group.	**R: 0/+** Poor nano-characterisation
Key findings: Fertility	**-**				
Key findings: Development	No significant effects were noted on locomotor, balance, affective, anxiety-like, or depressive-like behavior in the male adult exposed as a fetus. However, exposed rats showed impaired performance in the visible platform test and in Working Memory Correct error test.				Slight behavioural changes in prenatally exposed male rats
Key findings: Kinetics	**-**				
Elbastawisy et al. 2014	**Cc**: TiO_2 **Pu:** 99,5% **Ps**: 21 nm **Sh:** **Cr**: - **Sa**: 35-65 m^2/g **Sc**: - **Ch**: - **Ag:** mostly as agglomerates 114-122 nm **Em**: - **Ws**: -	Female albino Wistar han rats 15 females/group	**Route/ adm:** Oral gavage **Duration/period:** Daily GD6-GD12 **Exposure levels:** 5 g/kg bw Unclear whether this is the total dose or per day	Lung Morphological and histopathological evaluation of the lungs from female rats and the lungs from the offspring X-ray analysis of TiO_2 content in lung tissues.	**N: 5** **K: 2/3** **R: +** Unclear dose level
Key findings: Fertility	**-**				

Key findings: Development	**Offspring lung** Neonatal lungs from treated mothers revealed deficient septation, thickened mesenchyme between the saccules, pneumocytic apoptosis, atypical lamellar inclusions, and macrophage infiltration. The thickness of the primary septa was significantly increased ($p = 0.001$). The pulmonary response manifested as inflammatory lesions and delayed saccular development in neonates.				Adverse effects in the lungs of neonates after oral nano-TiO_2 exposure of female rats during gestation
Key findings: Kinetics	EDX analysis demonstrated the presence of TiO_2 in maternal and neonatal lungs.				
Warheit et al. 2015	Three nanoforms and three non-nanoforms characterized with the following parameters **Cc**: TiO_2 **Pu:** - **Ps**: 42-47 nm (nano) 153-213 nm (non-nano) **Sh:** spherical - near spherical all qulaitities **Cr**: + (both anatase and rutile) **Sa**: 50-82 m2/g (nano) 7.1-17.1 m2/g (non-nano) **Sc**: Ti, O, C on surface (nano); Ti, O,C,Al, K, Si,,K, P on surface (non-	Female Crl:CD(SD) rats (3 studies) Female Wistar rats (3 studies) 19-24 pregnant rats/group	**Route/ adm:** Oral gavage **Duration/period:** Daily, GD 5 GD 19. **Exposure levels:** 0, 100, 300, or 1000 mg/kg bw/day In all six studies	Six studies according to OECD 414 using three nanoforms and three non-nanoforms of TiO_2	**N: 7** **K: 1** **R: ++**

	nano) **Ch**: - **Ag:** > 99% of mass and particle number as agglomerates for all six forms **Em**: - **Ws**: -				
Key findings: Fertility	**-**				
Key findings: Development	There was no evidence of maternal or developmental toxicity at any dose level tested in any of the six studies. Based on these results, the no-observed-adverse-effect level (NOAEL) for titanium dioxide was 1000 mg/kg/day, the highest administered dose, in both the Sprague Dawley (Crl:CD(SD) and Wistar rat strains.				No toxic and developmental adverse effects caused by either the nano-forms or the non-nano forms
Key findings: Kinetics	-				
Bideskan et al. 2017	**Cc**: TiO_2 **Pu:** 99% **Ps**: < 100 nm **Sh:** spherical-like **Cr**: anatase **Sa**:>150m^2/g **Sc**: - **Ch**: - **Ag:** - **Em**:- **Ws**: -	Wistar female rats 6 female rats/group	**Route/ adm:** Oral gavage **Duration/period:** Daily during GD2 – GD21 or daily post-natal day 2-21 **Exposure levels:** 100 mg/kg bw/day	Histopathological examination of brains from one-day old pups indirectly exposed during gestation and from 22 days old offspring indirectly exposed during the lactation period.	**N: 5** **K: 2/3** **R: +**
Key findings: Fertility	**-**				

Key findings: Development	Exposure to TiO_2-NPs during pregnancy or lactation increased apoptotic cell number significantly ($P < 0.01$) in the offspring hippocampus. The immunolabeling of double cortin (DCX) protein as a marker of neurogenesis indicated that TiO_2 NPs reduced neurogenesis in the hippocampus of the offspring ($P < 0.05$).				Adverse effect in CNS in pups from females exposed in the gestation or the lactation period.
Key findings: Kinetics	The effect observed it taken as evidence for that TiO2NP can easily cross the placenta and the blood brain barrier.				No data is given for the presence of TiO2NP in the brain.
Stapleton and co-workers 2013-2019: Stapleton et al. 2013, 2015, 2018 Hathaway et al. 2017 Fournier et al. 2019	**Cc**: TiO2 **Pu:** - **Ps**: 21 nm **Sh:** spherical **Cr**: 80/20 anatase/rutile **Sa**: 48 m^2/g- **Sc**: - **Ch**:-56.6 mV - **Ag:** partly agglomerated **Em**: hydrodynamic dyameter in exposure chamber 130 nm **Ws**: -	Female Sprague Dawley rats	**Route/ adm:** inhalation **Duration/period:** Single or repeated exposure at various time-points during gestation **Exposure levels:** In the range of 9.4- 11 mg/m^3 for various duration (up to 6 h/day) corresponding to a lung deposition of 12.3 - 45 µg TiO2/ animal/day	Specialised *in vivo* and *ex vivo* examination of cardiac function of the heart and transcriptomic analysis of heart tissue in offspring exposed prenatally.	**N: 8** **K: 2** **R: ++**
Key findings: Fertility	**-**				
Key findings: Development	**In offspring exposed prenatally**: Significant epigenetic and transcriptomic changes occurred in cardiac tissue. Reduced vascular reactivity in fetal aorta and reduction of maximal mitochondrial respiration in the aorta tissue. It was concluded that microvascular dysfunction occurred in prenatally exposed offspring that that persist throughout multiple developmental stages.				

Key findings: Kinetics	**-**				
Mohammadipour et al. 2013	**Cc**: TiO_2 **Pu:** >99% **Ps**: 10 nm **Sh:** spherical **Cr**: anatase **Sa**: >150 m^2/g, **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Wistar rats 6 pregnant rats/ group	**Route/ adm:** Oral gavage **Duration/period:** Daily on lactation day 2 – 21 **Exposure levels:** 0 and 100 mg/kg bw/day	6 pups/ group (one from each litter) subjected to neurobehavioural testing from postanal day 60.	**N: 6** **K: 2/3** **R: +** Only one pup per litter tested.
Key findings: Fertility	**-**				
Key findings: Development	The Morris water maze and passive avoidance tests showed that the exposure to TiO_2 nanoparticles could significantly impair the memory and learning in the offspring during the three first day of testing. No difference observed at day 4 and 5 of testing.				Indication of slight reversible effects observed in neurobehavioral testing in rat offspring exposed dose via lactation.
Key findings: Kinetics					
	Development, mice				
Hougaard et al. 2010	**Cc**: TiO_2 (UV-titan L181) **Pu:** 70.8 wt%- **Ps**: average 20.6 nm **Sh:** elongated and needle-shaped	Time mated C57BL/6BomTac mice 22-23 mice/group	**Route/ adm:** Inhalation **Duration/period:** 1 h daily/ GD 8-18	Maternal lung inflammation Gestational parameters Neurobehavioural examinations of offspring	**N: 8** **K:** 2 **R: +/++**

	particles **Cr**: rutile **Sa**: 107.7 m^2/g- **Sc**: coated with polyalcohols, modified with Zr, Si, Al **Ch**: - **Ag:** mainly as agglomerates/aggregates **Em**: - **Ws**: -		**Exposure levels:** Aerosolized powder (1.7x 10^6 n/cm^3; peak-size: 97 nm); ~40 mg/m^3	Time to litter for obtaining F2-generation. Elemental Ti in tissues: Lung, liver, milk from adults, liver from pups	
Key findings: Fertility	-				
Key findings: Development	**Pregnancy** There were no effects on gestational parameters. **Neurobehavioural effects** 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). Maternal toxicity: Ti deposition in pulmonary tissues and lung inflammation in adult females **Time to litter** Time-to-litter for obtaining the F2-generation was not significantly altered for F1 male offspring.				Slight neurobehavioral alterations in offspring
Key findings: Kinetics	No increased Ti-level was found in liver from the pups (detection limit 0.4 mg/kg) or in milk (detection limit 1 mg/kg).				Low if any distribution to pups and in milk
Boisen et al. 2012	See Hougaard et al. 2010 above	Time mated C57BL/6BomTac mice 22-23 mice/group (P-generation)	**Route/ adm:** Inhalation **Duration/period:** 1 h daily/ GD 8-18	Examination for ESTR* mutations in F2 female offspring (N= 192) from mating F1 females exposed in utero. *sensitive mutation end-	**N: 8** **K: 2** **R: +**

			Exposure levels: Aerosolized powder (1.7x 10^6 n/cm^3; peak-size: 97 nm); ~40 mg/m^3	point having high spontaneous mutation rates.	
Key findings: Fertility	**-**				
Key findings: Development	No evidence for increased ESTR mutation rates in F2 females				
Key findings: Kinetics	**-**				
Jackson et al. 2013	See Hougaard et al. 2010 above	Time mated C57BL/6BomTac mice 22-23 mice/group (P-generation)	**Route/ adm:** Inhalation **Duration/period:** 1 h daily/ GD 8-18 **Exposure levels:** Aerosolized powder (1.7x 10^6 n/cm^3; peak-size: 97 nm); ~40 mg/m^3	11-14 animals (F1 offspring) examined for DNA strand breaks in liver on either PND2 or PND 22.	**N: 8** **K: 2** **R: +**
Key findings: Fertility	**-**				
Key findings: Development	Prenatal exposure to UV-titan did not affect the levels of DNA strand breaks in the livers of newborn or weaned offspring.				
Key findings: Kinetics	**-**				
Kyjovska et al. 2013	See Hougaard et al. 2010 above	C57BL/6J female mice	**Route/ adm:** inhalation	Body and testicle weight Sperm quality	**N:** 8 **K:**2 **R:** +/++

		12-13 fremale mice/groups	**Duration/period:** 1 h daily/ GD 8-18 **Exposure levels:** aerosolized powder (1.7x 10^6 n/cm3; peak-size: 97 nm); ~40 mg/m^3	Sperm content per g testicular parenchyma, daily sperm production (DSP) were assessed.	
Key findings: Fertility	-				
Key findings: Development	Maternal particulate exposure did not affect daily sperm production statistically significantly in the F1 generation, although TiO_2 tended to reduce sperm counts. Overall, time-to-first F2 litter increased with decreasing sperm production.				
Key findings: Kinetics	**-**				
Philbrook et al. 2011	**Cc**: TiO_2 **Pu:** 99.5% **Ps**: 50 nm **Sh:** particles **Cr**: rutile **Sa**: - **Sc**: - **Ch**: - **Ag:** as agglomerates **Em**: particle size 472 nm in 0.5% tragacanth gum solution **Ws**:-	CD-1 mice 11-14 pregnant females /group	**Route/ adm:** Oral gavage **Duration/period:** Once on GD 9 **Exposure levels**: 0, 10, 100 or 1000 mg/kg bw	Gestational and developmental parameters Histopathology of placentas, fetal livers and fetal kidneys.	**N:** 7 **K:** 2 **R:** ++
Key findings: Fertility	**-**				
Key findings:	There was no increase in the number of skeletal defects in fetuses prenatally exposed to TiO_2. No				Slight decrease in

Development	histopathological changes in placentas, fetal livers and fetal kidneys. No significant difference between the litter size or maternal weight gain from GD 9 to GD 19; nor were fetal resorptions, mean fetal weights or lengths significantly different from the control group. At the two highest dose levels TiO_2 negatively affected progeny development evidenced by a significant increase in the number of fetuses with external morphological defects (5.5% in mid-dose and 2.5 in high dose compared to 0 % in controls) and at the highest dose level a significantly greater percentage of fetuses was non-viable (7.6% nonviable compared to 1.7% in controls).				viability and morphological defects at very high dose levels.
Key findings: Kinetics	**-**				
Zhang et al. 2018	**Cc**: TiO_2 **Pu:** 99,7% **Ps**: < 25 nm **Sh:** **Cr**: anatase **Sa**: 45-55 m^2/g **Sc**: - **Ch**: 9.12 mV **Ag:** - **Em**: 10 nm in 0.5% tragacanth solution **Ws**: -	Female Kunming mice 10 pregnant mice/group	**Route/ adm:** Oral gavage **Duration/period:** Daily GD1-GD13 **Exposure levels:** 0, 1, 10 mg/kg bw/day	Organ weights Fertility parameters Histopathology of placenta Placental ultrastructure Immunochemistry Gene expression	**N:** 7 **K:** 2 **R:** +
Key findings: Fertility	**Organ weights** No changes in absolute and relative organ weight of ovaries.				Effects on placenta development in relation to gestational exposure at 1 and 10 mg/kg bw/day
Key findings: Development	No changes in resorption and number of viable embryos. **Placenta** Significantly reduced relative placenta weight at 1 mg/kg bw/day. Immunohistochemical staining and histopathological examinations revealed dysregulation of vascularization, proliferation and apoptosis resulting in significant impairment of growth and development of placenta in mice.				
Key findings:	**-**				

Kinetics					
			Kinetics		
Geraets et al. 2014	Five different commercial qualities of TiO_2 tested (available from JRC). Four in nanoform: **Cc**: TiO_2 **Pu:** - **Ps**: 6- 20 nm **Sh:** - **Cr**: anatase and rutile **Sa**: 60 – 320 m^2/g **Sc**: - **Ch**: - **Ag:** 38-138 nm **Em**: 108 – 367 nm in test suspensions **Ws**: -	Male and female Wistar rats A total of 36 males and 21 females were used for the study.	**Route/ adm:** Oral gavage and I.V. **Duration/period:** Single exposure and daily exposure for 5 days **Exposure levels:** Oral: cumulative doses in the range of 6.8 – 59.9 mg/kg bw I.v.: cumulative doses of 8.4- 71.9 mg/kg bw	Tissue distribution and blood kinetics. Levels measured as elemental Ti. 90 days recovery groups included.	**N: 6** **K: 1/2** **R: ++**
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	**Oral** Very limited bioavailability after oral exposure, though there was evidence that absorption is possible in the gastrointestinal tract as increased levels of titanium could be detected in some livers and mesenteric lymph nodes from exposed animals compared to control animals. **I.v.** After i.v. exposure no large differences in distribution between male and female animals were observed. The major difference between male and female animals was that in male animals Ti was not detectable in the testes 30 days after administration, whereas at day 90 Ti was still detectable				Very low distribution of various qualities of nano TiO2 after i.v. administration. No distribution found after oral exposure.

	in the female ovaries. 24 hours after the last i.v. dose less than 0.01% was found to be distributed to testes/ovaries.				
Wang et al. 2007	**Cc**: TiO_2 **Pu:** 92% **Ps**: 25 nm and 80 nm and 155 nm **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**:	CD-1(ICT) mice 10 male and 10 female mice/group	**Route/ adm:** Oral gavage **Duration/period:** Single dose **Exposure levels:** 5 g/kg bw	Body and organ weights. Histopathological examination. Organ tissue content of elemental Ti. .	**N: 3** **K: 2** **R: +** Only one dose level
Key findings: Fertility	No abnormal pathology changes in testicles/ovaries				
Key findings: Development	-				
Key findings: Kinetics	TiO_2 was mainly retained in the liver, spleen, kidneys, and lung tissues				
Other references considered less relevant and not evaluated further					
Ema, M., et al. (2016). "Developmental toxicity of engineered nanomaterials in rodents." Is a review. Relevant studies from this review already included in the table above. **Khoradmehr et al. (2015).** "Apoptotic cells and loss of follicle development were resulted after administration of Nano dioxide titanium on immature mouse ovary" only as abstract- not further evaluated. **Morgan, A. M., et al. (2017).** "Reproductive toxicity provoked by titanium dioxide nanoparticles and the ameliorative role of Tiron in adult male rats." No data/ characterisation of test item – not evaluated further.					

Scsukova, S., et al. (2015). "Effects of selected metal oxide nanoparticles on ovarian steroidogenesis: Use of whole ovary culture technique." only as abstract- not further evaluated.

Abu Zeid, E. H., et al. (2017). "Impact of titanium dioxide on androgen receptors, seminal vesicles and thyroid hormones of male rats: possible protective trial with aged garlic extract." – no indication that a nanoform of TiO_2 was used - not further evaluated

Stapleton, P., et al. (2015). "Intravital Microscopy of the Rat Uterus After Titanium Dioxide Nanomaterial Exposure." Conference abstract – *ex vivo* examanations of uterus – not further evaluated.

Orazizadeh, M., et al. (2014). "Effect of beta-carotene on titanium oxide nanoparticles-induced testicular toxicity in mice." No parameters/ characterisation given on the test material - not further evaluated

Patel, S., et al. (2018). "TiO2 nanoparticles induce omphalocele in chicken embryo by disrupting Wnt signaling pathway." Data on chicken embryo – not evaluated further

Rollerova E et al. (2015). "Titanium dioxide nanoparticles: some aspects of toxicity/focus on the development." Is a review. Relevant studies from this review already included in the table above.

Takahashi, Y., et al. (2010). "Prenatal exposure to titanium dioxide nanoparticles increases dopamine levels in the prefrontal cortex and neostriatum of mice."
Using subcutaneous administration – not further evaluated

Note: In addition to the above publications that were exluded for further examination the following pulblications from the research group of Fashui Hong and co-workers at Medical College, Soochow University, Suzhou, Republic of China were not included in the assesmnet as serious doubts have been raised about the quality and validity of the work from these reseraches and consequently articles from this group have been retracted from Journals e.g. from Particle and Fi bre Toxicology,
https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/s12989-015-0097-1 and
https://particleandfibretoxicology.biomedcentral.com/track/pdf/10.1186/s12989-015-0098-0.

Based on uncertainties regarding the validity of the data from this group the following pulblications were excluded for further assessment:

Hong, F., et al. (2018). "Nanosized titanium dioxide-induced premature ovarian failure is associated with abnormalities in serum parameters in female mice."

Hong, F., et al. (2016). "Exposure to TiO2 Nanoparticles Induces Immunological Dysfunction in Mouse Testitis."

Hong, F., et al. (2015). "Decreased spermatogenesis led to alterations of testis-specific gene expression in male mice following nano-TiO2 exposure."

Hong, F., et al. (2017). "Maternal exposure to nanosized titanium dioxide suppresses embryonic development in mice."

Zhao et al 2013. "Nanosized TiO2-Induced Reproductive System Dysfunction and Its Mechanism in Female Mice"

Zhao et al. 2015. "Mechanisms of nanosized titanium dioxide-induced testicular oxidative stress and apoptosis in male mice" - *retracted*

Gao et al 2012. "Ovarian dysfunction and gene-expressed characteristics of female mice caused by long-term exposure to titanium dioxide nanoparticles"

Gao et al 2013. "Titanium dioxide nanoparticle-induced testicular damage, spermatogenesis suppression, and gene expression alterations in male mice"

Evaluation and overview

Data availability

From the literature search 45 publications on titanium dioxide (TiO_2) NPs were identified for further examination in full text. Of these 10 references were not further assessed in the table, as they were excluded based on the inclusion/exclusion criteria as indicated in appendix B.1. Also, seven references from one specific group of researchers were excluded as serious doubts about the scientific validity of their work have been raised (further described in Appendix B2).
Of the remaining 28 publications, the most relevant and informative data could be extracted from the following publications (scored as R++ or R++/+):

Nano titanium dioxide data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Shahin & Mohammed 2017 (N:4, K:2. R+/++)	Warheit et al. 2015 (N:9, K:1, R++) Lee et al. 2019 (N:8, K:1, R++)	Geraets et al. 2014 (N:6, K1/2,R++) Lee et al. 2019 (N:8, K:1, R++)
Rats, inhalation	-	Nurkiewicz and Stapleton (2013-2019) (N:8, K:2, R++)	-
Mice, oral	Song et al. 2017 (N:9, K:2, R++)	Philbrook et al. 2011 (N:8, K2, R++)	-
Mice, inhalation/ resp. tract	Lauvås et al. 2019 (N8, K:2, R:++)	Hougaard et al. 2010 (N:8, K2, R+/++) Kyjovska et al. 2013 (N:8, K2, R+/++)	Hougaard et al. 2010 (N:8, K2, R+/++)

Nano-characterisation

Of the publications included in the table above, the N-scores for nano-characterisation of the test item were in the range of 2-9 with an average score of 6.2. Four references did not provide information on the crystal structure. Nine references included testing of the rutile crystal structure and nine references included testing of the anatase crystal structure. Eight references tested a mixture of anatase and rutile TiO_2NPs.

No information on water solubility was given in the publications. In the REACH registration of titanium dioxide (CAS 13463-67-7) covering both the anatase and the rutile forms, it is indicated that nanosized titanium oxide does not dissolve to any relevant extent under regular environmental conditions and test data indicates a water solubility < 6 µg/L (ECHA, January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/15560).

Kinetics

Geraets et al. 2014 tested five different commercial qualities of TiO_2NP, covering both the anatase and the rutile form (particle sizes in the range of 6-20 nm), and found that after i.v. exposure to adult rats only very low levels of elemental titanium could be detected in the organs of rats. No such distribution could be detected after oral exposure to 8.4 - 59.9 mg/ kg bw.

In an OECD 414 test where pregnant female rats were gavaged with 0, 100, 300 and 1000 mg/kg bw/day of TiO_2NP (anatase/rutile: 80/20) with a particle size of 21 nm, increased levels of elemental titanium were found at the highest dose level in placenta (0.6 mg Ti/kg at the highest dose level vs 0.2 mg Ti/kg in control) (Lee et al. 2019).

Hougaard et al. (2010) did not find distribution of elemental Titatnium (Ti) above the detection limit (0.2-5 mg Ti/kg) to milk from lactating mice or to liver from young pups following 1hr daily inhalation of TiO_2NP at 40 mg/m^3 during gestation day (GD) 8-18 of pregnancy.

Fertility

Shahin and Mohammed (2017) exposed adult male Wistar rats by daily oral gavage to 50 mg/kg bw/day of TiO_2NP (anatase form with a particle size of 25 nm) for either 7 days, 14 days, or 21 days. The exposure, in a duration-related manner, caused significant adverse responses in relation to testis and prostate weight; sex hormone levels; biomarkers indicating impaired spermatogenesis; biomarkers for lipid peroxidation and inflammation in testicular tissues; and on sperm parameters**.**

Song et al. (2017) examined testes and sperm quality in male mice after exposure to 0, 10, 50, or 100 mg/kg body bw/day TiO_2NP (anatase form with a particle size of 5-10 nm) by oral gavage for 28 days. Exposure did not affect the weight of the testicles and epididymis at any dose level. Sperm malformation and sperm cell micronucleus rate showed dose related and significant differences at the two highest dose levels. Exposure caused reduction in germ cell number and led to spherospermia, interstitial glands, malalignment, and vacuolization in spermatogenic cells at the two highest dose levels. Superoxide dismutase (SOD) activity significantly decreased at the highest dose level and the malondialdehyde significantly increased at the two highest dose levels, both of which are markers indicating cell damage in testis.

After intratracheal instillation of TiO_2NP (rutile, 20.6 nm) once weekly during seven weeks to adult male mice at a dose level of 63 µg/animal/dosing, no effects on weight of testis or epididymis, daily sperm production or plasma testosterone levels were found by Lauvås et al. (2019).

Developmental toxicity

Warheit et al. (2015) conducted a study following the OECD Test Guideline (TG) 414 using three non-nanoforms and three nanoforms of TiO_2 (both anatase and rutile forms with a particle size of 42-47 nm of the nanoforms). In all studies female rats were exposed by oral gavage to 0, 100, 300, or 1000 mg/kg bw/day of the test substance. No maternal toxicity nor developmental adverse effects were noted in any of the studies.

