Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

Titanium dioxide

EC Number: 236-675-5
CAS Number: 13463-67-7

CLH-O-0000001412-86-163/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

14 September 2017

CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

Substance Name: Titanium dioxide

EC Number: **236-675-5**

CAS Number: **13463-67-7**

Index Number: **-**

Contact details for dossier submitter:

ANSES (on behalf of the French MSCA)

14 rue Pierre Marie Curie

F-94701 Maisons-Alfort Cedex

reach@anses.fr

Version number: **2** **Date:** **May 2016**

CONTENTS

Part A.

Part B.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	Titanium dioxide
EC number:	236-675-5
CAS number:	13463-67-7
Annex VI Index number:	-
Degree of purity:	>87%
Impurities:	Confidential

Substance name:	Anatase (TiO_2)
EC number:	215-280-1
CAS number:	1317-70-0
Annex VI Index number:	-
Degree of purity:	>87%
Impurities:	Confidential

Substance name:	Rutile (TiO_2)
EC number:	215-282-2
CAS number:	1317-80-2
Annex VI Index number:	-
Degree of purity:	>87%
Impurities:	Confidential

There is only one registration dossier (EC no 236-675-5) for "titanium dioxide" which indicates that there is sufficient data to support a mono-constituent substance under REACH of "all crystal phases&hydrates of titanium dioxide including rutile, anatase, monohydrate and dehydrate". Crystal phase, morphology, lattice stabilizers or surface treatment included in the scope of this REACH registration dossier are not clearly reported. There is also one registration dossier for the specific crystal phase "rutile" TiO_2 under CAS 1317-80-2.

As further detailed in the dossier, TiO2 is considered poorly soluble particles and the main proposed mechanism of carcinogenicity by inhalation is thus based on the low solubility and biopersistency of the particles leading to pulmonary inflammation then oxidative stress. Secondary genotoxicity and cell proliferation result in carcinogenicity. Nevertheless, possible direct genotoxicity cannot be excluded.

Based on available evidence and information in the registration dossier (e.g. mechanism of carcinogenicity, characterization of the particles), the proposed scope for the Annex VI entry is: "**Titanium dioxide in all phases and phase combinations; particles in all sizes/morphologies**".

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	None
Current proposal for consideration by RAC	Carc. 1B – H350i
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 1B – H350i

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives				Not evaluated
2.2.	Flammable gases				Not evaluated
2.3.	Flammable aerosols				Not evaluated
2.4.	Oxidising gases				Not evaluated
2.5.	Gases under pressure				Not evaluated
2.6.	Flammable liquids				Not evaluated
2.7.	Flammable solids				Not evaluated
2.8.	Self-reactive substances and mixtures				Not evaluated
2.9.	Pyrophoric liquids				Not evaluated
2.10.	Pyrophoric solids				Not evaluated
2.11.	Self-heating substances and mixtures				Not evaluated
2.12.	Substances and mixtures which in contact with water emit flammable gases				Not evaluated
2.13.	Oxidising liquids				Not evaluated
2.14.	Oxidising solids				Not evaluated
2.15.	Organic peroxides				Not evaluated
2.16.	Substance and mixtures corrosive to metals				Not evaluated
3.1.	Acute toxicity - oral				Not evaluated
	Acute toxicity - dermal				Not evaluated
	Acute toxicity - inhalation				Not evaluated
3.2.	Skin corrosion / irritation				Not evaluated
3.3.	Serious eye damage / eye irritation				Not evaluated
3.4.	Respiratory sensitisation				Not evaluated
3.4.	Skin sensitisation				Not evaluated
3.5.	Germ cell mutagenicity				Inconclusive
3.6.	Carcinogenicity	Carc. 1B – H350i			Conclusive and sufficient for classification
3.7.	Reproductive toxicity				Not evaluated
3.8.	Specific target organ toxicity –single exposure				Not evaluated
3.9.	Specific target organ toxicity – repeated exposure				Not evaluated
3.10.	Aspiration hazard				Not evaluated

4.1.	Hazardous to the aquatic environment				Not evaluated
5.1.	Hazardous to the ozone layer				Not evaluated

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Danger

Hazard statements: H350i

Hazard pictogram: GHS08

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

There is no current harmonized classification for titanium dioxide.