A similar lack of findings was noted by Lee et al. 2019, that conducted an OECD 414 TG study in which female rats were exposed by oral gavage to 0, 100, 300 and 1000 mg/kg bw/day of TiO_2NP (anatase /rutile form: 80/20, particle size 21 nm).
Bowdridge et al. (2019) and Abukabda et al. (2019) exposed female rats to 12 mg/m3 (6h/day) of TiO_2NP (anatase/rutile: 80/20 with of particle size of 21 nm) by inhalation during gestation. Exposure resulted in increased placental weights and an impaired vascular reactivity in placenta considered as a sign of placenta dysfunction.

Stapleton and co-workers (2013-2019) exposed pregnant rats to TiO_2NP (anatase/rutile (80/20) with a particle size of 21 nm) by inhalation at approx. 10 mg/m^3 for up to 6 hr/day for different periods during gestation and found cardiovascular effects in offspring such as epigenetic and transcriptomic changes in cardiac tissue, reduced vascular reactivity in aorta, and reduction of maximal mitochondrial respiration in aorta tissue.

In Philbrook et al. 2011, female mice were exposed by a single oral gavage to 0, 100 or 1000 mg/kg bw of TiO_2NPs (rutile form, particle size of 50 nm) on day 9 of gestation. At the two highest dose levels TiO_2NPs negatively affected normal progeny development as assessed by a statistically significant increase in the number of fetuses with external morphological defects (5.5% at mid-dose and 2.5% at high dose compared to 0% in control) and at the highest dose level by a significantly greater percentage of non-viable fetuses (7.6% nonviable compared to 1.7% in controls). There was no significant difference between litter sizes, fetal resorptions, or mean fetal weight or length compared to the control group. Also, there was no increase in the number of skeletal defects in fetuses and no histopathological changes in placentas, fetal livers and fetal kidneys.

Hougaard et al. (2010) exposed female mice by inhalation to approx. 40 mg/m^3 of TiO2NP (particle number concentration 1.7x 10^6 n/cm^3, rutile form, particle size 20.6 nm), 1h/day during GD 8-18. Slight neurobehavioral alterations were observed in the offspring. In the same offspring, Kyjovska et al. (2013) found that the maternal particulate exposure did not affect daily sperm production in the F1 male offspring, although TiO_2 tended to reduce sperm counts/g testicular tissue.

Overall evaluation

The current data indicate that oral exposure to high dose levels of TiO_2NP (e.g. 1000 mg/kg bw/day to pregnant rats) may lead to a small systemic uptake and distribution (measured as elemental Ti) into maternal organs including the placenta, however, at very low

levels. Also, after inhalation during the gestation period in mice no increased Ti levels was found in milk or in livers from the pups. Sparse amounts of data are available regarding effects on reproduction/fertility.

Repeated oral dosing of male rats to 50 mg/kg bw/day of TiO_2NP resulted in decreased prostate and testis weight and further disrupted the hormone profile by signficantly decreased serum testosterone level and increased serum estradiol, Luteinezing hormone (LH) and Follicle stimulating hormone (FSH) levels. Normal sperm counts decreased from 88% (control) to 68% after 21 days of exposure. In male mice repeated oral exposure has lead to increased level of sperm malformation and historpathological changes in the germinal tissue at dose levels of 50 and 100 mg/kg bw/day.

In male mice intratraheal instialltion of TiO_2NP did not cause any effects on tested, epididymis, sperm count or plasma testosterone levels

Prenatal developmental testing according to OECD TG 414 has been performed using oral exposure of rats to both the anatase and the rutile crystalline form of TiO_2NP without any adverse reproductive/developmental outcome even at dose levels of 1000 mg/kg bw/day. In pregnant mice a single oral exposure of 0, 100 or 1000 mg/kg bw of TiO_2NP negatively affected normal progeny development at both dose levels (however, in an inverse dose related manner) and resulted in a significantly greater percentage of non-viable fetuses at the highest dose level.

In female rats inhalation of 12 mg/m^3 TiO_2NP in female rats during gestation was found to increase placental weights and impair the vascular reactivity in placenta. Also, adverse cardivasular effects in the offspring has been found after inhalation of approx. 10 mg/m^3 during gestation in rats.

Data gaps

Kinetics:
No data was found in the literature search examining uptake from inhalation of TiO_2NP and the following distribution into gonads, placenta or into organs of the fetus (other than the liver).

Fertility:
Some indicative findings especially on the male reproduction system suggest concern for effects on fertility, however, for example no one-generation guideline study on TiO_2NP is available neither in relation to oral nor inhalation exposure.

Development:
In relation to prenatal developmental toxicity it should be noted that TiO_2NP in various qualities have been covered by OECD TG 414 testing but only using oral exposure in rats. Therefore, data regarding inhalation and other species is lacking in order to make

confirmative conclusions on this endpoint. Thus, several studies where pregnant rats were exposed to TiO_2NP by inhalation indicate concern for cardiovascular effects in the offspring.

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B.3 Silver (AgNP)

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Silver (AgNP)					
Reference	**Test material, nano** **characterisation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score** **K-score** **R-score** **Comments**
Fertility data					
Baki et al. 2014 **(and Amraie et al., 2013)**	**Cc**: Ag **Pu:** - **Ps**: 70 nm **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Male Wistar rats 15 per group	**Route/ adm:** Oral gavage **Duration/period:** 45 days Every 12 h **Exposure levels:** 25, 50, 100, 200 mg/kg bw/day	Sperm parameters Serum hormone levels Examined after last gavage	**N:** 2 **K:** 2 **R:** +/++ Study published twice
Key findings: Fertility	**Sperm parameters** Decreased number of leydig cells, sperm progressive motility, and sperm with normal morphology and increased numbers of sperm with non-progressive motility and immotile sperm, at all dose levels. **Serum hormone levels** Decreased in serum testosterone at ≥ 50 mg/kg/day and incresed serum LH at ≥ 50 mg/kg/day				Effects on sperm motility at ≥ 25 mg/kg/day and serum hormone levels at ≥ 50 mg/kg/day

Key findings: Development	-				
Key findings: Kinetics	-				
Elsharkawy et al., 2019	**Cc**: Ag **Pu:** **Ps**: 8.93-33.4 nm **Sh:** spherical particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** **Em**: **Ws**: -	Male adult Sprague Dawley rats 30 males/group	**Route/ adm:** Oral gavage **Duration/period:** 6 months Administration twice/week **Exposure levels:** 0, 5.36, 13.4 mg/kg bw/exposure	5 rats from each group was examined after each month Sperm parameters Serum hormone levels	**N:**3 **K:**2 **R**:+/++ Low level of NP characterisation
Key findings: Fertility	**Sperm parameters** Significant decrease in sperm viability in rats in both exposure groups. A significant decrease in DNA chromatin integrity was obtained in rats exposed to 13.4 mg/kg in comparison with low dose and control. Histopathological changes were observed in both exposure groups. Vacuolations with a disturbance in the arrangement and the staining affinity of spermatogenic cells was seen at all doses. No effects on sperm morphology was detected. **Serum hormone levels** Significant decrease in testosterone level at month 6 after low dose exposure and after 4, 5, and 6 months at high dose exposure. A significant increase in LH level after high dose exposure was seen after 4, 5, and 6 months.				Effects on sperm viability and hormone levels
Key findings: Development	-				
Key findings: Kinetics	-				

Lafuente et al., 2016	**Cc**: Ag, Polyvinyl pyrrolidone (PVP) 0.2 wt% **Pu:** 99.95% **Ps**: 20-30 nm **Sh:** **Cr**: - **Sa**: - **Sc**: PVP coated **Ch**: –17.55 ± 4.16 mV **Ag:** - **Em**: - **Ws**: -	Male Sprague Dawley rats 6 males/group	**Route/ adm:** Oral gavage **Duration/period:** 90 days, daily **Exposure levels:** 0, 50, 100, 200 mg/kg bw/day	Sperm motility, viability and morphology were examined. Histological evaluations of testis and epididymis	**N: 5** **K: 2** **R: ++**
Key findings: Fertility	Increased abnormal sperm at 100 mg/kg bw/day, and an increase in neck and midpiece sperm abnormalities was found at 50 mg/kg bw/day. These effects were not seen at the other dose levels. No effects were found on sperm count and sperm motility and viability. No histopathological changes in testis or epididymis was detected, and no effects on bodyweight was observed.				Slight effects in sperm morphology after 50 and 100 mg/kg bw/day. No clear dose-response.
Key findings: Development	**-**				
Key findings: Kinetics	-				

Lee et al., 2013	**Cc**: Ag **Pu:** 99.98% **Ps**: 10 or 25 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Male Sprague Dawley rats 20 males/group	**Route/ adm:** Oral gavage **Duration/period:** 28 days **Exposure levels:** 0, 100, 500 mg/kg bw/day	Rats were examined at the end or 1, 2 or 4 months after end exposure Biochemical, hematological and histopathological examinations Weight of body and organs Determination of silver in tissues	**N: 4** **K: 2** **R: ++**
Key findings: Fertility	No histopathological changes in testes, and no changes in body or organs weights				
Key findings: Development	**-**				
Key findings: Kinetics	AGNPs exposure significantly increased Ag tissue levels in ovaries (at 500 mg/kg bw/day ca. 10 µg/g for 10 nm and ca. 9 µg/g for 25 nm AgNPs) and testes (ca. 800 ng/g at both dose levels). No decrease in Ag levels in testis and brain occured after cessation of AgNP exposure until 4 months after termination of exposure to 500 mg 10 nm AgNP/kg bw/day and 100 and 500 mg 25 nm AgNPs/kg bw/day				Low clearance of Ag in testes and brain after AgNP exposure
Mathias et al., 2014	**Cc**: Ag **Pu:** **Ps**: 86 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Weaned Wistar rats 10 males/group	**Route/ adm:** Oral gavage **Duration/period:** Postnatal day (PND) 23-58 **Exposure levels:** 0, 15, 30 µg/kg bw/day	Rats were examined on PND 102 for: Sperm parameters Sexual partner preference and sexual behaviour Serum concentrations of FSH, LH, testosterone and estradiol	**N: 3** **K: 2** **R: ++**

Key findings: Fertility	Increase in sperm abnormalities in both groups (~6% vs 15-20% at the low and high dose level, respectively) Reduced sperm integrity (~1% vs 5% non-intact acrosome and ~0.6% vs. 3-4% non-intact membrane at the low and high dose level, respectively) and mitochondrial activity No changes in growth and nor sexual behavior or serum hormone concentrations in any groups.				Increase in sperm abnormality after prepubertal exposure to very low doses (15 µg/kg bw/day)
Key findings: Development	Delayed onset of puberty of approximately two days in both groups.				
Key findings: Kinetics	-				
Miresmaeili, S. M., et al. 2013	**Cc**: Ag **Pu:** **Ps**: 70 nm **Sh:** spherical particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Wistar rats 8 males/group	**Route/ adm:** Oral gavage **Duration/period:** 48 days, every 12 h **Exposure levels:** 0, 25, 50, 100, 200 mg/kg bw/day	The rats were examined after the last gavage. Sperm parameters and histological examination of testes.	**N: 3** **K: 2** **R: +/++**
Key findings: Fertility	Decrease in spermatogonia cells at 200 mg/kg bw/day. Decrease in primary spermatocytes, spermatids and spermatozoa at 50 mg/kg bw/day and above				
Key findings: Development	**-**				
Key findings: Kinetics	-				

Sleiman et al., 2013	**Cc**: Ag **Pu:** **Ps**: 60 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em: -** **Ws: -**	Weaned Wistar rats 10 males/group	**Route/adm:** Oral gavage **Duration/period:** PND 23-53 **Exposure levels:** 15 or 50 µg/kg bw/day	The rats were examined on PND 53 or PND 90	**N: 3** **K: 2** **R: +/++**
Key findings: Fertility	Delayed preputial separation and reduced total sperm production and daily sperm production on PND 90 in both exposure groups. Reduced sperm reserve in epididymis and histopathological changes in testis on PND 53 and 90 in both groups was found. No changes in body weight				Impaired spermatogenesis and histopathological changes after low level prepubertal exposure
Key findings: Development	**-**				
Key findings: Kinetics	-				
Thakur et al. 2014	**Cc**: Ag **Pu:** - **Ps**: 5-20 nm **Sh:** spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	10-12 weeks old male Wistar rats 8 males/group	**Route/ adm:** Oral gavage **Duration/period:** 90 days **Exposure level:** 20 µg/kg bw/day	Testis tissues was processed for histology and transmission electron microscopic study.	**N: 3** **K: 2-3** **R: +**

	Synthesized, non-commercial AgNPs.				
Key findings: Fertility	Atrophy of seminiferous tubules, disorganization of germinal epithelium, loss, degeneration, and necrosis of spermatogenic cells; ultrastructural changes in spermatogonia and Leydig and Sertoli cells; TEM revealed accumulation of NPs near basement membrane. No effect on body weight or behavior.				Effects on spermatogenesis at low level of exposure to synthesized AgNPs
Key findings: Development	-				
Key findings: Kinetics	-				
Developmental toxicity					
Hong et al. 2014	**Cc**: Ag **Pu:** - **Ps**: 8.8 nm **Sh:** spherical **Cr**: - **Sa**: - **Sc**: citrate capped **Ch**: –17.55 ± 4.16 mV **Ag:** - **Em**: - **Ws**: -	Male and female SD rats 5/sex/group	**Route/ adm:** Oral gavage **Duration/period:** Daily administration Males: from 14 days before mating (total of 42 days) Females: from 14 days before mating to Lactation Day 4 (up to 52 days) **Exposure levels:** 0, 62.5, 125, 250 mg/kg bw/day	Test performed according to OECD 422 and GLP	**N: 5** **K: 1** **R: ++** Test performed according to OECD 422 and GLP Deviation: reduced number of males and females /group
Key findings: Fertility	No effects in relation to mating, fertility and implantation were detected				No effects
Key findings: Development	No effects on delivery and fetal development				No effects
Key findings:	In dams, Ag accumulated in liver, kidney and lungs				

Kinetics					
Amiri et al. 2011	**Cc**: Ag **Pu:** **Ps**: 10 nm, 30 nm **Sh:** spherical particles **Cr**: - **Sa**: - **Sc**: -30.5 ± 8 and -4.92±3.1 **Ch**: - **Ag:** monodispersed **Em**: 10.0 ± 4.4 nm and 28.8 ± 4.8 nm **Ws**:- Synthesized, non-commercial	Pregnant NMRI mice 6/group	**Route/ adm:** Oral gavage **Duration/period:** From GD 0 until delivery **Exposure levels**: AgNPs 0.26 mg/kg bw/day (2 particle sizes) $AgNO_3$: 0.26 mg/kg bw/day	PND 1: Male pups were sacrificed on for mitochondrial function, gene expression, and histopathological Study (3-6/grp). PND 60: Behavioural test (7-8/grp): (forced swimming test, splash test, open-field test, passive avoidance, and hole-board tests) and molecular assessments (same as above)	**N: 6** **K: 2** **R: ++** The study compares the effects of particulate and ionic silver
Key findings: Fertility	**-**				
Key findings: Development	Mitochondrial dysfunction and upregulation of the genes relevant to innate immune system in the brain on PND1 On PND 60 prenatal exposure to Ag-NPs provoked severe cognitive and behavioral abnormalities in male offspring.				Cognitive and behavioral abnormalities in male offspring, mitochondrial dysfunction and upregulation of genes relevant to innate immune system in the brain
Key findings: Kinetics	High concentration of silver present in the brain of pups at PND 1 (control/$AgNO_3$/10 nm AgNP/ 30 nm AgNP: 99.8/ 513.5/ 2213.5/ 1427 ng/g brain), respectively.				

Philbrook et al. 2011	**Cc**: Ag **Pu:** 99.8% **Ps**: 35.3 ± 5.8 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** as agglomerates **Em**: particle size 220 nm in 0.5% tragacanth gum solution **Ws**:-	CD-1 mice 11-14 pregnant females /group	**Route/ adm:** Oral gavage **Duration/period:** Once on GD 9 **Exposure levels**: 0, 10, 100 or 1000 mg/kg bw	Gestational and developmental parameters Histopathology of placentas, fetal livers and fetal Kidneys	**N:** 6 **K:** 2 **R:** + Pnly single exposure
Key findings: Fertility	**-**				
Key findings: Development	Increase in fetal mortality at 10 mg (9.6%), but not at 100 (5.48%) or 1000 mg (6.12%) compared to controls (3.3%) No effect on dams, fetal growth, or morphological development. No maternal toxicity.				Slight effects on fetal viability at 10 mg, but no dose-response
Key findings: Kinetics	TEM analysis showed AgNPs in fetal liver and kidneys.				

Yu et al. 2014	**Cc**: Ag **Pu:** - **Ps**: 6.45 ± 2.55 nm **Sh:** particle **Cr**: **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws:** -	Female SD rats (8-11 pregnant rats/ group)	**Route/ adm:** Oral, gavage **Duration/period:** Daily GD6-19 **Exposure levels:** 0, 100, 300, and 1000 mg/kg bw/day	Cesarean section was performed on GD 20 and the fetuses were examined for signs of embryotoxic and teratogenic effects	**N:3** **K:2** **R: ++**
Key findings: Fertility	**Fertility:** Pre-implantation loss at 1000 mg (25.5 ± 28.29 % vs. 2.4 ± 3.3 % in controls), however with large variation Increase in maternal brain weight at all dose levels. Decrease in maternal liver catalase and GR at all doses and glutathione at 1000 mg.				
Key findings: Development	No effect on dams, or fetal survival, growth, or morphological development.				No developmental effects were observed
Key findings: Kinetics	-				
Charehsaz et al., 2016	**Cc**: Ag **Pu:** - **Ps**: 55 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: -45 mV **Ag:** -	Pregnant female SD rats 10/group	**Route/ adm:** Oral gavage **Duration/period:** Daily, GD7-20 **Exposure levels:** 0, 0.2, 2, 20 mg/kg bw/day	Animals were sacrificed on PND 2. Ag level assessed in maternal and pup organs (2-3 pups per litter). Hepatotoxicity and oxidative stress parameters and	**N:4** **K:2** **R:++** The study compares the effects of particulate and ionic silver

	Em: - **Ws**: - Synthesized, non commercial		20 mg Ag/kg/day of AgNO3	histopathology were evaluated	
Key findings: Fertility	Ag in ionic form induced oxidative stress (significantly reduced SOD activity) liver in dams and increase in SOD was seen in the brain				
Key findings: Development	No difference in SOD levels were seen in pups. No histopathological changes were observed in brain, liver, heart, kidney and lung tissue of pups. No effect on gestational parameters including pregnancy length, maternal weight gain, implantations, birth weight and litter size at any dose level of AgNPs. Maternal weight gain was lower in dams receiving AgNO3 compared to the other groups. Mild to moderate neuronal cell loss and gliosis event in hippocampus of dams exposed to Ag in nanoparticulate or ionic form (however, without dose-response relationship).				Oxidative stress in brains of pups. Ionic form of Ag more potent compared to NPs
Key findings: Kinetics	In dams, AG concentration in tissue increased with increased dosage of AgNPs. Higher Ag levels were found in spleen, kidney, uterus, and erythrocytes. The concentration was generally higher in tissue after treatment with $AgNO_3$ compared to AgNPs. Ag found in offspring indicating transport across the placenta. Significantly higher Ag levels were found in kidney at all dose levels. $AgNO_3$ significantly elevated Ag levels in lung. Offspring tissues levels of Ag were generally similar or lower if their dams had been exposed to AgNO3 rather than the Ag-NPs. Only for plasma did AgNO3 offspring present with statistically significantly higher concentration than in the corresponding Ag-NP group.				Gestational exposure to Ag in both ionic and nanoparticle forms increased the levels of Ag in kidney, lung, and liver of the offspring. Indicate placental transfer.

Fatemi et al., 2017	**Cc**: Ag **Pu:** - **Ps**: 20 ± 4 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** agglomerates (state not given) **Em**: - **Ws**: -	Female Wistar rats 30/group	**Route/ adm:** Intra-gastric gavage **Duration/period:** Daily, GD1-19 **Exposure levels:** 0, 25 mg/kg bw/day	16 pups per group were sacrificed after birth and their livers collected. Oxidative stress	**N:4** **K:2** **R:+/++**
Key findings: Fertility	-				
Key findings: Development	Glutathione peroxidase (GPX) activity and glutathione (GSH) level were significantly decreased and malondialdehyde (MDA) and caspase 9 levels were significantly increased in the liver of offspring, but there was no significant change in caspase 8 content. In the liver, fatty degeneration and congested dilated sinusoids were observed in the histopathological examinations.				Prenatal exposure to AgNPs induce oxidative stress in liver of offspring
Key findings: Kinetics	-				
Fatemi et al., 2013	**Cc**: Ag **Pu:** - **Ps**: 20 ± 4 nm **Sh:** colloidal **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** agglomerates (state not given) **Em**: - **Ws**: -	Pregnant female Wistar rats 45/group	**Route/ adm:** Oral gavage **Duration/period:** Daily, from GD9 to end of gestation **Exposure levels:** 0, 25 mg/kg bw/day	Pups were sacrificed after weaning and their brains were collected. Silver accumulation, the amounts of malondialdehyde and glutathione, glutathione peroxidase activity, and the amounts of caspase 8 and 9 in the brains of offspring were determined	**N:4** **K:2** **R:+/++**
Key findings: Fertility	-				

Key findings: Development	AgNP exposure decreased offspring body weight, relative brain weights, and GPX activity and GSH levels in brain. Exposure furthermore increased microvascular structure, levels of MDA and Caspase 9 in brains of treated offsprings. No effect of exposure was observed on maternal body weight gain or gestation length.				Maternal exposure to silver nanoparticles induced oxidative stress and apoptosis in brains of their offspring
Key findings: Kinetics	Increase in Ag content in brain of treated offspring compared to controls				
Kovvuru et al., 2015	**Cc**: Ag **Pu:** - **Ps**: 5-150 nm (33.6 ± 22.9) **Sh:** spherical **Cr**: **Sa**: - **Sc**: PVP coated **Ch**: -21.1 mV **Ag:** both monomers and agglomerates **Em**: - **Ws**: -	Myh -/- mice with C57BL/6J background Number of animals/group not indicated	**Route/ adm:** Oral gavage **Duration/period:** Daily, GD9.5-13.5 **Exposure levels:** 500 mg/kg bw/day	Offspring were sacrificed at PND 20 and eyes examined for DNA deletions	**N:6** **K:2-3** **R:+** Group number not given, only one dose level
Key findings: Fertility					
Key findings: Development	Increased frequency of DNA deletions in Myh−/− and wildtype mice eyes of offspring				
Key findings: Kinetics					
Campagnolo et. al. 2017	**Cc**: Ag **Pu:** - **Ps**: 18 nm, unimodal	4-5 pregnant C57BL/6 mice/group	**Route/ adm:** Inhalation (nose only)	At GD14.5: Distribution of particles and Ag to maternal organs,	**N:** 6 **K: 2** **R: ++**

	Sh: spherical **Cr**: - **Sa**: 3.94 **Sc**: - **Ch**: - **Ag:** No agglomeration **Em**: Particle number concentration in air: $3.8x10^7$part/m^3; surface concentration: $3.9x10^{10}$ nm^2/m^3 **Ws**: Soluble in water Custom-generated by spark-generator		**Duration/period:** Daily for the first 15 days of gestation (GD0.5-14.5) **Exposure levels:** 642 µg/m^3 for 1 or 4 hours/day	placenta and fetus (by TEM/EDX and single particle ICP-MS) Gestation and litter effects Histology Expression of inflammatory genes in maternal lungs and the placenta Serum oestrogen	
Key findings: Fertility	-				
Key findings: Development	No changes in maternal weight gain, but expression of inflammatory mediators (e.g. IL-6, IL-1b, TNF-a and MCP-1) were significantly up-regulated in maternal lungs, somewhat highest in the 4 h/d exposure group. No histopathological changes in placentas, but expression of TNF-a and IL-1b were significantly increased. There were no changes in litter size, fetal weight and length. Statistically significant increase in fetal resorptions in the 4h/day exposure group, where also maternal serum oestradiol was significantly decreased.				Exposure increased fetal resorption for 4h exposure, and elevated gene expression of inflammatory mediators
Key findings: Kinetics	Approximately 15% of the inhaled AgNP were estimated to deposit in the maternal alveoles and 35% in the upper airways; 4 hours after the last exposure mass concentration in lungs was estimated deposited dose was 24.3 mg Ag/kg lung weight.				AgNPs and Ag were detected in placenta and fetus, mainly as

	AgNP was detected in maternal liver and spleen and in the placenta, in the order of µg/kg tissue. In the placenta, total mass concentration of AgNPs was 0.005 ± 0.001 mg/kg, and total amount of silver 0.082 ± 0.006 mg/kg. A low number of particles was present in fetuses, including the the head region, but the amount was below the detection limit of 13 nm. Total silver in fetuses was 0.012 ± 0.003 mg/kg, part of which probably included AgNPs smaller than 13 nm. No elevation of Ag was observed in the control group.				Ag
Kinetics data					
Kim et al. 2008	**Cc**: Ag **Pu:** 99.98% **Ps**: 52.7-70.9 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: -	Male and female Sprague Dawley rats, 10/group	**Route/ adm:** Oral, gavage **Duration/period:** 28 days **Exposure levels:** 0, 30, 300 and 1000 mg/kg bw/day	According to OECD 407 and GLP After 28 days of exposure, the blood biochemistry and hematology were investigated, along with a histopathological examination and silver distribution study.	**N:**4 **K:1** **R:++**
Key findings: Fertility	Some effects on liver markers indicating slight liver damage. No effects in blood chemistry and hematology parameters or genotoxicity effects on bone marrow.				
Key findings: Development	**-**				
Key findings: Kinetics	A statistically significant dose-dependent increase in the silver concentration in all examined tissues, including the testes, was found in all dose groups. Highest levels were found in stomach, kidney, liver, and lung followed by testes, brain and blood, respectively. Further, the kidneys showed a sex-dependent accumulation of silver, with a twofold higher accumulation in the female compared with the male rats across all the dose groups.				Dose-dependent increase in the silver concentration in testes

Kim et al. 2010	**Cc**: Ag **Pu:** 99.98% **Ps**: 56 ± 1.46 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: -	Male and female Fisher rats, 10/group	**Route/ adm:** Oral, gavage **Duration/period:** 13 weeks (90 days) **Exposure levels:** 0, 30, 125 and 500 mg/kg bw/day	According to OECD 408 and GLP After 90 days of exposure, clinical chemistry, hematology, histopathology, and silver distribution were studied.	**N:**4 **K:1** **R:++**
Key findings: Fertility	There was a significant decrease ($P < 0.05$) in the body weight of male rats after 4 weeks of exposure. Significant dose-dependent changes were found in alkaline phosphatase and cholesterol for the male and female rats, indicating that exposure to more than 125 mg/kg of silver nanoparticles may result in slight liver damage. Histopathologic examination revealed a higher incidence of bile-duct hyperplasia, with or without necrosis, fibrosis, and/or pigmentation, in treated animals.				
Key findings: Development	**-**				
Key findings: Kinetics	There was a statistically significant ($P < 0.01$) dose dependent increase in the silver concentration of all the tissue samples from the groups exposed to silver nanoparticles. Higher Ag levels in testis in males than in brain and blood at all dose levels and higher levels in testis compared to liver, kidney and lungs at 30 and 125 mg/kg bw/day.				High accumulation of Ag in testis after exposure compared to other tissues.

Loeschner et al. 2011	**Cc**: Ag **Pu:** 99.98% **Ps**: 14 ± 4 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: -2mV **Ch**: - **Ag:** - **Em**: **Ws**: - Non-commercial, synthesized with PVP	Female Wistar Hannover Galas rats with specific pathogen-free health status 7-9/group	**Route/ adm:** Oral **Duration/period:** 28 days **Exposure levels:** 10 ml/kg bw of 11.5 mg/ml AgNPs or AgAc Vehicle (PVP solution) The daily dose of silver in the AgNP and AgAc group was 12.6 and 9.0 mg/kg bw respectively	28- day study of tissue distribution and elimination of AgNPs and AgAc. Tissue examined: Liver, kidney, lung, muscle, brain, plasma, intestine	**N:4** **K:2-3** **R:+** No reproductive organs investigated to silver content
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	Organ distribution of silver was similar for AgNPs or AgAc. However, the absolute silver concentrations in tissues were lower following exposure to AgNPs. This was in agreement with an indication of a higher fecal excretion following administration of AgNPs. Besides the intestinal system, the largest silver concentrations were detected in the liver and kidneys. Silver was also found in the lungs and brain. Autometallographic (AMG) staining revealed a similar cellular localization of silver in ileum, liver, and kidney tissue in rats exposed to AgNPs or AgAc				

Melnik et al. 2012	**Cc**: AgNPs in PVP **Pu:** **Ps**: 34.9 ± 14.8 (8.4-80.9) nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: labelled with ^{110m}Ag radioactive isotope **Ch**: - **Ag:** - **Em**: **Ws**: -	Pregnant (3-4/ group) and lactating Wistar rats (n=5, 9 infants)	**Route/ adm:** Intra-gastrically, gavage **Duration/period:** GD 20 or 14-16th day of lactation **Exposure levels:** 1.69 (n=3) or 2.2 mg/kg bw/day (n=4) in pregnant rats; 2.11 mg/kg bw/day in lactating rats	Pregnant rats euthanized 24 h after exposure. Infant rats nursed by exposed dams were killed 48 h after exposure	**N:4** **K:2** **R:++**
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	Transfer of NPs across the placenta The average level of accumulated NPs in the fetus was 0.085-0.147% of the administered dose. In lactating females, the total accumulation of [^{110m}Ag]-labeled NPs into the milk exceeded 1.94 ± 0.29% of the administered dose over a 48-hour period of lactation; at least 25% of this amount was absorbed into the gastrointestinal tract of infant rats.				Transfer of NPs across the placenta, however low level recovered in fetus (1.94 ± 0.29%)

Park et al. 2010	**Cc**: Ag **Pu:** **Ps**: 22, 42, 71, 323 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: -	Male and female ICR mice 14 day study: 5/group 28 day study: 6/group	**Route/ adm:** Oral administration (not specified) **Duration/period:** Daily for 14 days or 28 days (42 nm only) **Exposure levels:** 14 d: 1 mg/kg bw/day 28 d: 0.25 mg/kg, 0.5 mg/kg and 1mg/kg	Ag concentration in tissue after 14 days exposure to 4 sizes of AgNPs Repeated dose toxicity after 28 days exposure to 42 nm AgNPs. Reproductive tissue not examined	**N:3** **K:2** **R:+** Four different particle sizes
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	14 d: o Ag was detected in any tissue after administration of large (323) nm Ag particles. Ag was detected in testes following exposure to the 22 and 42 nm AgNPs, but not the 71 nm AgNPs. For the latter size, Ag was detected in brain, lung, liver and kidney. The highest levels of Ag were detected for the smaller AgNPs.				
Lee et al. 2012	**Cc**: Ag **Pu:** - **Ps**: 7.9 nm **Sh:** particle **Cr**: **Sa**: 7.53 x 10^2 nm^2/particle **Sc**: Citrate coated **Ch**: - **Ag:** - **Em**: - **Ws**: -	Male and female Sprague Dawley rats (>4/ group)	**Route/ adm:** Oral, gavage **Duration/period:** From 14 days before mating to PND 4, daily **Exposure levels:** 250 mg/kg bw/day	Study based on OECD 422, but not followed overall. Offspring tissue were examined on PND 4	**N:5** **K:2-3** **R:+** Low level of information on study
Key findings:	-				

Fertility					
Key findings: Development	**-**				
Key findings: Kinetics	Roughly the same levels of Ag in lungs (42.0 µg/g), liver (37.3 µg/g), and brain (31.1 µg/g), with the highest levels of Ag in kidneys (132.4 µg/g) in offspring of exposed mothers. AgNPs in liver and brain of offspring were observed (analysed by TEM).				Transfer of Ag to fetus during pregnancy
Vidmar et al. 2018	**Cc**: AgPEG and AgCOONa NPs **Pu:** - **Ps**: 27.6 ± 2.1 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: coated with polyethylene glycol or sodium carboxylate **Ch**: - **Ag:** - **Em**: - **Ws**: Soluble in water Custom-synthesized to mimic commercial NPs	Human placenta Ex vivo (n=3/group)	**Route/ adm:** N/A **Duration/period:** 6h perfusion **Exposure levels in maternal media:** Ag mass concentrations: AgPEG: 12.48 µg/mL AgCOONa: 39.26 µg/mL, Particle mass concentrations including coating: AgPEG: 40 µg/mL and AgCOONa: 75 µg/mL	Translocation and accumulation of AgNPs in the human ex vivo placenta perfusion model	**N:5** **K:2** **R:++**
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	AgNPs and ionic Ag were detected in the fetal circulation in low but not negligible amounts. Slightly higher Ag translocation across the placental barrier and accumulation in placental tissue was observed for perfusion with AgPEG NPs compared to perfusion with AgCOONa NPs. Perfusion with $AgNO_3$ revealed the formation of Ag-containing NPs in both circulations over time, of				AgNPs and ionic Ag were detected in the fetal circulation in low

	which the amount and their size in the fetal circulation were comparable to those from the perfusion experiments with both synthetized AgNP types.	but not negligible amounts

Other references

Baki et al. (2012). ” The effect of silver nanoparticles (Ag-Nps) concentration on the number of leydig cells and sex hormones in wistar rats”. Only abstract from conference available.

Barcikowski et al. (2015). "Influence of gold, silver and gold-silver alloy nanoparticles on germ cell function and embryo development.". Review.

Conceicao et al.(2015). "Silver nanoparticles exposure in rats disrupts hypothalamus-pituitarythyroid axis”. Only abstract from conference available.

Han et al. (2016). “In vitro studies and investigation of specific mechanistic effects.” The study was therefore not considered relevant based on the selection criteria.

Talebi et al. (2014). "The detrimental effects of silver nanoparticles on sperm chromatin structure and DNA integrity in mice." Only abstract from conference available.

Wang et al. (2016). "Silver nanoparticles (AgNPs) induced changes of reproductive parameters and gene expression was involved in apoptosis in the murine male testis." Only abstract from conference available.

Buchtova et al. (2014). The paper "Embryonic Toxicity of Nanoparticles" is a review

Ema et al. (2017). “A review of reproductive and developmental toxicity of silver nanoparticles in laboratory animal”s. Review, includes papers listed in table above.

Ema et al. (2016). “Developmental toxicity of engineered nanomaterials in rodents”. Review, includes papers listed in table above.

Evaluation and overview

Data availability
Based on screening of the abstracts 34 publications were identified and were further examined in full text.

Of these 34 publications three publications (Ema et al. 2017; Buchtova et al., 2014; Barcikowski et al., 2015) were review articles, while five studies (Baki et al., 2012; Conceicao et al., 2015; Han et al., 2016; Talebi et al., 2014; Wang et al., 2016) were not considered relevant based on the screening criteria or limited data available (abstracts only).

Of the remaining 26 publications the most relevant and informative data could be extracted from 13 publications which were given the score R++. These covered one oral OECD 422 study in rats, four oral studies in male rats examining semen quality and testicular toxicity and three studies and one oral study in rats and mice, respectively, examining developmental toxicity. One study investigated developmental toxicity in mice, following maternal inhalation exposure. Further four studies were found that specifically addressed the kinetics of AgNPs; three were performed in rats and one in the *ex vivo* placenta perfusion model.

Nano silver data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Elsharkawy et al., 2019 (N:2, K:2, R:++) Hong et al. 2014 (N:3, K:1, R:++) Lafuente et al., 2016 (N:5, K:2, R:++) Lee et al., 2013 (N:4, K:2, R:++) Mathias et al., 2014 (N:3, K:2, R:++) Sleiman et al. 2013 (N:3, K:2, R:+/++)	Yu et al. 2014 (N:3, K:2, R:++) Charehsaz et al., 2016 (N:4, K:2, R:++) Hong et al. 2014 (N:3, K:1, R:++)	Kim et al. 2010 (N:4, K:1, R:++) Kim et al. 2008 (N:4, K:1, R:++) Melnik et al. 2012 (N:4, K:2, R:++) Charehsaz et al., 2016 (N:4, K:2, R:++) Lee et al., 2013 (N:4, K:2, R:++)
Mice, oral	-	Amiri et al. 2011 (N:6, K:2, R:++)	

Mice, inhalation/ resp. tract	-	Campagnolo et al. 2017 (N:6, K:2, R:++)	Campagnolo et al. 2017 (N:6, K:2, R:++)
Other			Vidmar et al. 2018 (N:4, K:2, R:++)

Nano-characterisation

Of the 13 publications considered most relevant for the present project (included in the table above), the N-scores for nano-characterisation of the test item were in the range of 2-6 with an average score of 4. One study had an N-score of 2 only. Solubility was only addressed in one of the assessed articles (kinetics study) where the water solubility of the polyethylene glycol or sodium carboxylate coated AgNP is described as soluble in water (Vidmar et al. 2019). No REACH registration for silver nanoparticles was found); however, Campagnolo et al. (2017) observes that particles diminish in size from the original size of 20 nm, indicative of dissolution. In the REACH registration for silver (Ag, CAS 7440-22-4), the water solubility is given as insoluble (< 0.1 mg/L) (ECHA, January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16155).

Kinetics

A dose-dependent increase in tissue Ag levels was observed in rats after exposure to AgNPs (56 ± 1.46 nm) at 30, 125 and 500 mg/kg bw/day for 28 and 90-days, respectively (Kim et al. (2010) and (2008)). Higher levels of Ag were observed in testis compared to liver, kidney and lungs at 30 and 125 mg/kg bw/day and in brain and blood at all dose levels after 90 days of exposure (Kim et al., 2010). Increased Ag levels in testis, ovaries and brain were observed in rats exposed to 10 and 25 nm AgNPs for 28 days, with very low clearance rate from testes and brain (Lee et al., 2013).

The transfer of AgNPs across the placenta and via milk during lactation was examined in rats exposed intra-gastrically on GD 20 or on lactating day 14-16. The rats were exposed to 1.69-2.2 mg/kg bw AgNPs of 34.9 ± 14.8 nm in diameter labelled with 110mAg radioactive isotope. Transfer of AgNPs across the placenta was found, however the average level of AgNPs accumulated in the fetus of was low (0.085-0.147% of the administered dose). In lactating females, the total accumulation of labeled NPs into the milk exceeded 1.94 ± 0.29% of the administered dose over a 48h period (Melnik et al., 2012). Charehsaz et al. (2016) exposed pregnant rats on GD7-20 to 20 nm Ag particles at 0, 0.2, 2, 20 mg/kg bw/day, or 20 mg of Ag/kg/day of AgNO3. Ag was found in offspring, indicative of transport across the placenta. Significantly higher Ag levels were found in offspring kidneys at all dose levels. Following inhalation exposure, AgNPs were detected in the placenta, with a total mass concentration of AgNPs of 0.005 ± 0.001 mg/kg. Total silver amounted to 0.082 ± 0.006 mg/kg. A low number of particles was present in fetuses, including the head region. Total silver in foetuses was 0.012 ± 0.003 mg/kg,

part of which probably included AgNPs smaller than 13 nm (Campagnolo et al. 2017). The transfer of AgNPs across the placenta was also investigated in the ex vivo human placenta model. Perfusions were performed with AGNPs synthesized to mimic commercial NPs. The AgNPs were coated with polyethylene glycol or sodium carboxylate. Ionic Ag was detected in the fetal circulation in low but not negligible amounts after 6 hours of perfusion (Vidmar et al 2018).

Fertility

Elsharkawy et al. (2019) exposed adult male rats to 0, 5.36 or 13.4 mg Ag/kg bw/day twice a week for 6 months as AgNPs (particle size 8.93-33.4 nm). Significant decrease in sperm viability as well as histopathological changes were observed at both exposure levels. Also, significant decrease in testosterone level and a significant increase in LH level were detected, however no effects on morphology was detected. Effects on spermatogenesis after prepubertal exposure to very low dose levels (15 µg/kg bw/day) was also found in Wistar rats exposed to AgNPs (86 nm) on PND 23-53/8 (Mathias et al., 2014; Sleiman et al., 2013). Lafuente et al. (2016) exposed male SD rats for a duration of 90 days to 0, 50, 100 and 200 mg/kg bw/day to PVP-coated AgNPs and found effects of sperm morphology at 50 and 100 mg/kg bw/day, but not at 200 mg/kg bw/day. Lafuente et al. (2016) did not find effects on sperm count and sperm motility and viability. In two other studies, no effects on testes weight and histopathological parameters were found in rats exposed to 20 and 25 nm AgNPs up to a dose level of 500 mg/kg bw/day 28 day (Lee et al., 2013). Also, Hong et al. (2014) in an OECD 422 study with oral gavage of male and female SD rats to 0, 62.5, 125, 250 mg/kg bw/day to AgNPs (8.8 nm) found no effects on reproductive parameters, following exposure for a total of 42 days. These studies did, however, not investigate sperm parameters or sperm morphology.

Developmental toxicity

In the majority of the studies examining developmental toxicity following oral exposure, no effects on fetal survival, growth and morphology were reported in the studies (Hong et al., 2014; Amiri et al., 2011; Yu et al 2014; Charehsaz et al., 2016).
In the OECD 422 study performed by Hong et al. (2014), no effects on development of the offspring nor on the on the exposed females were observed. Similarly, no effects were found in a prenatal developmental toxicity study in which rats were exposed GD6 to 19 to 0, 100, 300, and 1000 mg/kg bw/day of AgNPs with a particle size of 6.45 ± 2.55 nm (Yu et al., 2014).

In mice exposed prenatally to synthesized non-commercial AgNPs (10 nm, 30 nm) and ionic silver ($AgNO_3$) at a dose level of 0.26 mg/kg/day from GD 0 until delivery, cognitive and behavioural abnormalities, mitochondrial dysfunction and upregulation of the genes relevant to the innate immune system in the brain were detected accompanied by high concentration of silver present in the brain of male pups. The same effects were not seen in female offspring (Amiri et al. 2011).

Signs of increased oxidative stress in the brain of offspring were found by Fatemi et al. (2013) in rats prenatally exposed to AgNPs during gestation.

Campagnolo et al. (2017) exposed female mice by inhalation during the first two weeks of gestation and observed increased rate of resorptions and levels of inflammatory mediators in the placenta, and decreased oestradiol levels in maternal plasma.

Overall evaluation

In rats, exposure to AgNPs leads to measurable levels of Ag in testis, ovaries and other organs, and the clearance may be rather low in testis. Following exposure to AgNP during pregnancy, AgNPs may at low levels cross the placenta and lead to AgNP exposure of the fetuses. One study indicated that particles partly dissolved during the period of exposure (2 weeks), which may decrease particle size and increase translocation, either as dissolved Ag or as very small particles. Further evidence of penetration across the placenta is available from the human placenta *ex vivo* model. No studies of kinetics were found in mice. Also, no kinetic data was found in relation to inhalation exposure.

Toxicity in testes and germinal tissue and reduced sperm quality as well as changes in sex hormone levels have been found in male rats subjected to repeated exposure to AgNPs. However, no effects on fertility were observed in a combined repeated dose/reproductive toxicity study (OECD TG 422) with oral exposure of rats to AgNPs up to a dose level of 1000 mg/kg bw/day including male and female rats. Also, in this study no developmental effects were noted. This is in alliance with other developmental studies, were most indicate no effects on fetal survival, growth and morphology. There are some findings that indicate that maternal exposure to AgNPs may affect brain development and function and oxidative stress in the outcome.

Data gaps

Kinetics:
No data on uptake from inhalation of AgNPs and the following distribution into gonads, placenta or fetus was found from the literature search. Further, no data is available regarding reproductive and developmental toxicity from inhalation exposure to AgNP.
Fertility:
One OECD TG 422 study is available, where no effects were found, however there are available data regarding adverse effects on testes and spermatogenesis, which implies that more data is needed to clarify the effects.

Development:
No standard prenatal developmental toxicity testing (OECD TG 414) has been performed with AgNPs. Although one OECD TG 422 study was performed, fetal exposure and distribution to the fetal brain as well as indications of fetal neurotoxicity indicate a need for data that could be provided by an extended one-generation study with the inclusion of neurobehavioural and neuropathological endpoints.

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B.4 Zinc oxide (ZnONP)

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Zinc oxide (ZnONP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance (see Appendix B1)** **N-score (1-11)** **K-score (1-4)** **R-score (R0, R+, R++)** **Comments**
Fertility					
Wang et al. 2016	**Cc**: ZnO **Pu:** **Ps**: 40.9 nm (30-50 nm) **Sh:** spherical **Cr**: crystals, 0.26 lattice constant **Sa**: - **Sc**: - **Ch**: - **Ag:** **Em**: **Ws**: -	CD-ICR mice 12 males/group	**Route/ adm:** Oral, via diet **Duration/period:** 32 weeks of exposure from the age of 3 weeks to 35 weeks. **Exposure levels:** 0, 50 mg/kg diet, 500 mg/kg diet, 5000 mg/kg diet	**Examination:** Organ weights on liver, kidney, brain, spleen, heart, pancreas, and testis. Biodistribution of minerals (Zn, Fe, Cu and Mn) in above tissues and in addition also muscles and bones.	**N:4** **K:2** **R:+/0** Considered less relevant as dosing with the diet makes exposure to nanoparticles very uncertain.
Key findings: Fertility	Exposure had no effects on testis weight. Decrease ($P<0.05$) of Fe level in testis at exposure of 5000 mg/kg bw/ day.				

Key findings: Development	-				
Key findings: Kinetics	-				
Talebi et al. 2013	**Cc**: ZnO **Pu:** - **Ps**: only indicated as nanoparticles **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	NMRI mice 6-8 weeks old 8 male mice/group	**Route/ adm:** Oral/ gavage **Duration/period:** 35 days of exposure **Exposure levels:** 0, 5, 50, 300 mg/kg bw/day	Epididymal sperm parameters, testicular histopathology, morphometric analysis and spermatogenesis .	**N: 2** **K: 2** **R: +/++** Poor nano-characterisation of test item
Key findings: Fertility	Sperm number, motility and percentage of abnormality in sperm: significant ($p<0.01$) impairment at 50 and 300 mg/kg diet. **Histopathology:** epithelial vacuolization, sloughing of germ and detachment were significantly increased at 50 and 300 mg/kg diet. Exposure to 300 mg/kg ZnONP induced formation of multinucleated giant cells in the germinal epithelium. Exposure to 50 and 300 mg/kg ZnONP significantly decreased seminiferous tubule diameter, seminiferous epithelium height and induced maturation arrest.				Adverse effect on sperm cells and histopathological effects in testes.
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Tang et al. 2019	**Cc**: ZnO **Pu:** - **Ps**: 30 nm **Sh:** spherical particles **Cr**: - **Sa**: -	Male Kunming mice 10 male mice/group	**Route/ adm:** Oral gavage **Duration/ period:** Daily exposure during 30 days	Histopathological examination of testes. Sperm analysis. Serum testosterone levels. Determination of elemental Zn levels in testes.	**N: 3** **K: 2** **R: +/++**

	Sc: - **Ch**: - **Ag:** - **Em**: - **Ws**: -		**Exposure levels:** 0, 50, 150 and 450 mg/kg bw/day		
Key findings: Fertility	In the 150 mg/kg group, the seminiferous tubules were degenerated with larger distances, and there was significant vacuolization of the Sertoli cells. Increase in severity of these effects were noted at 450 mg/kg bw/day. With increased dosing to ZnO NPs expression of IRE1α, XBP1s, BIP, and CHOP ($P<0.05$) was upregulated. All are genes related to endoplasmic reticulum stress. Additionally, there was at highest dose level a significant down-regulation of the gene StAR ($P<0.05$), a key player in testosterone synthesis and in addition to this also a significant decrease in serum testosterone.				Oral exposure to ≥50 mg/kg bw/ day of ZnONPs damaged testicular tissue and spermatogenesis and decreased serum testosterone levels.
Key findings: Development	-				
Key findings: Kinetics	Non-conclusive findings regarding zinc content in testes and epididymis				
Radhi et al. 2019	**Cc**: ZnO **Pu:** 99.99% **Ps**: 50 nm **Sh:** cube shape **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Albino mice, 6 males/ group	**Route/ adm:** Oral, gavage **Duration/period:** 7 days (daily exposure) and 14 days (daily exposure) **Exposure levels:** 0, 100 and 200 mg/kg bw/day	Examination (microscopy, organ weights) of semen, testes, epididymis, seminal vesicle and prostate.	**N:4** **K:2** **R:++**
Key findings: Fertility	**Testes**: Decreased ($P<0.05$) organ weight in all exposed groups (not dose and duration related) **Epididymis:** Decreased ($P<0.05$) organ weight in all exposed groups (dose and duration related)				Adverse effects on testes, epididymis and sperm cells.