2.2 Short summary of the scientific justification for the CLH proposal

Commercially, titanium dioxide (CAS no 13463-67-7) particles range from non-nano (bulk) to nanosizes that can aggregate or agglomerate. Primary particles are single crystals that are bound in crystal planes. Aggregates are sintered primary particles that are connected by crystal faces. Agglomerates are multiple primary particles and aggregates that are held together by van der Waal's forces (IARC, 2006). Three main crystal structures are clearly described: rutile (CAS no 1317-80-2), anatase (CAS no 1317-70-0) and brookite (CAS no 12188-41-9). Anatase and rutile are tetragonal, brookite is orthorhombic. In all polymorphs, titanium is coordinated octahedrally by oxygen, but the position of the octahedral differs between polymorphs. The structure of rutile is the densest and its unit cell is the smallest. Anatase has four formula units per unit cell with a = 0.379 nm and c = 0.951 nm; rutile has two with a = 0.459 nm and c = 0.296 nm; brookite has eight with a = 0.917 nm, b = 0.546 nm and c = 0.514 nm. Only the structures of rutile and anatase are reported in commercial products (IARC, 2006; INRS 2013) and could also be mixture combination of anatase/rutile (P25). Titanium dioxide can be formulated in different shapes (spheres, nanorods, nanowires, nanotubes, thin films or nanoporous structures…). Dimension of all these forms (from nanosize to bulk size) vary widely depending on the manufacturer and uses of titanium dioxide. Titanium dioxide can also be modified by using various coatings (including aluminum oxide, silicon dioxide, calcium salts…) or dopant agents to enhance or maintain its properties.

In the current REACH registration database there is one registration for "titanium dioxide" with 130 members in April 2016. This registration stated that it intends to cover "all crystal phases&hydrates of titanium dioxide including rutile, anatase, monohydrate and dihydrate". However, the types and number of compositions considered to be covered in terms of crystalline phase, morphology and surface chemistry are not transparently (and exhaustively) reported. Due to this lack of transparency, the impact on the hazard profile when the parameters vary cannot be established from the information included in the registration dossier. However it is clearly stated in the registration dossier that all possible variations are considered equivalent in terms of hazard profile. Taking these statements into account, the approach applied in the REACH dossier was used to support the scope of the proposed entry in Annex VI of CLP.

In the context of dossier evaluation under REACH, a final decision has been issued by ECHA to the lead registrant with requests to transparently report the scope of the registered substance in terms of crystalline phase, morphology and surface chemistry. The information was considered by ECHA to be a prerequisite to the assessment of the data submitted in accordance with Annexes VII-XI of the REACH Regulation.

In this context, FR-MSCA focuses on a hazard for which commonalities can be proposed independent of crystalline phase, morphology and surface chemistry variability (and all possible combinations thereof).

Although it was initially foreseen to propose a harmonized classification for mutagenicity, this hazard category has been put aside from the proposal because the existing data show too many discrepancies that cannot be explained with the current state of the science. Indeed, the FR-MSCA was not able to

identify specific physicochemical parameter justifying the discrepancies along the mutagenic results and whether the differences reported in the results could be due to different study protocols having been employed. For this endpoint, further data are necessary to consolidate the existing data and see if specific forms are leading to more severe toxicity than others. Genotoxicity dataset on TiO_2 is therefore only presented as supporting data for carcinogenicity endpoint, and summarized in Annex I.

This CLH report therefore focuses on carcinogenicity of TiO_2. Indeed, because the carcinogenic mode of action of TiO_2 seems to be rather due to inflammatory process and oxidative stress, it is believed that biopersistence and solubility are relevant to explain this toxicological effect. All possible crystal modifications, morphologies and surface chemistries in all possible combinations of TiO_2 are expected to be biopersistent and of poor solubility, and therefore covered by this CLH dossier. Indeed TiO_2 in all these combination is considered to behave in the same way as other poorly soluble low toxicity particles (e.g. coal dust, diesel exhaust particulates, toner …). This statement does not preclude that some parameters (in particular shape and coating) might also lead to a more potent carcinogenicity or to other specific lesions *via* a specific mode of action. The proposal presented below is based on data considered sufficient by MSCA-FR to propose a general entry for classification of TiO_2 for Carcinogenicity by inhalation. In case new data is available, the entry may be modified upon submission of these data by the registrant.

Carcinogenicity

Human data do not suggest an association between occupational exposure to TiO_2 and risk for cancer. However, all these studies have methodological limitations and the level of exposure reported is debatable.

In experimental animal studies, lung tumours were reported after inhalation or intra-tracheal administration of TiO_2 (fine rutile, anatase/rutile P25 nano-TiO_2 and nano-rutile) in rats in an overload context. Overload is defined by an impairment of normal pulmonary clearance due to high accumulation of particles. Although inter-species variability was found in particle retention, the overload concept is relevant for humans, and in particular for workers exposed to high dust concentrations. Furthermore, it appears that lung retention and chronic pulmonary inflammation occurring in humans are consistent with the findings in rats. Although benign lung tumours (bronchioalveolar adenomas) were observed in both sexes, malignant tumours (squamous cell carcinomas and bronchioalveolar adenocarcinomas) were only reported in female rats. Cystic keratinizing tumours were also reported but the relevance to human remains unclear. Based on these effects, IARC (2006) concluded that there is sufficient evidence that TiO_2 is carcinogenic in animals.