	Seminal vesicle and prostate: Iincreased (P<0.05) organ weight in all exposed groups (dose and duration related) **Semen**: Increased percentage (P<0.05) of abnormal sperm in all exposure groups.				
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Jo et al. 2013	**Cc**: ZnO **Pu:** - **Ps**: 35 nm **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws:** -	SD rats 12 male and 12 female rats/ group	**Route/ adm:** Oral gavage **Duration/period:** Daily exposure Males: 6 weeks starting from 2 weeks before mating. Females: from 2 weeks before mating to day 4 of lactation **Exposure levels:** 0, 500 mg/kg bw/day	Refers to OECD TG 421 but using smaller group sizes and only one dose level. Histopathology: Testis, epididymis, ovary, uterus Fertility parameters Distributiom	**N:2** **K:2** **R:+** Only one -rather high - dose level used. Data both on fertility and development
Key findings: Fertility	In ZnONP exposed group increased organ weight of the maternal uterus that also showed histopathological lesions. Three males died in the exposed group. The mating perfomance, pregnancy rate, implantation rate was unaffected .				
Key findings: Development	Offspring: Increased implantation loss rate (52.8% vs 5.1% in control), decreased no of pups born per litter (5.8 vs 13.1) and reduced number of live pups on PND4 (1.3 vs 13.1) were noted.				Foetal toxicity and reduced number of live pups observed at high maternal dose levels.
Key findings: Kinetics	Distribution: ZnO distributed to mammary tissue of dams and liver and kidney in pups				

Developmental toxicity					
Lee et al. 2016	**Cc**: ZnO **Pu:** - **Ps**: <35 nm **Sh:** particles **Cr**: - **Sa**: - **Sc**: coated with 3-aminopropyl triethoxysilane **Ch**: - **Ag:** slightly aggregated in media **Em**: particle size checked by microscopy in media **Ws**: -	Sprague–Dawley rats (20-24 pregnant rats/ group)	**Route/ adm:** Intravenous injection **Duration/period:** Daily GD6-GD20 **Exposure levels:** 0, 5, 10, and 20 mg/kg bw/day	According to OECD 414	**N: 6** **K: 1** **R: +** High quality study. Included in the table although exposure route is not relevant for human exposure. However, indicate that maternal and developmental toxicity may occur at high enough systemic exposure.
Key findings: Fertility	-				
Key findings: Development	No effects in corpora lutea, resorption, placental weight, morphological alterations including external, visceral and skeletal malformations. **Post-implantation loss:** increased at 20 mg/kg bw/day **Fetal body weight:** decreased at 20 mg/kg bw/day **Maternal toxicity:** Histopathological analysis of treated dams revealed multifocal mixed cell infiltration and thrombosis in lung, tubular dilation in kidneys, and extramedullary hemopoiesis in liver. Two dams died at 20 mg/kg bw/day. **Conclusion**: Maternal toxicity: LOAEL 5 mg/kg bw/day. Developmental toxicity NOAEL: 10 mg/kg bw/day				
Key findings: Kinetics	**-**				

Hong et al. 2014a+b	**Cc**: ZnO **Pu:** 100% **Ps**: 20 nm **Sh:** particle **Cr**: **Sa**: - **Sc**: capped with L-serine **Ch**: positive **Ag:** - **Em**: - **Ws: -**	Crl:CD(SD) female rats (21-24 pregnant rats/ group)	**Route/ adm:** Oral, gavage **Duration/period:** daily GD5-GD19 **Exposure levels:** 0, 100, 200, and 400 mg/kg bw/day	Study performed according to OECD 414 (design and examinations) and in compliance with GLP.	**N:**6 **K:**1 **R:** ++
Key findings: Fertility	-				
Key findings: Development	**Gestational parameters:** no differences observed between groups (corpora lutea; number of implantation sites; implantation rate (%); resorption; dead fetuses; litter size; fetal deaths and placental weights; and sex ratio) **Fetal weight:** reduced at 400 mg/kg bw(day **Fetal abnormalities**: Significant increases in the number of fetuses with visceral variations at 400 mg/kg bw/day. **Maternal toxicity:** reduced food consumption at the two highest dose levels and decreased liver weight and increased adrenal gland weight at 400 mg/kg bw/day				Adverse effects on development at maternal toxic dose level. No effects on reproduction
Key findings: Kinetics	Zn content in fetuses from exposed groups not different from control fetuses				
Bara et al. 2018	**Cc**: ZnO **Pu:** - **Ps**: only indicated as nano **Sh:** spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** -	Female Swiss albino mice 4 female mice/group	**Route/ adm:** Oral gavage **Duration/period:** On 2 alternate days during GD 15-19 **Exposure levels:** 0, 50, 100, 300 mg/kg bw/day	Histological examination of placenta and examination of testes in offspring at 60 days of age.	**N: 2** **K: 2/3** **R: +** Poor characterisation of test item and low number of mice in each group

	Em: **Ws**: **Further test items:** Bulk ZnO (no particle size indicated) and mesoporous SiO2NP		Bulk ZnO only at 100 mg/kg bw/day		
Key findings: Fertility	-				
Key findings: Development	Dose of 300 mg/kg bw/day highly toxic to dams causing loss of pregnancy and neonatal death. No reduction of live pubs observed at lower dose levels and by exposure to bulk ZnO. No histopathological changes in placenta. Testis of male mice which were exposed prenatally to 50 and 100 mg/kg bw of ZnONPs showed gross pathological changes like prominent epithelial vacuolization, decrease in the seminiferous tubule diameter and low cellular adhesion of epithelia. No effects in male mice prenatally exposed to bulk ZnO.				Histopathological adverse effects on testes in male offspring from female mice orally exposed during gestation to ZnONP but not to larger bulk particles
Key findings: Kinetics	**-**				
Teng et al. 2019	**Cc**: ZnO NP (I); ZnO NP (II); ZnO (III); **Pu:** - **Ps**: 13.2 nm 57.1 nm 1900 nm **Sh:** I, II, III: particles **Cr**: - **Sa**: -	ICR mice 6-8 pregnant mice/group	**Route/ adm:** Oral gavage **Duration/period:** Daily exposure during GD1-GD10 (peri-implantation stage) or GD7-GD16 (organogenesis stage) **Exposure levels:** 7.2 mg/ mouse (corresponding to about 280 mg/kg bw/day	Pregnant mice were sacrificed on GD19. Reproductive parameters Fetal weight. Morphological abnormalities, histopathology of selected organs Biodistribution examined by determining elemental Zn level.	**N: 6** **K:1** **R: ++**

	Sc: - **Ch**: 32.2 mV 25.7 mV 16.9 mV **Ag:** - **Em**: particle sizes in medium **Ws:** 6.2-8.2% dissolution measured in gastric fluid.		assuming a BW of 26 g)		
Key findings: Fertility	-				Adverse effects on placenta
Key findings: Development	No obvious maternal toxicity was noted in maternal mice. Exposure to ZnONPs with a particle sizes of 13 and 57 nm caused pathological lesions in placenta (swelling of trophoblast giant cells and accumulation of neutrophils). ZnONPs (13 nm) caused decreased placental weight (g/fetus) and fetal developmental toxicity recorded as decreased viability, fetal weight, decreased decreased crown-rump and tail length. The organogenesis period (GD7-GD16) was more vulnerable to such toxicity compared with the peri-implantation period (GD1-GD10) of pregnancy. No such effects were found in relation to exposure to 57 nm and 1900 nm particles. No obvious morphological abnormality and visceral abnormality were observed after oral exposures to any of the ZnO particles.				Adverse developmental effects induced by ZnO (13nm) particles but not larger particle sizes
Key findings: Kinetics	Increased Zn content (determined as elemental Zn) was determined in placenta and in the fetuses exposed to 13 nm ZnONP in the period of organogenesis.				ZnO (13 nm) distributed in placenta and fetus.
Other references considered less relevant and not evaluated further					
Kielbik et al 2019. "Transfer of orally administered ZnO:Eu nanoparticles through the blood-testis barrier: the effect on kinetic sperm parameters and apoptosis in mice testes." Testing of fluorescent europium doped ZnONP for biomedical purposes. Not considered relevant for the purpose of this project **Singh et al. 2019.** "Zinc oxide nanoparticles impacts: cytotoxicity, genotoxicity, developmental toxicity" is a review: Relevant studies from this review are presented elsewhere in the table.					

Liu et al. 2017. "Oocyte exposure to ZnO nanoparticles inhibits early embryonic development through the γ-H2AX and NF-κB signaling pathways." Hens were exposed to ZnO NPs, and after fertilization their impacts on embryonic development and the underlying mechanisms were explored. Considered less relevant as the extrapolation of data from hens to humans may be controversial.

Mohamed & Abdelrahman 2019. "The possible protective role of zinc oxide nanoparticles (ZnONPs) on testicular and epididymal structure and sperm parameters in nicotine-treated adult rats (a histological and biochemical study)." The publication dose only contain data on exposure to ZnO nanoparticles in combination with nicotine for assessing the protective role of ZnO toward nicotine testicular toxicity. Not considered relevant

El-behery et al. 2019. "The efficacy of chronic zinc oxide nanoparticles using on testicular damage in the streptozotocin-induced diabetic rat model." This study not considered relevant as testicular effects was only studied on diabetic male rats orally dosed with ZnO nanoparticles.

Evaluation and overview

Data availability
Based on screening of the abstracts 15 publications were identified and were further examined in full text.

Of these 15 publications, one publication (Singh et al. 2019) was a review article, while four studies (Kielbik et al. 2019; Liu et al. 2017, Mohamed & Abdelrahman 2019, El-behery et al. 2019) were not considered relevant based on the screening criteria.

Of the remaining 10 publications most relevant and informative data could be extracted from 6 publications (scored with R++ or R+/++). These covered two oral studies in male mice examining semen quality and testicular toxicity, and on developmental toxicity, one oral study in mice and one in rats (conducted according to the OECD 414 study protocol, this study was covered by two publications).

Nano zinc oxide data with highest R-score

	Fertility data	Developmental toxicity data
Rats, oral	-	Hong et al. 2014 (a+b); (N:6, K:1, R:++)
Mice, oral	Radhi et al. 2019 (N:4, K:2, R:++) Talebi et al. 2013 (N:2, K:2, R:+/++) Tang et al. 2019 (N:3, K:2, R:+/++)	Teng et al. 2019 (N:6, K:1, R:++)

Nano-characterisation

Of the 10 publications included in the table above the N-scores for nano-characterisation of the test item were in the range of 2-6 with an average score of 3.9. It may be noted that three studies had an N-score of 2 only. Solubility has been determined in one publication where a dissolution of 6.2-8.2% was measured in gastric fluid (Teng et al. 2019). In the REACH -registration water solubility in the range of 1.1 - 47 mg/L is given for various nano-qualities of ZnO (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16139).

Kinetics

Teng et al. (2019) found increased Zinc (Zn) content in placenta and fetuses of mice when dams were orally exposed during GD7-GD17 to ZnONP with a particle size of 13 nm at an exposure level at about 280 mg/kg bw/day. This was not seen in dams exposed to 57 nm and 1900 nm ZnONPs. Zn content was measured after digestion of the organs in nitric acid, so no data on accumulation of particles can be concluded.

Hong et al. (2014 a+b), however, did not find increased Zn levels in fetuses from rats exposed to ZnONP (particle size 20 nm) during GD5-GD19 to 0, 100, 200, and 400 mg ZnONP/kg bw/day.

Fertility

Radhi et al. (2019) exposed male mice to 0,100 and 200 mg ZnONP/kg bw/day (particle size of 50 nm) for 7 or 14 days. In all exposed groups significantly reduced testes, epididymal, seminal vesicle and prostate weights were observed. The percent of abnormal sperm cells was also increased at both dose levels.

Talebi et al. (2013) exposed male mice to ZnONP for 35 days at 0, 5, 50 and 300 mg/kg bw/day (particle size not indicated). Significant impairment of sperm number and motility and increased percentage of abnormal sperm were noted in mice exposed to 50 and 300 mg/kg bw/day. Also, at the two highest dose levels histopathological changes was observed in testicular tissue. Similar results were found by Tang et al. (2019) following exposure of male mice to 50, 150 and 450 mg ZnONPs/kg bw/day for 30 days. In addition, Tang et al. (2019) observed a dose related decrease in serum testosterone levels and a downregulation of the *StAR* gene (involved in testosterone synthesis) in testes.

Developmental toxicity

At exposure of pregnant mice to ZnONP sized 13 and 57 nm at a dose level of 7.2 mg ZnONPs mg/dam (about 280 mg/kg bw/ day), pathological lesions was observed in the placenta (swelling of trophoblast giant cells and accumulation of neutrophils. ZnONPs (13 nm) further caused decreased placental weight (g/fetus) and fetal developmental toxicity recorded as decreased viability, fetal weight and crown-rump and tail length. The organogenesis was more vulnerable than the peri-implantation period. None of the effects were seen after exposure particles with a diameter of 1900 nm (Teng et al. 2019).

In pregnant rats exposed on GD5-GD19 to 0, 100, 200, and 400 mg ZnONP/kg bw/day (20 nm), significant increases in the number of fetuses with visceral variations was observed at 400 mg/kg bw/day. Reduced maternal food consumption and decreased liver weight and increased adrenal gland weight was observed at the two highest dose levels (Hong et al., 2014a+b). This study was conducted according to OECD 414 and in compliance with GLP.

Overall evaluation

Toxicity in testes and germinal tissue and reduced sperm quality has been found in two studies where male mice were subject to repeated exposure to ZnONP.
In pregnant mice exposure of ZnONP may result in increased zinc level in level in placenta and fetuses after exposure to 13 nm ZnONP but not for 57 nm particles. No increased Zn levels has been found in fetuses from rats to 20 nm ZnONPs.
A prenatal developmental toxicity in rats found visceral variations in pups only at maternal toxic doses, while in mice developmental toxicity and reduced number of live pups were seen at levels with no obvious maternal toxicity.

Data gaps

Kinetics:
No data on uptake from inhalation of ZnONP and the following distribution into gonads, placenta or fetus was found from the literature search.

Fertility:
Although data indicate concern for testicular toxicity no reproductive toxicity studies are available. Furthermore, there are no studies addressing potential placental toxicity.

Development:
Although an oral OECD TG 414 study has been conducted in rats showing no concern, conclusive data is missing as data from oral exposure in mice indicates some concern for developmental toxicity.
No data is available for the inhalational exposure route.

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B.5 Silicon dioxide (SiO2NP)

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Silica; Silicon dioxide (SiO_2NP)					
Reference	**Test material, nanocharacterisation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance (see Appendix B1) N-score (1-11) K-score (1-4) R-score (R0, R+, R++)** **Comments**
Fertility					
Ren et al. 2016; Zhang et al. 2016 (Two publication covering the same study)	**Cc**: SiO_2 **Pu:** - **Ps**: 57.7 nm **Sh:** near-spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** high degree of monodispersion **Em**:- **Ws: -**	Male C57 mice 20 male mice/group	**Route/ adm:** intratracheal instillation **Duration/period:** Every 3rd day for 45 days (total of 15 instillations). Parameters were also assessed at 75 days, i.e. after 30 days without exposure **Exposure levels:** No treatment, 0 (saline vehicle) or 2 mg/kg bw per instillation	Testes and epididymis were collected for histopathology, determination of oxidative stress. Sperm analysis. Further determination of the protein expressions of meiosis-regulating factors, Sohlh1/ cyclin A1/cyclin B1/CDK1/CDK2protein.	**N: 4** **K: 2** **R: ++**
Key findings: Fertility	Sperm quality was decreased at day 45 in exposed animals compared to the controls: sperm concentrations $84x10^4$ per/ml *vs*. $128x10^4$ per/ml; sperm motility was 33% *vs* 76%, and sperm abnormality rate 6.4% *vs* 2.4 %, respectively.				Adverse effects on testes and sperm parameters

	Microscopic studies of the SiO_2NP-treated animals showed a significant reduction in the number of primary spermatocytes and spermatids compared to the saline group. Histopathology indicated damage of the seminiferous epithelium. Moreover, in testicular tissue expression of Sohlh1/cyclin A1/cyclin B1/CDK1/CDK2 were greatly downregulated and the ROS level was significantly increased on day 45; these effects were reversed on day 75. It was concluded that SiO2NPs induced oxidative stress in testis and led to apoptosis and necroptosis of the spermatogenic cells.				
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Hassankhani et al. 2015	**Cc**: SiO_2 **Pu:** 100% **Ps**: 10-15 nm **Sh:** spherical **Cr**: - **Sa**: 180-270 m2/g **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws:** -	Wistar mice (age: 6-8 weeks) 4 males/group	**Route/ adm:** Oral gavage **Duration/period:** Dosing period not indicated **Exposure levels:** 333 mg/kg bw/day	Blood chemistry Histopathologcal examinations of testes epididymis. .	**N: 5** **K: 2/3** **R: 0/+**
Key findings: Fertility	In the testis, congestion, disruption and reduction of spermatogenesis, necrosis, and edema were reported in all the mice treated with SiO_2NPs.				Few animals and poor reporting. Low reliability.
Key findings: Development	**-**				
Al-Husseini & Al-Khauzy 2018	**Cc**: SiO_2 **Pu:** 99% **Ps**: 10-30 nm **Sh:** particles **Cr**: - **Sa**: -	Wistar rats 10 males / group	**Route/ adm:** Oral gavage **Duration/period:** Daily for 22 days	Histopathology on testes and epididymis. Sperm analysis.	**N: 4** **K: 2** **R: 0/+** Reliability considered low as

	Sc: treated with silane coupling agent **Ch**: - **Ag:** - **Em**: - **Ws**:-		**Exposure levels:** 0,1,10 and 100 mg/kg bw/day		all findings for all examined parameters in the four dose levels are reported to be significantly different from each other and in a very consistent dose-related manner. Very low standard errors for all parameters and significant effect at even the lowest dose level of 1 mg/kg bw/day.
Key findings: Fertility	Significant and dose related adverse effects (P<0.05) observed at all dose levels for all parameters examined i.e. sperm concentration, motility, abnormality, and viability. Histopathological results revealed changes in tissues of testes such as atrophy in some seminiferous tubules with expanded lining. Tubules in both the testis and epididymis were found empty from sperms and two highest dose levels with hyperplasia and damage in stereocilia of tubules lining cells of epididymis.				
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Wolterbeek et al. 2015	**Cc**: SiO_2, precipitated (same batch as test item used by Hofmann et al. 2015 from JRC repository) **Cc**: SiO_2, precipitated	Wistar (Crl:WI(Han) rat 28 rats/sex/group	**Route/ adm:** Oral gavage **Duration/period:** Males dosed daily during a 10-week premating period and during 2 weeks of mating.	Two-generation study according to OECD 416 .	**N: 7** (test item described in Hofmann et al. 2015) **K: 1** **R: ++** Two-generation study according to

	Pu: 96.5% **Ps**: 10-25 nm primary particle **Sh:** particles **Cr**: - **Sa**: 230 m^2/g **Sc**: - **Ch**: - **Ag:** sub 10-nm to 3 µm in test solution **Em**: test item occurred as agglomerates in test solution **Ws: -**		Females dosed during a 10-week premating period and during mating, gestation and lactation up to postnatal day 21. F1-generation pups were dosed by gavage from postnatal day 22 and onwards, also during a 10 week premating and through mating period **Exposure levels:** 0, 100, 300 and 1000 mg/kg body weight/day		OECD 416
Key findings: Fertility	No effects were observed on fertility and reproductive parameters including mating, fertility, fecundity and gestation indices. No parental toxicity was noted. A NOAEL of 1000 mg/kg bw/day for reproductive toxicity was concluded.				No adverse fertility effects seen up to an oral dose level of 1000 mg/kg bw/day
Key findings: Development	No treatment related effects were observed on any of the development parameters, including pre- and post implantation loss, gestation length, pup viability indices, pup weights, pup organ weights and the measures of sexual maturation (testes descending, preputial separation and vaginal opening). At sacrifice of the F0- and F1-generation animals, no toxicological relevant differences were observed on absolute- and relative organ weights and on microscopical examinations. A NOAEL of 1000 mg/kg bw/day for reproductive toxicity was concluded.				No adverse developmental effects seen up to an oral dose level of 1000 mg/kg bw/day
Key findings: Kinetics	**-**				
Development					
Hofmann et al. 2015	Test item from JRC repository. **Cc**: SiO_2, precipitated	Wistar rats 25 mated female rats/ group	**Route/ adm:** Oral gavage **Duration/period:**	According to OECD 414 .	**N: 7** **K: 1** **R: ++**

	Pu: 96.5% **Ps**: 10-25 nm primary particle **Sh:** particles **Cr**: - **Sa**: 230 m2/g **Sc**: - **Ch**: - **Ag:** sub 10-nm to 3 µm in test solution **Em**: test item occurred as agglomerates in test solution **Ws: -**		Daily during GD6-GD16 **Exposure levels:** 0, 100, 300, or 1000 mg/kg bw/d		According to OECD 414
Key findings: Fertility	-				
Key findings: Development	Exposure did not affect numbers of corpora lutea, implantations, resorptions, live and dead foetuses. Also, no compound-related increase in the incidence of malformations or variations was observed in the fetuses. No effects were seen on fetal or placental weights. No maternal toxicity was observed. A no observed adverse effect level (NOAEL) of 1000 mg/kg bw/d was concluded for developmental effects.				No adverse developmental effects seen up to an oral dose level of 1000 mg/kg bw/day (NOAEL)
Key findings: Kinetics	**-**				
Kinetics					
Lee et al. 2014	**Cc**: SiO_2 (I and II) **Pu:** - **Ps**: spehrical I: 15 nm (TEM) II: 89 nm (TEM) **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**:	Sprague Dawley rats 6 rats/sex/group	**Route/ adm:** Oral gavage **Duration/period:** Single dose **Exposure levels:** 0, 500, 1000 mg/kg bw	Organs analysed for tissue Si distribution: brain, heart, kidneys, liver, lungs, spleen, and testes or ovaries 1 and 6 hours, and 1, 2, 3, and 7 days postadministration .	**N: 4** **K: 2** **R: ++**

	I: - 60 mV II: - 76 mV **Ag:** - **Em**: - **Ws**: -				
Key findings: Fertility	-				
Key findings: Development	**-**				
Key findings: Kinetics	Orally administered silica distributed predominantly to the kidneys, liver, lungs, and the spleen in rats. The organ distribution was not affected by particle size or animal sex. The silica nanoparticles were found to retain their particulate form, although more decomposition was observed in kidneys, especially for 15 nm particles. No increased Si levels were found in ovaries and testes.				SiO_2 was taken up and distributed to various organs after oral exposure, however, no distribution to the testes and ovaries.
Poulsen et al. 2015	**Cc**: SiO_2 (two particle sizes) **Pu:** - **Ps**: I:25 nm II: 50 nm **Sh:** pseudo-spherical **Cr**: - **Sa**: I; 159 m^2/g (BET) II: 87 m^2/g (BET) **Sc**: uncoated **Ch**: - **Ag:** - **Em**: stability and particle size	*Ex vivo* human placenta	**Route/ adm:** Perfusion **Duration/period:** 360 minutes **Exposure levels:** 100 µg/mL At maternal circulation **Flow:** Maternal circulation: 9 mL/min Fetal circulation: 3 mL/min	Collection of samples from both circulations after 0, 2, 30, 60, 120, 180, 240, 270, 300, 330 and 360 min of perfusion.	**N: 6** **K: 2** **R: ++**

	measured **Ws**: -				
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	The percentage of silica NPs reaching the fetal perfusate after 6 h was limited to 4.2 ± 4.9% and 4.6 ± 2.4% for 25 and 50 nm NPs, respectively.				Penetration of the placenta demonstrated ex vivo

Other references considered less relevant and not evaluated further

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Evaluation and overview

Data availability

Based on screening of abstracts, 13 publications were identified for further examination in full text. Of the 13 references two of the

references were only available as conference abstracts (Narciso et al. (2017) and Yaman et al. (2016)), and two other references were reviews (Celá et al. (2014) and Murugadoss et al. (2017)). The publication by Bara et al. (2018) was not considered relevant due to lack of nano-characterisation.

Of the remaining 8 publications most relevant and informative data could be extracted from five publications (scored with R++). These covered two oral studies in rats which were conducted according to OECD TG 416 (two-generation study by Wolterbeek et al. (2015)) and OECD TG 414 (prenatal developmental toxicity study by Hoffmann et al. (2015)). One study on reproductive toxicity in mice (Ren et al., 2016). Further, two studies on kinetics are included, one in rats (Lee et al., 2014) and one in the ex-vivo human placenta model (Poulsen et al., 2015).

Nano silicon oxide data with highest R-score

	Fertility data	Developmental toxicity data	Kinetic data
Rats, oral	Wolterbeek et al. 2015 (N: 7, K: 1, R: ++)	Wolterbeek et al. 2015 (N: 7, K: 1, R: ++) Hofmann et al. 2015 (N: 7, K: 1, R: ++)	Lee et al. (2014) (N: 4, K: 2, R: ++)
Mice, resp. tract	Ren et al. 2016 + Zhang et al. 2016 (N: 4, K: 2, R: ++)		
Human placenta			Poulsen et al. 2015 (N: 6, K: 2, R: ++)

Nano-characterisation

Of the 8 publications for detailed examination, the N-scores for nano-characterisation of the test item were in the range of 3-7 (average 5.7). No data is given on any of the publications regarding water solubility of SiO_2NP. In the REACH registration of nano silicon dioxide the water solubility of all non surface-treated SAS products (silica gel, colloidal, precipitated and pyrogenic SAS) is indicated to be in the range of 100 mg/L or higher (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/15556/4/9).

Kinetics

Lee et al. (2014) found that oral administration of SiO_2NP (particle sizes of 15 nm or 89 nm) was predominantly distributed to the kidneys, liver, lungs, and the spleen in rats exposed to 500 and 1000 mg/kg bw of SiO_2NP. The SiO_2NPs were found to retain their particulate form, although more decomposition was observed in kidneys especially for 15 nm particles. No increase in content of silicium was observed in testes and ovaries, indicating no or very low distribution to these organs. No data on the kinetics of SiO_2NP from inhalation or exposure to the respiratory tract was found in the data search.

Poulsen et al. (2015) used the human placenta *ex vivo* model, and found penetration of SiO_2NP to the fetal circulation, of 4.2 ± 4.9% and 4.6 ± 2.4% for 25 and 50 nm NPs after 6 hours of perfusion with a concentration of 100 mg SiO_2NP /L in the maternal circulation compartment.

Fertility

Ren et al. (2016) and Zhang et al. (2016) (the same study reported twice) found increased malformation of sperms and decreased sperm motility and concentration in the epididymis in mice after intratracheal instillation of SiO_2NP (57.7 nm) at a dose level of 2 mg/kg bw/instillation every third day for a period of 45 days. SiO_2NP exposure was associated with induction of oxidative stress in the testis and led to apoptosis and necroptosis of the spermatogenic cells.

Wolterbeek et al. (2015) conducted an OECD TG 416 two-generation study in which rats were orally dosed to SiO_2NP (primary particle size 10-25 nm and a surface area of 230 m^2/g, and mainly as agglomerates in the test solution) at dose levels of 0, 100, 300, or 1000 mg/kg bw/day. No effects were found for any reproductive or developmental toxicity parameters in this study.

Developmental toxicity

Hofmann et al. (2015) conducted an OECD TG 414 prenatal developmental study in which rats were orally dosed to SiO_2NP (same test item/batch as in Wolterbeek et al. (2015)). No effects were found for any developmental parameters in this study.

Overall evaluation

The current data indicates that orally administered SiO_2NP does not reach the testes or the ovaries in rats. An *ex vivo* study with a human placenta indicates that SiO_2NP may have the potential for a low degree of translocation across placenta, at least at the late stage of pregnancy.

In mice exposure to monodispersed SiO_2NP by tracheal instillation, histopathological findings in testes and adverse effects on semen quality indicate that SiO_2NP may interfere with male fertility.

In contrast, oral exposure to even high dose levels of agglomerated SiO_2NPs (at dose levels up to 1000 mg/kg bw/day) did not result in adverse effects on fertility or fetal development when tested in rats according to OECD TGs 416 and 414.

Data gaps

Kinetics:
No data on uptake from inhalation of SiO_2NP and the following distribution into gonads, placenta or fetus was found in the literature search.

Fertility:
No data available in relation to inhalation exposure. Intratracheal administration in mice indicates concern about toxicity on testes and spermatogenesis.

Development:
No data available in relation to inhalation exposure.

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B.6 Carbon nanotubes (CNT) + graphene

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility*

Carbon nanotubes CNT + graphene					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance (see Appendix B1) N-score (1-11) K-score (1-4) R-score (R0, R+, R++) Comments**
Fertility, male mice					
Skovmand et al., 2018	Graphene oxide (GO) Flammruss 101 (F) Printex90 (P90) Diesel exhaust particle, SRM1650b (DE) **Cc**: Graphene oxide (GO), carbon black (F or P90) or diesel exhaust particles (DE) with high level of PAHs **Pu**: **Ps**: GO: 2-3 µm plates	Mature male NMRI mice N=105 (15/group)	**Route:** Intratracheal instillation **Duration/ period:** Weekly exposure for seven consecutive weeks. **Exposure levels:** Graphene oxide: 18 µg/mouse/i.t. Flammrus 101 Printex90 SRM1650b: 0.1 mg/mouse/i.t. each	Lungs: Pulmonary inflammation Sperm parameters: Epididymal sperm count Sperm motility Epididymal sperm viability and morphological abnormalities Daily sperm production Sperm integrity (damage) Plasma testosterone	**N:4** **K:2** **R: ++**

	of 2-3 layers of GO F: 95 nm P90: 14 nm DE: 18-30 nm **Sh:** GO: flat plates **Cr:** - **Sa:** GO: 338-411 m^2/g F: 23.8 m^2/g P90: 295-338 m^2/g DE: 108 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:-** **Ws_-** Further information published previously				
Key findings: Fertility	**Lungs:** Neutrophil numbers in the bronchoalveolar fluid were increased by 51 fold in mice exposed to GO. **Sperm:** No significant changes in epididymal sperm parameters, daily sperm production or plasma testosterone levels were found. **Conclusion:** Despite the sustained pulmonary inflammatory response, the exposure to graphene oxide, Flammruss 101, Printex 90 and the diesel particle SRM1650b in the present study did not appear to affect semen parameters, daily sperm production or testosterone concentration in male NMRI mice.				**No effect on sperm parameters or plasma testosterone levels**
Key findings: Development	-				
Key findings: Kinetics	-				
Vasyukova et al., 2015	CNM Taunit (95-98% MWCNT)	C57B/6 × DBA2 mice	**Route:** Oral gavage	Male fertility: Fertilizing capacity index.	**N:4** **K:2** **R: ++**

	Cc: Carbon (MWCNTs (95–98 wt %), fullerenes, graphite, and amorphous carbon) **Pu:** 95-98% MWCNT **Ps**: Diameter 11-28 nm Length: 5-10 µm **Sh:** Tube **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - Ws:-	Mature males: 7/group (5 groups)	**Duration/ period:** Daily exposure during 30 consecutive days **Exposure levels:** 0.3, 3, and 30 mg/kg bw/day	Testis: pathological examination Sperm parameters: spermatogenesis index Hormone levels Treated males were bred with untreated females	
Key findings: Fertility	**Male fertility** A dose-dependent statistically significant decrease of fertilizing capacity of 15-40% was registered for the male mice were seen at all doses. A decrease in plasma testosteron level was also seen at 30 mg/kg/ day, albeit an increase in testosteron level was seen at the lowest dose. **Testis:** No structural mor morphological changes of the testes were detected. **Sperm parameters:** No effects observed on spermatogenesis index **Hormone levels:** No changes in hormeone levels (FSH ans LH)				Effects on male fertility
Key findings: Development	-				
Key findings: Kinetics	**-**				
Developmental studies, rats					
Lim et al., 2011a+b	MWCNT, CM-95 (Hanwha Nanotech) **Cc**: Carbon **Pu:** 95 % D, 5% Fe	SD rats 12 /group	**Route:** Oral gavage **Duration/period:** GD 6-19, daily	During the test period, clinical signs, mortality, body weights, food consumption, serum biochemistry, oxidant-antioxidant	**N:4** **K:2** **R: ++**

	Ps: 10–50 µm by ~20 µm **Sh:** tube **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws:** -		**Exposure levels:** 0, 40, 200 or 1000 mg/kg bw/day	status, gross findings, organ weights, and Caesarean section findings were examined.	
Key findings: Fertility	**-**				
Key findings: Development	Decreased maternal thymus weight at 1000 mg. No effect on fetal growth, viability, or morphological development				The no-observed adverse effect level of MWCNTs is considered to be 200 mg/kg/day for pregnant rats, and 1000 mg/kg/day or more for embryonic development.
Key findings: Kinetics	**-**				
Developmental studies, mice					
Hougaard et al., 2013	MWCNT (NM-400) **Cc**: Carbon **Pu**: ~84% **Ps**: Diameter: 10 nm Length < 1 µm **Sh:** Tube (curved) **Cr:** - **Sa:**	Mature, female C57BL/6J mice N=60 (30/group)	**Route:** Intratracheal instillation. **Duration:** One day pre-mating **Exposure levels:** Total dose: 67 µg	Time-to-delivery of first litter Gestation parameters Littering females Pups/litter Implantations and perinatal loss Lung and liver of dams Assessed 6 weeks and 4	**N:5** **K:2** **R: ++**

	Sc: - **Ch:** - **Ag:** - **Em:** zeta size: 89 nm and hydrodynamic number size-distributions peaked at 51 nm **Ws:**-			months after exposure Offspring Behavioural testing (males) Daily sperm production	
Key findings: Fertility	A short delay in the delivery of the first litter was observed in exposed females. (Histopathological changes were observed in the lungs and livers of exposed adult females six weeks and 4 months after exposure)				Long lasting effects in lung and liver of exposed dams. Uncertain whether this is associated with delay in delivery
Key findings: Development	Litter parameters, behavior and daily sperm production were similar in control and exposed groups				
Key findings: Kinetics	**-**				
Johansson et al., 2017	MWCNT (NM-400) **Cc**: Carbon **Pu**: ~84% **Ps**: diameter: 10 nm Lenth 295 nm **Sh:** tube (curved) **Cr:** - **Sa:** 298 m^2/g **Sc:** - **Ch:** - **Ag:** -	Experiment 1: C57BL/6 J mice N=50 (5/group) Experiment 2: Naïve 57BL/6JBomTac mice, 100 females:Control (n= 30), low (n= 20), medium (n = 20) and high dose (n = 30)	**Route:** Intratracheal instillation. **Duration:** 1: Day 15 of smearing (out of 4 weeks) of naïve females 2: One day prior to cohabitation with a mature, unexposed male	Exp. 1: Vaginal smear Exp. 2: Time to delivery of first litter Litter parameters	**N:5** **K:2** **R: ++**

	Em: Hydrodynamic number size-distributions peaked at 33 and 51 nm **Ws**:-		**Exposure levels:** Total doses: 1: 67 µg 2: 2, 18 or 67 µg		
Key findings: Fertility	Compared to normal estrous cycling determined prior to exposure, exposure to MWCNT significantly prolonged the estrous cycle during which exposure took place (by approximately 2 days, i.e., from 5.3 days before exposure to 7.2 days for exposed cycles), but significantly shortened the estrous cycle immediately after the exposed cycle ($p < 0.001$). No consistent effects were seen on time to delivery of a litter.				Lung exposure has effect on estrous cycle
Key findings: Development	No consistent effects were seen on litter parameters, such as litter size, sex ratio, implantations and implantation loss				
Key findings: Kinetics	**-**				
Fujitani et al., 2013	MWCNT **Cc**: Carbon **Pu**: - **Ps**: nanotube (no specific information given) **Sh:** - **Cr:** - **Sa:** - **Sc:** - **Ch:** - **Ag:** - **Em:** **Ws**:-	Pregnant ICR mice (5-15/group)	**Route:** Intratracheal instillation. **Duration:** Single exposure on GD 9 **Exposure levels:** 3, 4 and 5 mg/kg bw		**N:1-2** **K:2** **R: +** No characterisation of test item
Key findings: Fertility	Increased early and late resorptions of fetuses were at 4 and 5 mg/kg bw /day, albeit not statistically significantly so.				

Key findings: Development	Body weight of fetuses was significantly lower at 5 mg/kg bw/day. Malformations seen in fetuses at 4 and 5 mg/kg bw /day (short or absent tail, and deduction or deformity of limb, fusion of ribs, fusion of vertebral bodies and arches)	Statistically significant increase in malformations at 4 and 5 mg/kg bw /day
Key findings: Kinetics	**-**	
Other studies		
Akhavan et al. (2015) Excluded due to irrelevant exposure route (intravenous injection) Liu, Y. and C. Chen (2016). "Effect on reproductive system of carbon nanomaterials." Book chapter. Only abstract available. Sawosz et al. (2014). "Toxicity of pristine graphene in experiments in a chicken embryo model." Chicken eggs were exposed to pristine graphene. Considered less relevant due to difficulties in extrapolation of data to human exposure Qie et al. (2018). "Effects of MWCNTs-COOH on Follicular Development in Female Mice." Article in Chinese. Only abstract available in English Wang et al. (2015). "Adsorption of bisphenol a to a carbon nanotube reduced its endocrine disrupting effect in mice male offspring." Not considered relevant as the focus of the study is to investigate the effect of BPA/ MWCNT–COOH compared to BPA alone. Buchtova et al. (2014). "Embryonic Toxicity of Nanoparticles." Review on the effects of various nanomaterials including MWCNT on embryonic development. No additional references found. Ema et al., (2016a). " Reproductive and developmental toxicity of carbon-based nanomaterials: A literature review". Review on the reproductive and developmental toxicity of carbon based nanoparticles. The review includes the studies included above. Ema et al., (2016b). "Developmental toxicity of engineered nanomaterials in rodents" The review includes the studies included above. OECD, DOSSIER ON MULTIWALLED CARBON NANOTUBES (MWCNT), ENV/JM/MONO(2015)12/PART3. 04-Jun-2015. The studies used in the evaluation are included above.		

Evaluation and overview

Data availability

Based on screening of the abstracts 16 publications were identified and were further examined in full text. One publication considered graphene, the remaining MWCNT. The majority of the studies are investigations relevant for airway exposure to carbon nanotubes, and there are three studies on oral exposure.

From the OECD testing programme on manufactured nanomaterials a compilation and evaluation (from 2015) of data of MWCNT is available. The studies used in the evaluation (Lim et al., 2011a+b; Fujitani et al., 2013) were also found in the present data search and were included in the evaluation.

Of the 16 publications three publications (Buchtova et al. 2014; Ema et al., 2016a+b) are review articles, while 5 studies (Akhavan et al., 2015; Liu, Y. 2016; Sawosz et al., 2014; Qie et al., 2018; Wang et al., 2015) was not considered relevant based on the screening criteria or limited information available.

The data was therefore extracted from the remaining five studies. These covered four studies in mice, three studies with intratracheal administration (Skovmand et al., 2018; Johansson et al., 2017; Hougaard et al., 2013) and one oral study (Vasyukova et al., 2015). The final study administered MWCNTs by the oral route in rats (Lim et al., 2011a+b). The main focus of the studies is reproductive toxicity in males and females and developmental toxicity. Besides gestational parameters, male testes and sperm parameters and behaviour were addressed in one study. No guideline studies were found.

Carbon nanotubes and graphene data with highest R-score

	Fertility data	Developmental toxicity data
Mice, oral	Vasyukova et al., 2015 (N:4, K:2, R:++)	Vasyukova et al., 2015 (N:4, K:2, R:++)
Mice, respiratory tract	Skovmand et al., 2018 (N:6, K:2, R:++) Johansson et al., 2017 (N:5, K:2, R:++) Hougaard et al., 2013 (N:5, K:2, R:++)	Johansson et al., 2017 (N:5, K:2, R:++) Hougaard et al., 2013 (N:5, K:2, R:++)

Rat, oral		Lim et al., 2011a+b (N:4, K:2, R:++)

Nano-characterisation

Of the five publications included in the table above, the N-scores for nano-characterisation of the test item were in the range of 46 with an average score of 4.8. Solubility was not addressed in any of the studies. Some of the studies refer to more in-depth characterization data in other publications, but these were not considered in the scoring. Two REACH registrations for MWCNT are available (Graphite and MWCNT). In the REACH registrations, the water solubility is given as < 2 mg/L at 20 °C and a pH of 7.5 - 9.2 for MWCNT and 0 mg/L (insoluble) for graphite. (ECHA January 2020, MWCNT https://echa.europa.eu/registration-dossier/-/registered-dossier/13454#SubNav4; ECHA January 2020, Graphite: https://echa.europa.eu/registration-dossier/-/registered-dossier/16080).

Kinetics

There was no data on particle kinetics.

Fertility

Males: Skovmand et al., (2018) exposed mature male NMRI mice to four different types of carbonaceous nanomaterials including graphene oxide (18 µg/mouse/i.t. for 7 weeks). The study is also described in section 4.1.7 on carbon black. The mice were exposed weekly for 7 weeks, and testes were examined for effects in sperm counts and motility, as well as for daily sperm production and sperm integrity. Despite the sustained pulmonary inflammatory response, semen parameters were unaffected in the male NMRI mice. Vasyukova et al., 2015 performed an oral study in male C57B/6× DBA2 mice, exposed by oral gavage to 0.3, 3, and 30 mg MWCNT/kg/day on 30 consecutive days. No effects on the testes or any of the sperm parameters investigated were observed. No changes in hormone levels (FSH and LH) were seen. The treated males were bred with untreated females C57B/6 × DBA2 mice. A dose-dependent decrease of fertilizing capacity of 15-40% was registered at all dose levels.

Females: In the study of lung exposure to MWCNT, Hougaard et al. (2013) exposed mature, female C57BL/6J mice to 67 µg MWCNT by intratracheal instillation one day prior to mating. A short delay in the delivery of the first litter (5 days) was observed for exposed females. In a follow-up study, naïve female C57BL/6J mice were intratracheally exposed once to 67 µg MWCNT. Compared to normal estrous cycling determined prior to exposure, exposure to MWCNT significantly prolonged the estrous cycle (by approximately 2 days, i.e., from 5.3 days before exposure to 7.2 days for exposed cycles). However, the estrous cycle immediately after the exposed cycle was significantly shortened ($p < 0.001$). Another group of females was intratracheally exposed to 2, 18 or 67 µg MWCNT on the day before cohabitation with unexposed males. No consistent effects were seen on time to delivery of a litter (Johansson et al. (2017).

Developmental toxicity

In Lim et al (2011a+b), Sprague-Dawley rats were exposed to 40, 200 or 1000 mg/kg MWCNT/kg bw by oral gavage from GD6 to GD9. No effects on fetal growth, viability, or morphological development were observed. A decrease in maternal thymus weight was found at 1000 mg. The no-observed adverse effect level of MWCNTs was therefore considered to be 200 mg/kg/day for exposed dams, and 1000 mg/kg/day or more for embryonic development (Lim et al., 2011a+b).

In the study of intratracheal lung exposure to MWCNT, Hougaard et al. (2013) exposed mature, female C57BL/6J mice to 67 µg MWCNT by intratracheal instillation one day prior to mating. Litter parameters, behaviour and daily sperm production were similar in control and exposed offspring. No consistent effects were seen on litter parameters, such as litter size, sex ratio, implantations and implantation loss following exposure of female mice by intratracheal administration to 2, 18 or 67 µg MWCNT on the day before start of cohabitation with unexposed males (Johansson et al. (2017). Fujitani et al. (2012) exposed pregnant ICR mice on day 9 of the gestation to 3, 4 and 5 mg/kg body weight. Fetuses were examined for external and skeletal anomalies on day 18 of gestation. The incidences of fetal malformations in the groups given 4 or 5 mg/kg body weight were statistically higher compared to controls. No or very low-level malformations were seen after instillation of 3 mg/kg bw/day. (However, this study is considered of lower relevance as very poor characterisation of the MWCNT was given).

Overall evaluation

nanomaterials including graphene oxide did not alter semen parameters, but 30 days of oral exposure to MWCNTs decreased fertilizing capacity of males. Female exposure to MWCNT on the day prior to cohabitation with an unexposed male increased time-to-delivery of a first litter in one study, but not in another study. Intratracheal exposure to MWCNT did interfere with estrous cycling, increasing the length of the exposed cycle but decreasing the length of the following cycle. Overall, these findings indicate that exposure to MWCNTs may interfere with adult fertility, but no firm conclusions can be drawn on the basis of the present studies.
No findings regarding effects on development were observed in two studies using instillation of MWCNT to female mice on the day prior to cohabitation with naïve males (~2.5 mg/kg).

However, one study (with very poor characterization of the MWCNT) found indications of developmental effects in mice after one intratracheal instillation during gestation (at and above 4 mg/kg).

Data gaps

Kinetics:
No data on the distribution of carbon nanotubes into gonads, placenta or fetus was found from the literature search.

Fertility:
Only sparse and very scattered published data with the testing of only mice is available concerning fertility effects of carbon nanotubes

and graphene exposure. Standard OECD testing determining fertility of carbon nanotubes and graphene using relevant exposure routes (oral or inhalation exposure) is therefore needed.

Development:
Limited data of the developmental effects of carbon nanotubes and graphene is available. Thus, standard OECD testing determining prenatal developmental toxicity of carbon nanotubes and graphene using relevant exposure routes (oral or inhalation exposure).