Although the full mode of action is still unclear, an inflammatory process and indirect genotoxic effect through ROS production seems to be the major mechanism to explain the effects induced by TiO_2. It is considered that this mode of action is principally due to the biopersistence and poor solubility of the TiO_2 particles. However, a genotoxic effect by direct interaction with DNA cannot be excluded since TiO_2 was found in the cell nucleus in various *in vitro* and *in vivo* studies. The proposed mechanism is already described for other substances such as aluminium oxide, insoluble nickel salts and iron oxides, acting as poorly soluble low toxicity particles, which elicit lung tumors in rats following prolonged exposure at sufficiently high concentrations.

Therefore, classification as Carc. Cat 1B – H350i is justified for TiO_2 considering the increase of both malignant and benign lung tumours in one species, reported in two studies by inhalation and two studies by instillation after exposure to TiO_2. Since the data provided cannot distinguish if a specific characteristic is linked to such effect, this classification applied to all existing possible crystal modifications, morphologies and surface chemistries in all possible combinations of TiO_2. The proposed classification focus on inhalation route because only local tumours were found after respiratory exposure and no carcinogenic concern was identified by oral and dermal routes. This last assumption is based on the negative results in different carcinogenicity studies that might be explained due to limited absorption reported in other studies and due to the hypothesized mode of action requiring a sufficient accumulation of particles to induce inflammation and proliferative lesions.

2.3 Current harmonised classification and labelling

There is no current harmonized classification for titanium dioxide.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

The following C&L inventory information is available for the general entry of Titanium dioxide (CAS 13463-67-7) on 11/04/2015.

Classification	Number of notifiers
Not classified	2387
Acute Tox 4 – H332	63
Acute Tox 4 – H312	4
Acute Tox 4 – H302	14
Skin Irrit 2 – H315	11
Eye Irrit 2 – H319	71
STOT SE 2 – H371	10
Resp Sens 1B – H334	1
STOT SE 3 – H335	76
STOT RE 1 – H372	69
STOT RE 2 – H373	1
Muta 2 – H341	1
Carc 1B – H350	9

Carc 2 – H351	115
Aqua Chronic 4 – H413	22

An additional C&L inventory is available for the specific crystalline form, Anatase (CAS 1317-70-0) on 11/04/2015.

Classification	Number of notifiers
Not classified	200
Acute Tox 4 – H302	5
Carc. 2 – H351	5
Skin Irrit 2 – H315	2
Eye Irrit 2 – H319	2
STOT SE 3 – H335	2

An additional C&L inventory is available for the specific crystalline form, Rutile (CAS 1317-80-2) on 05/04/2016.

Classification	Number of notifiers
Not classified	417
Acute Tox 4 – H302	5
Carc. 2 – H351	4
Skin Irrit 2 – H315	1
Eye Irrit 2 – H319	1
STOT SE 3 – H335	1

In conclusion, it can be noted that several notifiers titanium dioxide as a carcinogenic substance, including the anatase forms.

RAC general comment
The EC/CAS inventory listing for Titanium dioxide (236-675-5/13463-67-7) covers any chemical that has "TiO2" as its molecular formula and is therefore the broadest possible identifier for TiO2 chemicals. This EC/CAS inventory listing has been used in the name and numerical identifiers for registration for the REACH registration, as the scope of the registration is all TiO2 chemicals. EC/CAS inventory listings for two specific TiO2 crystal structures are available: Anatase (215-280-1/1317-70-0) and Rutile (215-282-2/1317-80-2). There is also

a CAS inventory listing available for Brookite (12188-41-9). These EC/CAS inventory listings are not included in the Annex VI entry as they are covered by the EC/CAS inventory listing for Titanium dioxide.

Carcinogenicity was the only endpoint proposed for harmonised classification and labelling (CLH) by the dossier submitter (DS).

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Available data show that TiO_2 has CMR property, i.e. carcinogenicity that is not currently harmonised and justify a harmonised classification and labelling according to article 36 of CLP.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

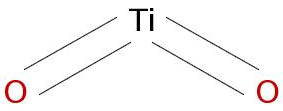
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	236-675-5
EC name:	Titanium dioxide
CAS number (EC inventory):	13463-67-7
CAS number:	13463-67-7
CAS name:	Titanium oxide (TiO_2)
IUPAC name:	dioxotitanium
CLP Annex VI Index number:	-
Molecular formula:	TiO_2
Molecular weight range:	79.8

Structural formula:



1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Titanium dioxide EC no.: 236-675-5	98.0 % (w/w)	>= 87.0 — <= 100.0 % (w/w)	For purity, materials were tested as uncoated and untreated material.