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B.7 Carbon black (CB)

***Legend**: Cc: chemical composition Pu: Purity Ps: particle size/size distribution Sh: Shape Cr: crystal structure Sa: surface area Sc: surface chemistry Ch: surface charge Ag: agglomeration Em: characterisation in experimental media Ws: water solubility NP: nanoparticles*

Carbon black (CB)					
Reference	**Test material, nano-characterisation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance (see Appendix B1)** **N-score (1-11)** **K-score (1-4)** **R-score (R0, R+, R++)** **Comments**
Fertility – male mice					
Skovmand et al., 2018	Flammruss 101 (F) Printex90 (P90) Graphene oxide SRM1650b **Cc**: carbon black (F or P90), graphene oxide (GO), or diesel exhaust particles (DE) with high level of PAHs **Pu**: **Ps**: F: 95 nm P90: 14 nm GO: 2-3 µm DE: 18-30 nm **Sh:** particle	Mature male NMRI mice N=105 (15/group)	**Route:** Intratracheal instillation **Duration:** Weekly exposure for seven consecutive weeks. **Exposure levels:** graphene oxide: 18 µg/mouse/i.t. Flammrus 101 Printex90 SRM1650b: 0.1 mg/mouse/i.t.	Lungs: Pulmonary inflammation was determined Weight of reproductive organs: Testis Epididymis Sperm parameters: Epididymal sperm concentration Sperm motility Epididymal sperm viability and morphological abnormalities Daily sperm production Sperm integrity (damage)	**N:4** **K:2** **R: ++**

	Cr: - **Sa:** F: 23.8 m^2/g P90: 295-338 m^2/g DE: 108 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:** **Ws:-**			Plasma testosterone	
Key findings: Fertility	**Lungs:** Neutrophil numbers in the bronchoalveolar fluid showed sustained lung inflammatory response in the nanoparticle-exposed groups one week after the last instillation. **Sperm parameters and plasma testosterone:** No significant changes in epididymal sperm parameters, daily sperm production or plasma testosterone levels were found **Conclusion:** Despite the sustained pulmonary inflammatory response, an eight week exposure to graphene oxide, Flammruss 101, Printex 90 and the diesel particle SRM1650b did not appear to affect semen parameters, daily sperm production or testosterone concentration in male NMRI mice.				No effect on sperm parameters or plasma testosterone levels
Key findings: Development	-				
Key findings: Kinetics	-				
Yoshida et al., 2009	Carbon Black (CB), (Printex90, Printex25 and Flammrus101) **Cc**: CB **Pu**: **Ps**: 14, 56, 95 nm **Sh:** particles **Cr:** - **Sa:** 300, 45, 20 m^2/g	ICR mice (male) Age: 6 weeks N=78 (15-16/group)	**Route:** Intratracheal administration **Duration:** Weekly exposure for 10 weeks **Exposure levels:** 0.1 mg/mouse/i.t. in 4 groups: 14-nm CB	Animals were killed one day after the last examination Body and organ weights: The weights of body, testes, epididymis and seminal vesicle (including prostate, seminal vesicle and coagulating gland) were bilaterally measured for each animal.	**N:4** **K:2** **R: ++**

	Sc: - **Ch:** - **Ag:** - **Em: -** **Ws: -**		56-nm CB 95-nm CB and 14 N group (using 14 nm CB with same particle number concentration as in the 56-nm CB group)	Testes histology Daily sperm production Serum testosterone	
Key findings: Fertility	**Body and organ weights** No effects were observed **Testes histology** Vacuolation of the seminiferous tubules was observed in 14-nm CB, 56-nm CB, and 95-nm CB groups. **Daily Sperm production** Daily sperm count was significantly decreased in all CB treated groups: 14 nm CB: decrease by 33% ($p < 0.001$) 56 nm CB: decrease by 33% ($p < 0.001$) 95 nm CB: decrease by 23% ($p < 0.05$) 14 N group: decrease by 23% ($p < 0.05$) **Serum testosterone** Serum testosterone concentration was significantly higher in the 14 nm CB (7.5-fold of control) and 56 nm CB groups (7.51-fold of control) compared to controls **Author conclusion** These results suggest that carbon nanoparticle-exposure has adverse effects on the mouse male reproductive function. Furthermore, the effects of nanoparticles on the male reproductive system depended on particle mass rather than particle number.				Decrease in daily sperm production
Key findings: Development	Not investigated				
Key findings: Kinetics	Not investigated				

Developmental – mice					
Kyjovska et al., 2013	Carbon black (CB): Printex90 **Cc**: CB **Pu:** - **Ps**: 14 nm **Sh:** particle **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: Hydrodynamic SD: 50-60nm Zeta size: 140 nm **Ws:** Low	F0: C57BL/6J mice 15/group F1: C57BL/6J 14-15/group Mated with CBA/J mice F2: CBA/J)/(C57BL/6J	**Route:** Intratracheal instillation **Duration:** Gestation days 7, 10, 15 and 18 of F0 dams **Exposure levels:** 67 µg CB/animal/day The total exposure dose corresponded to 16 days at the 8-h occupational exposure limit according to Danish Regulation which is 3.5 mg/m^3 for CB	Time to pregnancy: Time to preganancy of prenatally exposed mice mated with naïve CBA/J mice were recorded. Testis: Testis were weighed after removal of adipose tissue Sperm parameters: Daily sperm production (DSP) Sperm content per g testicular parenchyma (SC/g). Sperm parameters for 1 male per litter were investigated in F1 and F2 generations.	**N:4** **K:2** **R: ++**
Key findings: Fertility					
Key findings: Development	**Testis:** No effect on testis weight was found **Sperm parameters:** F1 males prenatally exposed to CB displayed no significant differences in the assessed reproductive parameters. F2 male offspring from C57BL/6J males whose mothers were instilled with CB during pregnancy (paternal germline; PF2, (CBA/J)/(C57BL/6J)) had statistically significantly lower sperm content SC/g compared to controls (P = 0.04) and tended to have lower DSP (P = 0.057). In contrast, F2 males from C57BL/6J females exposed to CB during foetal life (maternal germline; MF2, (C57BL/6J)/(CBA/J)) presented similarly to corresponding controls for all studied parameters				Lower sperm content in F2 male offspring from exposed F1 males (male germline), but not in F2 male offspring from F1 females (female germline).

	Fertility: The time it took breeding couples of a prenatally CB exposed F1 C57BL/6J male and a naïve CBA/J female to deliver a first F2 litter was *slightly extended* compared to F1 control C57BL/6J males cohabiting with naïve CBA/J females (29.2 ± 7.7 and 24.8 ± 6.2 days, respectively), although not statistically significant. No correlation between sperm content/daily sperm production and time-to-first F2 litter was found.				
Key findings: Kinetics	**No data**				
Skovmand et al., 2019	Carbon Black (CB), Printex90 **Cc**: CB **Pu**: **Ps**: 14 nm **Sh:** spherical particle **Cr:** - **Sa:** 183 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:** suspended particle mass concentration of 4.79 ± 1.86 and 33.87 ± 14.77 mg/m^3 **Ws: -**	Time-mated outbred NMRI mice N=60 (20/group) Generations F0-F4	**Route:** Whole body inhalation **Duration:** Gestational days 4 to 18 (only dams for the first generation of pups) **Exposure levels:** 4.6 and 37 mg/m^3 for 45 min per day Exposure corresponds to1 and 8 h, respectively, at the Danish 8 h time weighted average occupational exposure limit of 3.5mg/m^3 for carbon black	Lungs: Lung inflammation in the dams was assessed on day 11 (for time-mated females without litters) and day 28–29 post-exposure (for dams with litters). Weight of reproductive organs: Testes Epididymis Sperm parameters (one male per litter, all four male offspring generations): Sperm motility Daily sperm production Sperm chromatin structure (1^{st} generation only) Plasma testosterone	**N:5** **K:2** **R: ++**
Key findings: Fertility					

Key findings: Development	No changes in gestation lengths, number and loss of implantations, offspring weights, litter size and, sex ratio, for exposed females and offspring compared to control females and offspring in any of the generations was seen. **Lungs:** No inflammation and no acute phase response detected 11 and 28/29 days post exposure **Sperm parameters**: No changes detected in any sperm parameters **Author conclusion:** No significant changes were observed in body and reproductive organ weights, epididymal sperm parameters, daily sperm production, plasma testosterone or fertility.				No effect on male fertility
Key findings: Kinetics	-				
Umezawa et al., (2018) (same study as Skovmand et al 2019 above)	Carbon Black (CB), (Printex90) **Cc**: CB **Pu**: >99% **Ps**: 14 nm **Sh:** particles **Cr:** - **Sa:** 182–338 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:-** **Ws:**	Time-mated NMRI mice N=60 (20/group)	**Route:** Inhalation **Duration:** Gestational days 4 to 18 (45 min/day) **Exposure levels:** 0, 4.6 or 37 mg/m^3 Exposure corresponds to1 and 8 h, respectively, at the Danish 8 h time weighted average occupational exposure limit for carbon black of 3.5 mg/m^3	Lung inflammation in the dams was assessed on day 11 (for time-mated females without litters) and day 28–29 post-exposure (for dams with litters). Behaviour: Open field test One male and one female per litter (90 days of age) GFAP (glial fibrillary acidic protein) One male per litter, 6 weeks of age (n=5) PV+ (parvalbumin-positive) interneurons (high dose only) Brains were collected from male and female offspring on PND 25, and from adult female offspring that had undergone open field testing and given birth to a 2nd generation (n=5).	**N:5** **K:2** **R: ++**

Key findings: Fertility	Not investigated	
Key findings: Development	**Lung inflammation** No inflammation in exposed females 11 and 28/29 days post exposure **Open field test** In the open field test, behaviour was dose-dependently altered following maternal exposure to Printex90, at 90 days of age. Prenatally exposed female offspring moved a longer total distance, and especially prenatally exposed males spent significantly longer time in the central zone of the maze. **GFAP (glial fibrillary acidic protein)** Glial fibrillary acidic protein (GFAP) expression levels were dose-dependently increased in astrocytes around blood vessels in the cerebral cortex and hippocampus in six weeks old males, indicative of reactive astrogliosis. Also enlarged lysosomal granules were observed in brain perivascular macrophages (PVMs) in the prenatally exposed offspring. **PV+ (parvalbumin-positive) interneurons** The number of parvalbumin-positive interneurons and the expression levels of parvalbumin were decreased in the motor and prefrontal cortices at weaning and 120 days of age in the prenatally exposed offspring (only assessed in the high dose group). In the offspring, the described effects were long-lasting as they were present at all time points investigated. **Conclusion:** The present study reports for the first time that maternal inhalation exposure to Printex 90 carbon black induced dose-dependent denaturation of PVM and reactive astrocytes, similarly to the findings observed following maternal exposure to Printex 90 by airway instillation.	Maternal inhalation exposure to printex90 induced dose-dependent denaturation of PVM and reactive astrocytosis and altered field test behavior in offspring
Key findings: Kinetics	Not investigated	

Yoshida., et al. 2010	Carbon nanoparticles (CB) **Cc**: CB **Pu**:	Pregnant ICR mice n=40 (20/group)	**Route:** Intratracheal instillation	Gestation parameters: Gestation length Litter size Gender ratio	**N:4** **K:2** **R:++**

	Ps: 14 nm **Sh:** particles **Cr:** - **Sa:** **Sc:** - **Ch:** - **Ag:** - **Em:** 50-500 nm in supsension **Ws:** -		**Duration:** GD 7 and 14 **Exposure levels:** 0.2 mg/mouse/instillation	Male offspring was examined at age 5, 10, or 15 weeks for: Body weight Sperm parameters: Sperm characteristics Daily sperm production Blood parameters	
Key findings: Fertility					
Key findings: Development	**Gestation parameters** No significant differences were seen in gestation length, litter size, fertility or gender ratio **Body weight** No effects on bodyweight **Testes** The testes of male mice exposed to CB as fetuses exhibited vacuolation of seminiferous tubules and low cellular adhesion of seminiferous epithelia **Sperm parameters** The daily sperm count was significantly decreased in the CB treated group at all three ages (5 weeks: 47% [P<.001]; 10 weeks: 34% [P<.001]; and 15 weeks: 32% [P<.001]; Fig. 3). There were no significant changes in sperm cytomorphology. **Blood parameters** The CB tended to increase serum T at ages of 10 and 15 weeks after birth. However, there were no significant changes in serum T between the control and CB groups at all three ages **Conclusion:** These findings suggest that fetal nanoparticle exposure affects the reproductive function of male offspring.				Effect on testis and DSP in male offspring
Key findings: Kinetics	-				

Boisen et al., 2013 (study also described in Kyjovska et al., 2013)	Carbon Black (CB), (Printex90) **Cc**: 99% of C, 0.8% of N and 0.01% of H2 **Pu**: >99% **Ps**: 14 nm **Sh:** particles **Cr:** - **Sa:** - **Sc:** - **Ch:** - **Ag:** - **Em:** hydrodynamic number size distribution peaked at ~50nm in vehicle and the average zeta-size was approximately 140 nm **Ws:** -	C57BL/6JBomTac mice (n=11 CB and 14 controls) Female F1 offspring were mated with naïve CBA males to get the 2nd generation (n = 9 CB and 14 controls) F2 Offspring analysed = 178 CB and 258 controls)	**Route:** Intratracheal instillations **Duration:** GD 7, 10, 15 and 18 **Exposure levels:** 67 µg/animal per instillation (cumulative dose 268 µg/animal)	**Germline mutations** Expanded simple tandem repeat (ESTR) germline mutation rates in the resulting F2 generation were determined from full pedigrees (mother, father, offspring) of F1 female mice	**N:5** **K:2** **R: +**
Key findings: Fertility	(See Kyjovska et al. 2013)				
Key findings: Development	No effects were observed: ESTR mutation rates in CB-exposed F2 female offspring were not statistically different from those of F2 female control offspring				
Key findings: Kinetics	-				
Onoda et al., 2017b	Carbon black (CB): Printex90	Pregnant ICR mice N=40 (10/group) Age: 11 weeks	**Route:** Intranasal instillation	Brain tissue: Brains were collected from four male offspring/dam at 6	**N:5** **K:2** **R: ++**

	Cc: CB **Pu**: >99% **Ps**: 14 nm **Sh:** particle **Cr:** - **Sa:** 300 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:** - **Ws: -**	Placentae collected on GD 13 (n=5/group)	**Duration:** On gestational days 5 and 9 **Exposure levels:** 2.9, 15, or 73 µg/kg bw/instillation	weeks of age (one pup per litter for each outcome was used, n=5) Placentae: Placentae were collected from pregnant dams on gestational day 13 and examined by microarray analysis**.**	
Key findings: Fertility	Not investigated				
Key findings: Development	There were no significant between-group differences in litter size or sex ratio of offspring at birth. No differences in offspring body weight was found. **Brain tissue in offspring:** - Increase in glial fibrillary acidic protein (GFAP) expression in the cerebral cortex in middle and high dose from 1 to 2.23 and 2.77 (GFAP/β-actin), respectively - Increased in aquaporin-4 expression in the brain parenchyma region around blood vessels. - Altered expression levels in the cerebral cortex of mRNAs associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production. - The changes in the expression profiles of GFAP and Aqp4 in offspring after maternal CB-NP exposure were similar to those observed in naïve mice of a more advanced age. **Placentae:** Differentially expressed genes in placental tissue after CB-NP exposure did not populate any specific gene ontology category.				Increase in GFAP in cerebral cortex at 15 µg/kg bw and above
Key findings: Kinetics					
Onoda et al., 2014	Ultra fine Carbon Black (CB), Printex90 **Cc**: CB (0.82% nitrogen and 0.01%	Pregnant ICR mice N=10 (n=5/group) Age: 11 weeks	**Route:** Intranasal instillation **Duration:** Gestational days 5 and 9	**Brain tissue:** Brains were collected from 4-6 male offspring/dam at 6 and 12 weeks of age (one pup per litter for each outcome was used)	**N:7** **K:2** **R: ++**

	hydrogen) **Pu**: >99% **Ps**: 14 nm **Sh:** particle **Cr:** - **Sa:** 295-338 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:** Agglomerated particles with peak size of 84.2 nm **Ws**: insoluble		**Exposure levels:** The total dose of CB was190 mg/kg bw		
Key findings: Fertility	Not investigated				
Key findings: Development	There was no significant difference between control and CB exposed offspring in number and sex ratio of pups at birth or their body weight at 6 and 12 weeks of age. **Brain tissue:** Enlarged granules of Perivascular macrophages (PVM) and decreased number of PAS-positive PVMs in CB-exposed offspring. These results suggested that in offspring, the presence of ‘‘normal’’ PVMs decreased at a wide area of the CNS due to maternal CB exposure. Increase in astrocytic GFAP expression level was seen, which was closely related to the enlargement of granules PVMs in offspring. Changes in phenotypes of PVM and astrocytes were seen in the CB group: Honeycomb-like structures in some PVM granules and swelling of astrocytic end-foot were observed under electron microscopy. **Authors conclude:** The phenotypic changes in PVMs and astrocytes indicate that maternal CB exposure may result in changes to brain blood vessels and be associated with increased risk of dysfunction and disorder in the offspring brain.				Increase in GFAP in cerebral cortex
Key findings: Kinetics	**-**				

Jackson et al. (2012a+b; 2011) (part of the study also described in Kyjovska et al., 2013)	Carbon Black **Cc**: 99% C, 0.8% N and 0.01% H2 **Pu**: 99% **Ps**: 14 nm (GM size 65 nm) **Sh:** particles **Cr:** - **Sa:** 295-338 m^2/g **Sc:** - **Ch:** - **Ag:** Aggregates of <100 nm to 20–30 mm **Em: -** **Ws: -**	Time mated female C57BL/6 6Bom-Tac mice Inhalation: n=44 Instillation: n=80 (17-24/group)	**Route:** Inhalation or intratracheal instillations **Duration:** Inhalation: GD 8 to 18 (1 h/day) Instillation: GD 7, 10, 15 and 18 **Exposure levels:** Inhalation: 42 mg/m^3 Instillation: 11, 54 and 268 µg/animal (cumulative doses) Exposure corresponds to1 and 8 h, respectively, at the 8 h time weighted average occupational exposure limit of 3.5 mg/m^3 in Denmark	DNA damage (Comet assay) Toxicogenomics (Instillation only) Behavioural tests and sexual maturation (Instillation only)	**N:6** **K:2** **R: ++**
Key findings: Fertility	-				
Key findings: Development	**DNA damage**: Inhalation exposure induced an increase in DNA strand breaks in the liver of mothers and their offspring, whereas intratracheal instillation did not (Jackson et al., 2012a) **Toxicogenomics (Instillation only):** changes in the expression of several genes and proteins associated with inflammation in maternal lungs (26–27 days post exposure). A significant hepatic response was also observed in male and female offspring exposed prenatally to CB at the mRNA level, only studied at the highest dose level (more pronounced in the female offspring) (Jackson et al., 2012b) **Behavioural tests (Instillation only):** The female offspring prenatally exposed to 268 µg Printex 90 / animal displayed altered habituationpattern during the Open field test (Jackson et a., 2011)				Maternal inhalation exposure to Printex90 induced liver DNA damage in the mothers and in the in utero exposed offspring

	Female offspring prenatally exposed to 11 µg Printex 90/ animal entered puberty significantly earlier compared to controls (time of vaginal opening on PND 35.4 vs 37.7, p = 0.01) compared with controls. This was not seen at the higher dose levels (Jackson et a., 2011) Neither inhalation nor instillation affected gestation and lactation.				
Key findings: Kinetics	-				
Shimizu, R., et al. 2014	Carbon Black **Cc**: CB **Pu**: **Ps**: 14 nm **Sh:** particles **Cr:** - **Sa:** 300 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em:** 50-500 nm in suspension **Ws**: -	Pregnant ICR mice 26 exposed 28 control animals	**Route:** Intranasal instillation **Duration:** GD 5 and 9 **Exposure levels:** 95 µg/kg/time Total dose 190 µg/kg bw	**Spleen** Splenocyte phenotypes and gene expressions	**N:5** **K:2** **R: +**
Key findings: Fertility	-				
Key findings: Development	**Spleen:** CD3(+) (T), CD4(+) and CD8(+) cells were decreased in the spleen of 1-5-day-old offspring in the treated group. Expression level of Il15 was significantly increased in the spleen of newborn male offspring, and Ccr7 and Ccl19 were increased in the spleen of female offspring in the CB-NP group. Splenic mRNA change profiles by CBNP were similar between male and female offspring. **Conclusion:** Exposure of pregnant mothers to CB-NP partially suppressed the development of the immune system of offspring mice. The decrease in splenic T cells in the treated group recovered 14 days after birth.				
Key findings: Kinetics	-				

El-Sayed et al., 2015	Carbon Black (CB), (Printex90) **Cc**: CB **Pu**: >99% **Ps**: 14 nm **Sh:** particles **Cr:** - **Sa:** 300 m^2/g **Sc:** - **Ch:** - **Ag:** - **Em: -** **Ws: -**	Pregnant ICR mice N=8-11	**Route:** Intranasal instillation **Duration:** GD 9 and 15 **Exposure levels:** 95 µg/kg bw/day (Total dose =190 mg/kg bw).	The thymus and spleen were collected from the offspring on postnatal day (PND) 1, 3 and 5. **Thymus** Surface molecules and gene expressions was investigated. **Spleen** Surface molecules and gene expressions was investigated.	**N:5** **K:2** **R: +**
Key findings: Fertility	Not investigated				
Key findings: Development	**Thymus:** Increase in total thymocytes and their immunophenotypes ($CD4^-CD8^-$ and $CD4^+CD8^+$ cells) **Spleen** Increase in total lymphocytes at PND 5 in males **Conclusion:** These data suggest that respiratory exposure to CB-NP during middle and late gestation may have allergic or inflammatory effects in male offspring and may provide initial information on the potential developmental immunotoxicity of nanoparticles.				
Key findings: Kinetics	Not investigated				
Other studies					
Saber et al (2013). "Particle-Induced Pulmonary Acute Phase Response Correlates with Neutrophil Influx Linking Inhaled Particles and Cardiovascular Risk." Not considered relevant as reproductive endpoints were not investigated. Liu, Y. and C. Chen (2016). "Effect on reproductive system of carbon nanomaterials." Book chapter. Only abstract available.					

Zhang, L., et al. (2019). "Pregnancy exposure to carbon black nanoparticles exacerbates bleomycin-induced lung fibrosis in offspring via disrupting LKB1-AMPK-ULK1 axis-mediated autophagy." Investigates effects on lung fibrosis and was considered out of scope based on the criteria. Chaudhuri et al (2017) Review. "Evaluating the evidence on genotoxicity and reproductive toxicity of carbon black: a critical review." The review includes the studies from Yoshida et al, (2009). Shimizu et al (2014), Onada et al (2014), El-Sayed et al (2015) and Kyjovska et al. (2013) described above. Ema et al., (2016). "Review on the reproductive and developmental toxicity of carbon based nanoparticles." The review includes the studies included above.	

Evaluation and overview

Data availability

Based on screening of the abstracts 21 publications were identified and were further examined in full text. All studies are investigations relevant for inhalational exposure to carbon black. The majority of the studies use intranasal or intratracheal administration of carbon black nanoparticles (Printex90).

Of these 21 publications two publications (Chaudhuri et al (2017; Ema et al., 2016) are review articles, while three studies (Liu et al., 2008; Zhang et al., 2019; Saber et al., 2013) were not considered relevant based on the screening criteria or only abstract available. Of the remaining publications most relevant and informative data could be extracted from 10 studies. Some covered by more than one reference (see table below). These covered three inhalation studies, three intranasal instillation studies and four studies with intratracheal administration. All studies are performed in mice. The main focus of the studies is developmental toxicity, with focus on offspring brain development and male reproductive function. The remaining studies investigated the effects on male reproductive function following exposure to carbon black in adulthood. No guideline studies were found.

Carbon black data with highest R-score

	Fertility data	Developmental toxicity data
Mice, respiratory tract	Skovmand et al., 2018 (N:5, K:2, R:++) Yoshida et al., 2009 (N:4, K:2, R:++)	Kyjovska et al., 2013 (N:4, K:2, R:++) Onoda et al., 2017b (N:5, K:2, R:++) Onoda et al., 2014 (N:6, K:2, R:++) Umezawa et al., 2018 (N:5, K:2, R:++) Skovmand et al., 2019 (N:5, K:2, R:++) Yoshida et al. (2010) (N:3, K2, R++) Jackson et a. (2011, 2012a+b) (N:6, K:2, R:++)

There is one full REACH registration (1 000 000 - 10 000 000 tonnes per annum) of carbon black available. Carbon black is not classified in the REACH dossier. The toxicological data referred to in the dossier for reproduction and developmental effects is the publication by Jackson et al. (2012a) (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16056).

Nano-characterisation

Of the 10 studies included in the table above, the N-scores for nano-characterisation of the test item were in the range of 3-6 with an average score of 4.4. Only one study had an N-score of only 3. Solubility in water was described in two publications as low (Kyjovska et al. 2013) and insoluble (Onoda et al., 2014). The solubility in the REACH dossier is given as below 1 mg/L, which was the detection limit (ECHA January 2020: https://echa.europa.eu/registration-dossier/-/registered-dossier/16056). Several available characteristics on carbon black is, however, summed up in Jackson et al. (2012a+b; 2011).

Kinetics

No data on kinetics.

Fertility

Skovmand et al., (2018) exposed mature male NMRI mice by intratracheal instillation to four different types of carbonaceous nanomaterials, including two types of carbon black particles (Printex90 and Flammrus 101) as well as graphene oxide and diesel exhaust particles. The mice were exposed once a week for seven weeks, and testes were examined for effects in sperm concentration and motility as well as daily sperm production and sperm integrity. Despite the sustained pulmonary inflammatory response, an eight-week exposure

to graphene oxide, Flammruss 101, Printex 90 and the diesel particle SRM1650b in the present study did not appear to affect semen parameters, daily sperm production or testosterone concentration in male NMRI mice.
Yoshida et al., (2009) found a decrease in the daily sperm production and testosterone levels of male ICR mice after exposure to carbon black, 0.1 mg/mouse by intratracheal administration once a week for 10 weeks. Three different sizes were tested (14, 56, 95 nm CB) and further one group received 14 nm CB, where the particle number concentration is the same as that of 56-nm. Furthermore, vacuolation of the seminiferous tubules was observed in 14-nm CB, 56-nm CB, and 95-nm CB groups. The effects of nanoparticles on the male reproductive system seemed to depend on particle mass rather than on particle number (Yoshida et al., 2009). It should be noted that the vehicle used contains 0.05% tween 80, which has lipophilic and hydrophilic properties and may enhance permeability through cellular membranes because of their effects on tight junctions.

Developmental toxicity

Developmental toxicity was studied for effects on three organ systems in the offspring, i.e. the male reproductive system, the central nervous system and the immune system.

Three studies investigated the effects of maternal exposure to carbon black on male reproductive function in the offspring:
In Kyjovska et al. (2013), the fertility of the in utero exposed offspring were investigated in C57BL/6J mice mated with CBA/J mice. The C57BL/6J mice were exposed in utero on gestation days 7, 10, 15 and 18 via maternal exposure by intratracheal instillation of 67 µg CB (Printex90)/day. The time it took breeding couples of a prenatally CB exposed F1 C57BL/6J male and a naïve CBA/J female to deliver a first F2 litter was slightly extended compared to F1 control C57BL/6J males cohabiting with naïve CBA/J females, although not statistically significant and no correlation between sperm content/daily sperm production and time-to-first F2 litter was found.

The same group performed a study in NMRI mice, exposed to Printex90 particles by whole body inhalation on GD 4 to 18. The dams were exposed to 4.6 and 37 mg/m^3 for 45 min per day. No changes in gestation length, number and loss of implantations, offspring weights, litter size and sex ratio for exposed females and offspring compared to control females and offspring were seen. Also, no significant changes were observed in body and reproductive organ weights, epididymal sperm parameters, daily sperm production, plasma testosterone or fertility of the male offspring examined though four generations (F1-F4) (Skovmand et al. 2019).

Signs of toxicity in testes and reduced DSP was found by Yoshida et al., (2010), after prenatal exposure to 14-nm carbon nanoparticles was administered intratracheally on days 7 and 14 of gestation. Contrary to this, Skovmand et al. (2019) did not find any effect in the investigated sperm parameters, which were sperm motility, daily sperm production and sperm chromatin structure. The study by Skovmand et al (2019) was an inhalation study, which is considered more relevant for human extrapolation.

Several studies have found effects on the level of glial fibrillary acidic protein (GFAP) expression in the cerebral cortex after CB exposure.

Umezawa et al., (2018) found dose dependent increase in expression of glial fibrillary acidic protein (GFAP) in astrocytes around blood vessels in the cerebral cortex and hippocampus, indicative of reactive astrogliosis, and enlarged lysosomal granules were observed in

brain perivascular macrophages in 5 week old offspring after prenatal exposure in NMRI mice. The dams were exposed by inhalation to 0, 4.6 or 37 mg/m^3 carbon black Printex 90 on GD 4 to 18 (45 min/day). The authors also observed altered offspring behavior in the open field test, and decreased number of parvalbumin-positive interneurons were decreased in the motor and prefrontal cortices at weaning (this was only investigated at the highest dose level (Umezawa et al., 2018). Very similar findings regarding expression of GPA were observed in Onoda et al. (2017b), when pregnant ICR mice were exposed intranasally to carbon black Printex 90 at 2.9, 15, or 73 µg/kg on GD days 5 and 9. Brains were again collected from male offspring at 6 weeks of age (one pup per litter for each outcome was used). An increase in GFAP expression in the cerebral cortex was detected together with increased aquaporin-4 expression in the brain parenchyma region around blood vessels and altered expression levels in the cerebral cortex of mRNAs associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production. These changes are similar to what is observed with aging (Onoda et al 2017b). This was also found in a similar study performed previously by Onoda et al. (2014).

Altered open field test behaviour after CB exposure was also found in Jackson et al. (2011) after maternal intratracheal instillation on GD 7, 10, 15 and 18 to a cumulative dose of Printex 90/animal. In this study maternal inhalation exposure to Printex90 of 42 µg/m^3 on DG8-18 also induced liver DNA damage in the mothers and the in utero exposed offspring (Jackson et al 2012a).

Two studies found effects on the offspring immune system after maternal exposure to a total of 190 µg/kg bw Printex90 by intranasal instillation on GD 5 and 9 (El-Sayed et al., 2015; Shimizu et al., 2014).

Overall evaluation

Fertility following exposure of adult animals
Intratracheal instillation of carbon black (0.1mg/mice) weekly for 10 weeks induced testicular toxicity, with decreased daily sperm production and testosterone levels of male ICR mice, but not in NMRI mice exposed to the same dose for 7 weeks. No other effects on fertility was observed. Apart from mouse strain, there are several other differences between the two studies, the most important is probably the vehicle composition, as the ICR mouse study consisted of saline with 0.05% tween 80 compared to nanopure water in the NRMI study. Hence tween possesses both lipophilic and hydrophilic properties and is therefore able to partition between lipid and protein structures. Tween is also known to enhance permeability by altering tight junctions and cellular membranes (Skovmand et al., (2018). It is, however, not known, whether this is the underlying reason for the observed difference between the studies.

Gestational exposure by the maternal airways did not seem to affect gestational and litter parameters. Findings in two studies indicated that maternal exposure could interfere with offspring development of the immune system. Equivocal results regarding the effects of maternal CB exposure and effects on sperm parameters in male offspring were observed.

Exposure of pregnant mice to carbon black via the airways results in changes in protein expression in the brain of the offspring (Onoda et al., 2014 and 2017b; Umezawa 2018). These changes were in form of increased expression of the protein GFAP. GFAP increases naturally in the brain with age, but the changes induced in one of the studies were similar to levels normally observed in much older animals (Onoda et al. 2017b). Summing up, this kind of change have been observed in several studies, in two different mouse strains and

following inhalation exposure below the Danish occupational exposure limit as well as in intranasal instillation exposure. Related changes have been observed in two additional studies, not described in detail here (Onoda et al 2017a+c). Other CNS changes included altered mRNA expression levels in the cerebral cortex associated with angiogenesis, cell migration, proliferation, chemotaxis, and growth factor production. Finally, changes in parvalbumin positive interneurons bear high resemblance to observations in established animal models of maternal inflammation. Hence, the heavily reduced expression of PV+ in the cortex are furthermore indicative of a schizophrenia-like phenotype (Umezawa et al., 2018). However, more data is needed to elucidate the effects of the altered expression levels.

Data gaps

Kinetics:
No data on the distribution of carbon black into gonads, placenta or fetus was found from the literature search.

Fertility:
Only data on inhalational exposure in mice is available concerning fertility effects. No standard OECD testing of carbon black using relevant exposure routes (oral in particular) is available.

Development:
Limited data of the developmental effects of carbon black is available. Thus, standard OECD testing determining prenatal developmental toxicity of carbon black using relevant exposure routes (oral or inhalation exposure) are missing. The histopathological changes observed in offspring of carbon black exposed mothers raise concern about the long-term functional consequences hereof, e.g. due to increased neurodegeneration.

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B.8 Other nanomaterials

Aluminium, Cadmium, Cerium, Cobalt, Copper, Gold, Iron, Lead, Mangan oxide, Nickel, Platinum, Polystyrene and Selenium

The evaluation and overview of this appendix will due to the many nanomaterials and very few references per nanomaterial be structured in another way than in the other appendices. Thus, an overview of the data is first given showing data availability, type of data, animal species, exposure route and N-, K-, R- scoring:

Data availability

	Kinetics	Fertility	Development
Aluminium	Zhang et al. 2018 (N:5; K:2; R:++) Mice nasal drip exposure	-	Zhang et al. 2018 (N:5; K:2; R:++) Mice nasal drip exposure
Cadmium oxide	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.	Blum et al. 2012 + 2014 (N:4; K:2; R:++) Mice inh.
Cerium oxide	Geraets et al. 2012 (N:8; K:1-2; R:++) Rats inh.	Qin et al. 2019 (N:5;K:2; R:++) Mice oral	-
Cobalt (tricobalt tetraoxide)	-	Hussien & Mohamed 2018 (N:3; K:2/3; R:+) Mice oral	-
Copper	Adamcakova-Dodd et al. 2015 (N:5; K:2; R:++) Mice inh.	Kalirawana et al. 2018 (N:3; K:2; R:++) Rats oral	Adamcakova-Dodd et al. 2015 (N:5; K:2; R:++) Mice inh.
Gold	Myllynen et al. 2008 (N:4; K:2; R:++) ex vivo human placenta Gupta et al. 2018 (N:4; K:2; R:++) Rat oral	Gupta et al. 2018 (N:4; K:2; R:++) Rat oral	-
Iron	Park et al. 2017 (N:4; K:2; R:++) Mice intratracheal instillation	-	Park et al. 2017 (N:4; K:2; R:++) Mice intratracheal instillation

Mangan oxide	-	Negahdary et al. 2015 (N:3; K:2/3; R:0/+) Rats oral	-
Nickel	-	Kong et al. 2014 + 2016 + 2019 (N:7; K:1; R:++) Rats oral (OECD TG 415) Hu et al. 2019 (N:7; K:2; R:+) Mice oral	-
Platinum	-	-	Park et al. 2010 (N:2-3; K:2-3; R:+) Mice oral
Polystyrene	Wick et al. 2010 (N:7; K:2; R:+) ex vivo human placenta Grafmüller et al. 2015 (N:6; K:2; R:+) ex vivo human placenta	-	-
Selenium	-	Liu et al. 2017 (N:2; K:2; R:+) Rats oral	-

Aluminium					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Development					
Zhang et al. 2018	**Cc**: Al **Pu:** - **Ps (**declaration/ measured**)**: 13/20.9 nm 50/112 nm 10/ 8.6 µm **Sh:** spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: 49 mV (13 nm) 44 mV (50 nm) **Ag:** nanoforms partly agglomerated **Em**: - **Ws**: -	ICR mice 6 female mice/group	**Route/ adm:** Respiratory tract/nasal drip **Duration/period:** Three times daily from 14 days before mating to day of giving birth **Exposure levels:** Drip of 25-35 µL x 3/day corresponding to 50 mg/kg bw/day	Offspring; new-born and adolescent testing: Neuromotor and Behavioural testing (righting reflex, cliff avoidance, endurance, open field, Morris Water Maze). Al content in hippocampus. Oxidative stress (Superoxide dismutase (SOD) and malondialdehyde (MDA)) in cerebral cortex. Neurotransmitter enzyme activity (Choline acetyltransferase (ChAT) and total cholinesterase (TChE)).	**N: 5** **K: 2** **R: ++**

Key findings: Fertility	**-**	
Key findings: Development	Newborn pups delivered by AlNP-treated mice had significantly lower BW on PD1 compared with controls. The offspring delivered by AlNP-treated females displayed stunted neurodevelopmental behaviours (significant impairments in righting reflex, cliff avoidance and endurance tests. Also, the offspring of AlNP treated mice demonstrated significantly increased anxiety-like behavior with impaired learning and memory performance at 1 month of age (strongest response for the groups exposed to 13 and 50 nm particles) Finally, increased oxidative stress (decreased superoxide dismutase activity) and decreased neurotransmitter activity (choline acetyltransferase activity) in the cerebral cortex were found in offspring with strongest response related to maternal exposure to 13 nm and 50 nm AlNP.	Nasal exposure of pregnant female mice to AlNPs -but not micro-sized particles – resulted in impaired performance in neurobehavioral testing and induced oxidative stress and alteration of neurotransmitter levels in offspring.
Key findings: Kinetics	Aluminum contents in the hippocampus of newborns from AlNP-treated groups were significantly higher than those from the bulk-Al group and control groups (the smaller the particles, the higher the Al content).	Inverse size-related distribution of Al from AlNP to hippocampus of newborn mice prenatally exposed.

Evaluation

The study by Zhang et al. (2018) demonstrates a nano-size particle related induction of neurotoxic effects in relation to behaviour, oxidative stress and neurotransmitter levels in offspring from dams exposed to AlNPs via the upper respiratory tract.

By oral exposure no developmental toxicity effects was found from prenatal developmental testing of rats at dose levels up to 1000 mg/kg bw/day of AlO_3NP (abstract by Lee et al (2015).
(Comment: It has to be noted that aluminium is very poorly absorbed (<< 1%) by the oral exposure route).

Other references

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Cadmium					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Development					
Blum et al. 2012; **Blum et al. 2014**	**Cc**: CdO freshly generated from Cd electrodes **Pu:** 99% **Ps**: 11-15.3 nm **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: measured mass conc, number conc, surface conc. in air. Nm **Ws**:	CD-1 female mice 5-21 mice/ group	**Route/ adm:** Inhalation **Duration/period:** 2.5 h daily or every other day during GD 5-17 **Exposure levels:** 100 µg/m^3 (1.8 x 10^7 particles/cm^3; 9.9 x 10^9 nm^2/cm^3) 230 µg/m^3 (8.6 x 10^6 particles/cm^3; 9.7 x 10^9 nm^2/cm^3)	Content of elemental Cd in maternal organs and in fetuses and neonatal pups. Organ weight Fertility parameters, hormone levels and growth Urine from dams and pups Expression of mRNA in kidneys from dams and pups .	**N: 4** **K: 2** **R: ++**
Key findings:	Serum 17b-estradiol (E2) decreased by ~50% in mice exposed to the higher CdO NP concentration				

Fertility	compared with air-treated controls. Daily inhalation of 230 mg CdO NP/m3 decreased the incidence of pregnancy by 23%, but had no effect on numbers of fetal resorptions, average litter size and male/female sex ratio.	
Key findings: Development	Neonatal growth lagged significantly behind control pups and decreased even further with increasing pup age at the highest dose level. CdO NP concentration did not lead to structural defects in offspring. Creatinine levels in Cd-exposed offspring not markedly different from values in control offspring. Neonatal Kim-1 mRNA expression in kidneys of offspring increased between postnatal days (PND) 7 and 14, indicating milk being the apparent source of Cd for the offspring.	
Key findings: Kinetics	The cadmium levels significantly increased in the placenta at both dose levels. Mammary glands from mice exposed at 230 CdO NP/m^3 contained approximately fivefold higher Cd levels than controls. Significantly increased levels of cadmium in neonates up to PND10.	Distribution of Cd from CdNP into mammary, gland, uterus and fetuses

Evaluation

Only relevant data for this project was found for cadmium oxide, CdO. The study reported by Blume et al (2012 + 2014) indicate adverse effects of CdONPs on fertily and development.

The authors indicate that dissolution and liberation of Cd-ions may have caused the effects. This is considered plausible as e.g. $CdCl_2$, a very watersoluble salt, is subject to EU-harmonised classification as Repr. 1B, H360FD. The less soluble substance CdO is - irrespective whether it is in nano-form or not - subject to an EU harmonized classification as Repr. 2, H361fd.

Other references

Scsuka et al. (2015). "Effects of selected metal oxide nanoparticles on ovarian steroidogenesis: Use of whole ovary culture technique". Only as a conference abstract – not further evaluated.

Zhou et al (2014). "Reproductive toxicity of nano-cadmium sulfide and normal-sized cadmium sulfide on male mice". Publication only in Chinese – not evaluated further.

References

Blum et al. (2012). "Cadmium associated with inhaled cadmium oxide nanoparticles impacts fetal and neonatal development and growth". Toxicol Sci 126 (2) 478-86.

Blum et al. (2015). "Effects of Maternal Exposure to Cadmium Oxide Nanoparticles during Pregnancy on Maternal and Offspring Kidney Injury Markers Using a Murine Model". Journal of Toxicology and Environmental Health - Part A 78 (12), 711-724.

Scsuka et al. (2015). "Effects of selected metal oxide nanoparticles on ovarian steroidogenesis: Use of whole ovary culture technique". Toxicology Letters 238 (2), 08-048.

Zhou et al (2014). "Reproductive toxicity of nano-cadmium sulfide and normal-sized cadmium sulfide on male mice". Huanjing Yu Jiankang Zazhi 31, (4), 299-301. Publication in Chinese.

Cerium, cerium oxide (CeO_2)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Qin, F., et al. (2019)	**Cc**: Cerium oxide **Pu:** > 99% **Ps**: < 25 nm **Sh:** - **Cr**: cubic crystals **Sa**: - **Sc:** - **Ch**: - **Ag:** - **Em**: 27.62 ± 3.01 nm **Ws**: - Chemically synthesized (Non-commercial)	Male C57BL/6J mice 7 weeks-old 12/group	**Route/ adm:** Oral **Duration/period:** 32 days **Exposure levels:** 0, 10, 20, 40 mg/kg bw/day	Epididymis were examined for sperm motility and DNA integrity. Blood were tested for testosterone levels. Testicular tissues were collected to determine the elemental Ce content and daily sperm production (DSP)	**N: 5** **K: 2** **R: ++**
Key findings: Fertility	CeO_2 NPs (20 and 40 mg/kg bw/day) increased elemental Ce content in testis. Histopathological examination of testis showed degeneration of cells and sperm DNA damage was increased. Decreased testis weight, DSP and sperm motility were also seen at these levels. Reductions were observed for testosterone level in blood and marker enzymes activities,				Increased sperm damage and decreased testis weight, sperm motility and daily sperm production

	and mRNA expression levels of several steroidogenesis genes such as Star, P450scc, P450c17, 3β-Hsd, and 17β-Hsd. Also gene and protein expressions of SF-1 was altered.				
Key findings: Development	**-**				
Key findings: Kinetics	-				
Kinetics					
Geraets, L., et al. (2012)	Sigma-Aldrich, Umicore, and Antaria cerium oxide nano particles **Cc**: Cerium oxide **Pu:** - **Ps**: < 5000 nm (Sigma-Aldrich), 40 nm (Umicore) and 5–10 nm (Antaria) **Sh:** spherical particles **Cr**: crystalization **Sa**: 3.73 ± 0.01 27.15 ± 0.19 63.95 ± 0.30 m^2/g **Sc:** - **Ch**: - **Ag:** Aggregates **Em**: Powder aerosolization resulted in comparable mass median aerodynamic	Male Wistar rats 3/group	**Route/ adm:** Inhalation (nose-only) **Duration/period:** 28-day study in rats, 6 h day **Exposure levels:** Total estimated inhaled dose (nose-only): <5000 nm: 4.24 mg 40 nm: 1.54 mg 5-10 nm: 0.83 mg Air concentrations: 55.00, 19.95, and 10.79 mg/m^3, respectively	Tissue distribution of cerium oxide	**N: 8** **K: 1-2** **R: ++**

	diameter (1.40, 1.17, and 1.02 mm) **Ws**: Very poor at neutral pH Chemically synthesized (Non-commercial)				
Key findings: Fertility	-				
Key findings: Development	-				
Key findings: Kinetics	After a single exposure, approximately 10% of the inhaled dose were found in lung tissue. No consistent difference of size was observed. Cerium oxide also distributed to other tissue (liver, kidney, and spleen, brain, testis, and epididymis). Slow clearance: insignificant amounts of cerium oxide were eliminated from the body at 48- to 72-h post-exposure. Rats exposed to Sigma-Aldrich cerium oxide particles showed significant decreases in brain cerium levels. Umicore exposed rats showed significant decreases in lung and liver cerium levels after recovery. Elimination of Antaria cerium oxide particles appeared to be slower.				CeO_2 was deposited in tissue including testis with poor clearance. No effect of size was found.

Evaluation

Data show evidence of CeO_2 deposition after 28-day exposure in males, with slow clearance in rats exposed to micro and nano CeO_2 particles by nose-only inhalation (Geraets, L., et al. 2012). Mice exposed orally to CeO_2 NPs for 32 days had an increased sperm damage and decreased testis weight, sperm motility and daily sperm production after exposure to 20 and 40 mg/kg bw day. No studies of developmental toxicity are available.

Other references

Kobyliak, N. M., et al. (2015). "Antioxidative effects of cerium dioxide nanoparticles ameliorate age-related male infertility: Optimistic results in rats and the review of clinical clues for integrative concept of men health and fertility." EPMA Journal, 6(1): 1878-5077. Investigated the use of citrated coated CNPs in infertility treatment of male rats. Considered out of scope for the present study.

References

Geraets, L., et al. (2012). "Tissue distribution of inhaled micro- and nano-sized cerium oxide particles in rats: Results from a 28-day exposure study." Toxicological Sciences 127(2): 463-473.

Qin, F., et al. (2019). "SF-1 mediates reproductive toxicity induced by Cerium oxide nanoparticles in male mice." J Nanobiotechnology 17(1): 41.

Cobalt					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Hussien & Mohamed 2018	**Cc**: Co_3O_4 (tricobalt tetraoxide or cobalt (II,III oxide)) **Pu:** 99.5% **Ps**: < 50 nm **Sh:** - **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: -	Male Swiss albino mice 3 adult males/group	**Route/ adm:** Oral gavage **Duration/period:** Once daily for 3 days **Exposure levels:** 0, 20 mg/kg (/day? not clearly stated))	Micronucleus (MN) assay on bone marrow, Comet assay. Semen evaluation, epididymis	**N:3** **K: 2/3** **R: +**
Key findings: Fertility	Co_3O_4NPs administration significantly increased the number of micronucleated polychromatic erythrocytes (PCEs)/1000 PCEs and DNA damage in bone marrow cells. Further, significantly decreased sperm motility and sperm concentration was found in the dosed group compared to the control group.				
Key findings: Development	-				
Key findings: Kinetics	**-**				

Evaluation
Adverse effects on semen quality is observed to be induced by nanoparticles of tricobalt tetraoxide by Hussien & Mohamed (2018). This could likely be a consequence of dissolution of the free cobalt ion from the large surface area of the nanoparticles, as soluble cobalt, i.e. cobalt dichloride, is subjected to EU-harmonised classification as Repr 1B, H360F. In the REACH registration of tricobalt tetraoxide a water solubility of 1.62 mg/L at 20°C is given (particle size of test substance not indicted) which indicates that the substance is not insoluble. It is however not known if toxicity differs between nanosized Co-particles and Co in other forms.
References
Hussien & Mohamed (2018). "The protective role of omega-3 against genotoxicity and reproductive toxicity of cobalt oxide nanoparticles acute treatment in male mice". Asian Journal of Pharmaceutical and Clinical Research 11 (5), 423-428.

Copper (CuNP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Kalirawana et al. 2018	**Cc**: Cu **Pu:** - **Ps**: 40 nm and 60 nm **Sh:** **Cr**: - **Sa**: - **Sc**: Polyvinylpyrrolidone coated **Ch**: - **Ag:** - **Em**:	Male albino Wistar rats 6 males/group	**Route/ adm:** Oral gavage **Duration/period:** Once daily for 45 days **Exposure levels:** 0, 1, 2 mg/kg bw/day	Testes weight, Epididymal sperm parameters Level of male sex hormones Reproductive function	**N:3** **K:2** **R: ++**

	Ws:-				
Key findings: Fertility	Significant decrease ($p \leq 0.01$) in the weight of the testes, attributable to the loss of germ cells and elongated spermatids. Spermatogenic potential reduced by reducing the number of sertoli cells. A significant decrease ($p \leq 0.05$) in the motility and density of the caudal epididymal sperm in all exposed groups. Testosterone were significantly reduced with the treatment with Cu NPs. The control rats showed a 100% fertility rate by mating exposure test. Rats administered with copper nanoparticles at doses of 1 and 2 mg kg/bw/day revealed 60 and 70% negative fertility with the 40 nm copper NP and 40 and 50% of negative fertility with 60 nm copper NP treated rats as compared to the control group.				Repeated oral exposure to CuNPs caused severe negative impact on sperm and fertility in male rats at exposure to 1 and 2 mg/kg bw/day.
Key findings: Development	**-**				
Key findings: Kinetics	**-**				
Development					
Adamcakova-Dodd et al. 2015	**Cc**: Cu **Pu:** - **Ps**: 15.7 nm **Sh:** - **Cr**: - **Sa**: 14.6 m^2/g **Sc**: Cu2O and CuO on surface **Ch**: - **Ag:** **Em**: 35.6 nm in exposure chamber **Ws**: -	Female (C57Bl/6 J) mice 9 pregnant female mice/group 10 non-pregnant female mice/group	**Route/ adm:** Inhalation **Duration/period:** 4 hrs/day on GD 3–19 **Exposure levels:** 3.5 mg/m^3	Cytokine/chemokine concentrations were determined in BAL fluid and plasma of dams/non-pregnant mice and pups. Lungs and placentas were evaluated for histopathological changes. Gene expression of the Th1/Th2 profiles were analysed in spleens of pups.	**N: 5** **K: 2** **R: ++**
Key findings: Fertility	-				
Key findings: Development	During the time of exposure, pregnant exposed mice gained significantly less weight than pregnant controls. Histopathological evaluation of placentas did not identify changes related to exposure. Survival rate of 7-week-old pups prenatally exposed to Cu NPs was significantly lower than in control pups (73 vs. 97 %). Expression of several Th1/Th2 and other genes related to the immune response in offspring spleens				

	were significantly up- or down-regulated, indicating strong immunomodulatory effects.	
Key findings: Kinetics	No translocation of Cu into the placenta or the fetus was found by inductively coupled plasma-mass spectroscopy.	
Evaluation		
Only sparse data on CuNP is available, however, indicating concern for developmental and reproductive toxicity. Inhalation of 3.5 mg/m3 to CuNP in female pregnant mice during GD 3-19 resulted in a significant reduced survival rate of the offspring. In male rats repeated oral exposure to CuNP caused severe negative impact on sperm production that affected fertility at exposure levels of 1 and 2 mg/kg bw/day.		
Other references		
Zhang et al. (2018). "Copper nanoparticles show obvious in vitro and in vivo reproductive toxicity via ERK mediated signaling pathway in female mice". Int. Biol. Sci. 14(13), 1834-1844. Mouse study using i.v. exposure to CuNP" – not further evaluated.		
References		
Kalirawana et al. (2018). "Reproductive toxicity of copper nanoparticles in male albino rats". International Journal of Pharma Research and Health Sciences 6 (1), 2258-2263. **Adamcakova-Dodd et al. (2015).** " Effects of prenatal inhalation exposure to copper nanoparticles on murine dams and offspring". Part Fibre Toxicol 12, 18pp **Zhang et al. (2018). "**Copper nanoparticles show obvious in vitro and in vivo reproductive toxicity via ERK mediated signaling pathway in female mice". Int. Biol. Sci. 14(13), 1834-1844.		

Gold (AuNP)					
Reference	**Test material, nanocharacterisation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Gupta et al. 2018	**Cc**: Au **Pu:** - **Ps**: 5-20 nm (15 ±	Wistar rats 8 males/group	**Route/ adm:** Oral gavage	Rats were sacrificed 12-16 hours after the last administration.	**N: 4** **K: 2** **R: ++**

	5 nm) **Sh:** round or spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** well dispersed in solution **Em**: - **Ws**: - Chemically synthesized (Non-commercial)		**Duration/period:** 90 days **Exposure levels:** 0, 20 µg/kg bw/day	Testes examined for presence of gold (TEM*) and histopathology was performed	
Key findings: Fertility	No mortality, morbidity or gross behavioural changed were observed. Mild degeneration of testicular tissue, but histopathological analysis showed presence of all stages of testicular cells.				
Key findings: Development	**-**				
Key findings: Kinetics	Gold nanoparticles (AuNPs) were distributed and accumulated in the majority of the testicular tissue. Electron micrographs showed aggregates of gold nanoparticles in interstitial spaces of the testis including seminiferous tubules. Large aggregates were detected near Leydig cells and crossing the outer membrane of Leydig cells and inside Leydig cells, but Leydig cells appeared structurally intact. GNPs were also detected in Sertoli cell cytoplasm and membrane bound GNPs were detected close to developing spermatids as well as in germ cell cytoplasm entrapped in lysosomal bodies.				AuNP present in testicular tissue, indicate that AuNP can cross the blood-testis barrier
Kinetics					
Myllynen et al. 2008.	**Cc**: Au **Pu: -** **Ps**: 10, 15 or 30 nm **Sh:** - **Cr**: - **Sa**: - **Sc**: coated with	Human placenta One perfusion per size	**Duration/period:** Open perfusions – once through (18 min) Closed perfusions: 6 h perfusions	Open perfusions: Samples every 3 min from both reservoirs Closed perfusions: Samples were taken from maternal and fetal reservoirs every half hour	**N: 4** **K: 2** **R: ++**

	polyethylene glycol (PEG) **Ch**: - **Ag:** Monodispersed **Em**: **Ws**: Synthesized – non commercial NPs		**Level in maternal compartment:** Open perfusions (one through), total amount: 7.9×10^{11} 15nm AuNP 7.8×10^{10} 30nm AuNPs Closed perfusions: 9.1×10^{9} 10 nm AuNPs/ml 2.0×10^{9} 15nm AuNP/ml	for the first 2 h, and once per hour thereafter.	
Key findings: Fertility	-				
Key findings: Development	**-**				
Key findings: Kinetics	No transfer of gold nanoparticles to the fetal compartment (detection limit 0.045 ppb). AuNP present in placental tissue mainly in the trophoblastic cell layer.				No transfer of AuNPs across the placenta

Evaluation

The study by Gupta et al. (2018) is a 90-day study in 90 days Wistar rats exposed to gold nanoparticles (15 ± 5 nm) at 20 µg/kg bw/day. No mortality, morbidity or gross behavioural changed were observed. AuNP distributed and accumulated in the majority of the testicular tissue, with no to mild toxicity in testes. The study finds that AuNP is present in testicular tissue, which indicate that AuNP can cross the blood-testis barrier

Placental transfer of AuNPs was investigated in *ex vivo* human placenta perfusion model by Myllynen et al. (2008). Perfusions were performed with AuNPs in three sizes (10, 15 or 30 nm). No transfer of AuNPs across the placenta was found, but AuNPs were found in placental tissue.

Other references

Barcikowski, S., et al. (2015). "Influence of gold, silver and gold-silver alloy nanoparticles on germ cell function and embryo development." Beilstein Journal Of Nanotechnology 6: 651-664. Is a review article with main focus on in vitro studies.

Buchtova, M., et al. (2014). "Embryonic Toxicity of Nanoparticles." Is a review article with on embryonic development.

Semmler-Behnke, M., et al. (2007). "Uptake of 1.4 nm versus 18 nm gold nanoparticles in secondary target organs is size dependent in control and pregnant rats after intratracheal or intravenous application. Only available as a conference abstract.

Tian, X., et al. (2013). "Intrauterine inflammation increases materno-fetal transfer of gold nanoparticles in a size-dependent manner in murine pregnancy." Small 9(14): 2432-2439. Investigates placental transfer og GNPs with intrauterine infection. The exposure route was i.v. The study was therefore not considered relevant for the present project.
References
Gupta, H., et al. (2018). "Evaluation of histopathological and ultrastructural changes in the testicular cells of Wistar rats post chronic exposure to gold nanoparticles." Indian Journal of Biotechnology, 17(1): 9-15. **Myllynen, P. K., et al. (2008).** "Kinetics of gold nanoparticles in the human placenta." Reproductive Toxicology, 26(2): 130-137.

***TEM = Transmission electron microscopy

Iron					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Development					
Park et al. 2017	**Cc**: FeNP **Pu:** - **Ps**: - **Sh:** rod-shaped **Cr**: - **Sa**: - **Sc**: - **Ch**: 11.9 ± 2.6 and −219.1 ± 14.0 in PBS and Gambles solution, respectively **Ag:** - **Em**: Hydrodynamic Diameter: 209.4 ±	ICR mice, male and female 10 parents/dose/sex 12 offspring/dose/sex	**Route/ adm:** Intratracheal instillation **Duration/period:** Single instillation 14 days pre-mating and in offspring at 5 weeks of age **Exposure levels:** 1, 2, and 4 mg/kg	Modified OECD 421	**N: 4** **K: 2** **R: ++** Preconceptional exposure

	98.0 and 45.1 ± 2.6 nm in PBS and Gambles solution, respectively **Ws**: - Chemically synthesized (Non-commercial)				
Key findings: Fertility	Two female parent mice died at 4 mg/kg. Expression of MHC class II molecules were enhanced in the parental mice exposed to the highest dose of FeNPs.				
Key findings: Development	Increased mortality and significant hematological- and biochemical- changes were observed in offspring at 4 mg/kg, especially in females. The sex ratio (male/female) of the offspring mice increased in the groups exposed to FeNPs (no statistical information on significance).				Adverse effects observed at 4 mg/kg. Females may be more sensitive
Key findings: Kinetics	Iron accumulation was observed in the ovary and the testis of parent mice exposed to the highest dose of FeNPs.				Iron accumulation observed in the ovary and the testis of adult mice
Evaluation					
One study with intratracheal instillation was identified. Increased mortality and significant hematological- and biochemical- changes were observed in offspring of mice exposed to 4 mg/kg 24 days prior to mating, i.e. preconceptionally.					
Other references					
Valdiglesias et al. 2015 "Effects of IronOxide Nanoparticles: Cytotoxicity, Genotoxicity, Developmental Toxicity, and Neurotoxicity" is a review. No new studies were identified.					
References					
Park et al. 2017. Deleterious effects in reproduction and developmental immunity elicited by pulmonary iron oxide nanoparticles Environmental Research 152 (2017) 503–513					

Lead
Not evaluated further, see below.
References
Scsuka et al. (2015). "Effects of selected metal oxide nanoparticles on ovarian steroidogenesis: Use of whole ovary culture technique". Conference Abstract. Toxicolology Letters SUPPL. 1, pp. S211

Cao et al. (2016). "Rat Testis Damage Caused by Lead Sulfide Nanoparticles After Oral Exposure". Journal of Nanoscience and Nanotechnology 16(3):2378-2383.

Mangan oxide (Mn_2O_3NP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Negahdary et al. 2015	**Cc**: Mn_2O_3 **Pu:** **Ps**: 70 nm **Sh:** spherical **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: Mn_2O_3NP specifically synthesised for the testing	Male Wistar rats 10 rats/group	**Route/ adm:** Oral gavage **Duration/period:** Once daily for 14 days **Exposure levels:** 0, 100, 200 and 400 ppm solutions (dose volume not indicated!)	Blood hormone levels. Histopathology of testes .	**N:3** **K: 2/3** **R: 0/+**
Key findings: Fertility	Significant reduction in luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone, and reduced numbers of spermatogonial cells, primary spermatocytes, spermatids and Leydig cells were observed after treatment with Mn_2O_3 nanoparticle at 400 ppm compared with controls. It was concluded the Mn_2O_3 NP at a dose concentration of 400 ppm can reduce sex hormones, sperm production and damage the testicular cytology.				Mn_2O3NP may induce sex hormones, sperm production and cytotoxic damages in the testes of male rats.

Key findings: Development	**-**	
Key findings: Kinetics	**-**	
Evaluation		
Very limited data available on MnNPs. In a study with limited reporting (no specific dose level) effects on sex hormones and sperm production and toxicity in testes was reported after oral exposure in male rats.		
References		
Negahdary et al. (2015). "Toxic effects of Mn2O3 nanoparticles on rat testis and sex hormone". Journal of Natural Science, Biology and Medicine 6(2), 335-339.		

Nickel (NiNP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Kong et al. 2014 + 2016 + 2019	**Cc**: Ni **Pu:** 99% **Ps**: 30-100 nm **Sh:** spherical **Cr**: - **Sa**: > 8 m^2/g **Sc**: - **Ch**: - **Ag:** agglomeration **Em**: in medium 400-879 nnm **Ws**: Other test item:	Sprague-Dawley rats 10 males and 20 females/ group	**Route/ adm:** Oral gavage **Duration/period:** Daily exposure of both sexes from 10 weeks before mating. Females further exposed daily during gestation and lactation period. **Exposure levels:** 0, 5, 15, 45 mg/kg	One generation reproduction toxicity study according to OECD TG 415 Histopathological examination of organs Sperm analysis. Sex hormone levels. Indicators for reactive oxygen species (ROS), oxidant and antioxidant enzymes, and cell apoptosis-related factors	**N: 7** **K: 1** **R: ++** Nanoparticles compared with micrometer particles Tested according to OECD TG 415

	3.3 µm Ni particles		bw/day (3.3 µm Ni particles only tested at 45 mg/kg)		
Key findings: Fertility	Mating success and pregnancy rate was not significantly affected by the NiNP exposure. Exposure affected sex hormone levels in female rats and caused histopathological changes in the ovaries. In male rats histopathological changes in the testes and decreased sperm quality were observed. Such findings were also observed for Ni particles with a diameter of 3 µm (only one dose level at 45 mg/kg bw/day), but for hormone levels nanosized particles seemed to induce more pronounced effects than microsized particles. Based on mechanistic indicators it was concluded that oxidative stress and cell apoptosis may play a role in inducing male and female reproductive toxicity by nickel.				Identical adverse reproductive effects were found for the nanoform and the nano and non- nanoform of nickel.
Key findings: Development	Exposure to Ni particles negatively impacted birth survival rates at all dose levels and at a higher degree for nanoparticles compared to microparticles.				
Key findings: Kinetics	-				
Hu et al. 2019	**Cc**: Ni **Pu:** 99% **Ps**: 30-100 nm **Sh:** spherical **Cr**: - **Sa**: > 8 m^2/g **Sc**: - **Ch**: - **Ag:** agglomeration **Em**: in medium 400-879 nnm **Ws**: - Other test item: 3.3 µm Ni micro particles (NiMP)	Male ICR mice 12 mice/group	**Route/ adm:** Oral gavage **Duration/period:** Once daily for 30 days **Exposure levels:** 0, 5, 15, 45 mg/kg bw/day (3.3 µm Ni particles only tested at 45 mg/kg)	Organ weight and hisptopathological examination of testes and epididymis. Sperm analysis.	**N: 7** **K: 2** **R: +** Nanoparticles compared with micrometer particles
Key findings: Fertility	Ni NPs mainly induced damage to the reproductive system of male mice by affecting spermatogenesis and testicular structure. There was a significant decrease in the percentage of progressive sperm count in mid-dose NiNP-group and high dose following exposure to the 3.3 µm Ni				

	particles. Regarding sperm motility it was found that Ni MPs had a similar negative impact as Ni NPs. Pathological results showed cell apoptosis and disordered arrangement of cells in the seminiferous tubules of the NiNP exposed groups being most pronounced in the high dose group (histopathological data on NiMP not given).	
Key findings: Development	**-**	
Key findings: Kinetics	**-**	

Evaluation

Oral exposure to NiNP induced testicular toxicity and negatively affected sperm quality in male rats and male mice. In rats where female rats were exposed during gestation decreased birth survival rate was observed.
It is to be noted that nickel powder is subject to EU-harmonised classification, but without classification for reproductive toxicity, whereas soluble nickel salts e.g. $NiCl_2$ among several human health end-points is subject to a harmonized classification as Repr. 1B, H360D. Thus, the reproductive toxicity of metallic nickel as micro- and nanoparticles is most likely due to the dissolution and liberation of free Ni-ions from the particle surfaces of metallic nickel. The data indicate that metallic nickel as nanoparticles may cause adverse effects on fertility and development as well.

References

Kong et al. (2014). "Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats". International journal of molecular science 15 (11), 21253-21269.

Kong et al. (2016). "Mechanisms involved in reproductive toxicity caused by nickel nanoparticle in female rats". Environmental Toxicology 31 (11), 1674-1683.

Kong et al. (2019). "Mechanisms underlying nickel nanoparticle induced reproductive toxicity and chemo-protective effects of vitamin C in male rats". Chemosphere 218, 259-265.

Hu et al. (2019). "Study on the damage of sperm induced by nickel nanoparticle exposure". Environmental Geochemistry and Health https://doi.org/10.1007/s10653-019-00364-w

Platinum					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Development					
Park et al. 2010	**Cc**: Platinium **Pu:** - **Ps**: 20.9±11.4 nm **Sh:** particles (no specific shape) **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: - **Ws**: - Chemically synthesized (Non-commercial)	Male and female ICR mice 11/sex/group	**Route/ adm:** Oral **Duration/period:** M: 14 days before mating, daily F: 14 days before mating until PND4, daily **Exposure levels:** 0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg	Maternal and pup toxicity were evaluated.	**N: 2-3** **K: 2-3** **R: +** Low level of reporting and NP characterisation
Key findings: Fertility	-				
Key findings: Development	PNPs did not affect blood biochemical parameters or mortality in dams during the experimental period. Histopathological signs were not observed in dams and pup number and incidence of malformations and stillbirth did not differ between the control and treated groups. Increased pup mortality (6, 6, and 8 dead on PND 4 vs. 2 in control) and decreased offspring growth rate (1.07±0.09 g in the 0.25 mg/kg-treated group, 1.10±0.04 g in the 0.5 mg/kg-treated group, and 1.02± 0.06 g in the 1 mg/kg-treated group, while the body weight of the pups in the control group was 1.27± 0.09 g) during the lactation period at all doses. No information on statistical significance is provided.				Increased pup mortality and decreased infant growth rate during the lactation period.

Key findings: Kinetics	-	
Evaluation		
One study with oral administration was identified, where exposure to 0.25, 0.5 and 1 mg/kg in pregnant dams from two weeks prior to mating and until postnatal day 4 decreased infant growth rate during lactation. Information on statistical significance is however not provided.		
References		
Park et al. 2010. Effects of Platinum Nanoparticles on the Postnatal Development of Mouse Pups by Maternal Exposure. Environmental Health & Toxicology 25 (4), 279-286.		

Polystyrene					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Kinetics					
Wick et al. 2010	**Cc**: fluorescent polystyrene beds **Pu:** - **Ps**: 50/ 80/ 240/ 500 nm particles **Sh:** spherical **Cr**: - **Sa**: 1.9/ 5.4/ 4.5/ 2.0 nm^2 **Sc**: - **Ch**: -59/-56/-33/-42 mV **Ag:** both as dispersed and as	Human placenta	**Route/ adm:** exposure in materanal perfusion media **Duration/period:** Up to 6 hours of perfusion **Exposure levels:** Perfusion medium: 25 µg/mL For the various particle sizes particle number	*Ex vivo* human placental perfusion model. Analytical determination of maternal and foetal circulation .	**N: 7** **K: 2** **R: +** (exposure route comparable to i.v. exposure).

	agglomerates **Em**: zeta potential, mass conc. and surface conc. determined in medium **Ws**: -		concentrations in the range of $3.5x10^8$ – $3.9x10^{11}$ particles/mL was measured.		
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	At 180 min the following particle concentrations were found in the foetal perfusate: 50nm: 8.9 µg/mL; 80 nm: 7.5 µg/mL; 240 nm: 2.0 µg/mL; 500nm: 0.3 µg/mL. Thus, fluorescent polystyrene particles with diameters up to 240 nm were taken up by the placenta and were able to cross the placental barrier without affecting the viability of the placental explant.				NPs to a high degree were able to penetrate the human placenta *ex vivo.*
Grafüller et al. 2015	**Cc**: polystyrene beds Non-functionalized and carboxylate-modified (-COOH) **Pu:** - **Ps**: 50, 240, 300 nm **Sh:** spherical **Cr**: - **Sa**: 4.94×10^{14} - 3.27×10^{15} nm2/mL - **Sc**: - **Ch**: - **Ag:** both as dispersed and as agglomerates **Em**: Surface area		**Route/ adm:** Exposure in materanal as well as fetal perfusion media **Duration/period:** Perfusion up to 6 hours **Exposure levels:** Particle number concentrations In the range of 1.88×10^9 - 5.45×10^{11} particles/mL for the various sizes.	*Ex vivo* human placental perfusion model. Determination of both maternal to fetal transfer and fetal to maternal transfer. .	**N:6** **K: 2** **R: +** (exposure route comparable to i.v. exposure).

	and particle no determined **Ws**:-				
Key findings: Fertility	**-**				
Key findings: Development	**-**				
Key findings: Kinetics	Transport of polystyrene particles in the fetal to maternal direction was significantly higher than for the maternal to fetal direction. Regardless of their ability to cross the placental barrier and the direction of perfusion, all polystyrene particles accumulated in the syncytiotrophoblast of the placental tissue. The data indicated that the syncytiotrophoblast is important in regulating nanoparticle transport across the human placenta, most likely as an active, energy-dependent transport pathway. COOH functionalised beads were transferred across the placenta in significantly lower amounts than non-functionalised particles.				

Evaluation

Experiments with e*x vivo* human placental perfusion models indicate size-dependent transfer of polystyrene nanoparticles across the placenta. Also, COOH functionalised beads were transferred across the placenta in significantly lower amounts than non-functionalised particles. Fetal to maternal transfer direction found to be more efficient than maternal to fetal transfer indicating that transplacental transport most probably is facilitated by active transport.

Other references

Grafmüller et al. 2013. ”Transfer of engineered nanoparticles across the human placenta”. Toxicology Letters 221 (1), 24-26. Only as conference abstract. Not included in the table.

References

Grafmüller et al. 2013. ”Transfer of engineered nanoparticles across the human placenta”. Toxicology Letters 221 (1), 24-26.

Grafmüller et al. (2015). “Bidirectional transfer study of polystyrene nanoparticles across the placental barrier in an ex vivo human placental perfusion model“. Environmental Health Perspectives 123 (12), 1280-1286.

Wick et al. (2010). “Barrier capacity of human placenta for nanosized materials”. Environmental Health Perspectives 118(3), 432-436.

Selenium (SeNP)					
Reference	**Test material, nanocharactersation**	**Species/ strain. No/group**	**Exposure specifications**	**Study design/ examinations (tissues/ organs/ parameters)**	**Quality and relevance** **N-score (1-11)** **K-score (1-4)** **R-score (0,+,++)** **Comments**
Fertility					
Liu et al. 2017	**Cc**: Se **Pu:** - **Ps**: 80 nm **Sh:** **Cr**: - **Sa**: - **Sc**: - **Ch**: - **Ag:** - **Em**: **Ws**: SeNP specifically synthesised for the testing	Male Sprague-Dawley rats 10 rats/group	**Route/ adm:** Oral gavage **Duration/period:** Once daily for 14 days **Exposure levels:** 0.2, 0.4, or 0.8 mg Se/kg bw/day and 2.0, 4.0, or 8.0 mg Se/kg bw/day	Biochemical parameters gene expression of GPx1 and GPx4 in testes histopathological evaluation of testes sperm analysis	**N: 2** **K: 2** **R: +**
Key findings: Fertility	The three lowest doses significantly promoted sperm motility and movement parameters. Levels of 4.0 and 8.0 mg Se/kg bw/day significantly reduced the testis weight, sperm concentration and motility, and caused histopathological injury of testis and epididymis tissues to various degrees.				
Key findings: Development	**-**				
Key findings: Kinetics	**-**				

Evaluation
Very limited data available on SeNPs. Some initial findings suggest adverse effects on sperm production and testes in male rats.
References
Liu et al. (2017). "Effects of Selenium Nanoparticles on Reproductive Performance of Male Sprague-Dawley Rats at Supranutritional and Nonlethal Levels". Biological Trace Element Research 180 (1), 81-89.