*These data are taken from the REACH registration dossier for EC no 236-675-5. See also, for information, public data of FAO and IARC below

Table 7: Impurities

Impurity	Typical concentration	Concentration range	Remarks
Confidential			

See also, for information public, data of FAO and IARC below

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
Aluminium oxide EC no.: 215-691-6	Stabiliser	1.0 % (w/w)	>= 0.0 — < 2.0 % (w/w)	

*These data are taken from the REACH registration dossier. See also, for information, public data of FAO and IARC below

FR-MSCA acknowledge that the sum of minium purity, the maximum content of the impurity and the additive, does not reach 100%. However, no more data are available on this point.

Data from FAO CTA 2006 chapter 4 characterization

Composition

Titanium dioxide can be prepared at a high level of purity. Specifications for food use currently contain a minimum purity assay of 99.0% (FCC, 2003; Japan, 2000; JECFA, 2006). Maximum limits for Loss on Drying (Japan, 2000; JECFA, 2006) and Loss on Ignition (FCC, 2003; Japan, 2000; JECFA, 2006) have also been established.

Data from FAO CTA 2006 chapter 4 characterization

Maximum Specified Limits for Impurities in Titanium Oxide

Impurity	JECFA (2006)	FCC (2003)	Japan (2000)
Aluminium oxide/silicon dioxide	2%	2.0%	---
Acid-soluble substances	0.5% (1.5% for products containing alumina or silica)	0.5%	0.50%
Water-soluble matter	0.5%	0.3%	0.25%
Antimony	2 mg/kg	1 mg/kg	(a)
Arsenic	1 mg/kg	2 mg/kg	1.3 mg/kg as As_2O_3

Cadmium	1 mg/kg	---	(a)
Lead	10 mg/kg	10 mg/kg	(a)
Mercury	1 mg/kg	1 mg/kg	(a)

(a) 10 mg/kg total Heavy metals (as lead).

Data from American Chemistry Council (2005) available in IARC monograph 93(table 1.1)

Types of coating used for common grades of titanium dioxide pigment (normally titanium dioxide-rutile)

Surface treatment type	Composition, range (wt %)	Application
Alumina/TMP	Al_2O_3, 1.0–5.5 Total carbon, <0.3	Paint/coatings
Alumina/zirconia/TMP	Al_2O_3, 1.0–5.0 ZrO_2, 0.3–1.0 Total carbon, <0.3	Paint/coatings
Alumina/silica/siloxane	Al_2O_3, 1–6 SiO_2, 0.3–3 Total carbon, <0.3	Plastics
Alumina/silica/TMP	Al_2O_3, 1.0–6.0 SiO_2, 0.5–13.0 Total carbon, <0.3	Paint/coatings/plastics
Alumina/TME	Al_2O_3, 1.0–3.5 Total carbon, <0.3	Paint/coatings
Alumina/zirconia/TME	Al_2O_3, 1.0–5.0 ZrO_2, 0.3–1.0 Total carbon, <0.3	Paint/coatings
Alumina/silica/TME	Al_2O_3, 1.5–5.0 SiO_2, 1.5–3.5 Total carbon, <0.3	Paint/coatings
Alumina/silica/silane	Al_2O_3, 1.0–6.0 SiO_2, 0.3–3 Total carbon, <0.3	Plastics

TME, trimethylol ethane; TMP, trimethylol propane; wt, weight

1.3 Physico-chemical properties

The below information are extracted from registration dossier. These data have not been assessed in the context of this CLH dossier.

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Solid, crystalline, white, odourless inorganic substance.	-CRC Handbook of Chemistry and Physics Lide, D.R. (Ed.) CRC Press, 4-96	
Melting/freezing point	Melting point of anatase: 1560 °C, rutile: 1843 °C, brookite: 1825 °C	-CRC Handbook of Chemistry and Physics Lide, D.R. (Ed.) CRC Press, 4-96 -RÖMPP Online, Version 3.1 – Titandioxid Georg Thieme Verlag, Dokumentkennung RD-20-01896	
Boiling point	ca. 3000 °C	-CRC Handbook of Chemistry and Physics Lide, D.R. (Ed.) CRC Press, 4-96 -RÖMPP Online, Version 3.1 – Titandioxid Georg Thieme Verlag, Dokumentkennung RD-20-01896	
Relative density	Relative density: anatase = 3.9, rutile = 4.26, brookite = 4.17	-CRC Handbook of Chemistry and Physics Lide, D.R. (Ed.) CRC Press, 4-96	
Water solubility	Not soluble	-Brouwers T 2009 -CRC Handbook of Chemistry and Physics Lide, D.R. (Ed.) CRC Press, 4-96	The water solubility of titanium dioxide was below the LOD of 1µg/L at pH 6, 7 and 8
Solubility in organic solvent	Not soluble		Hazardous Substances Data Bank
Partition coefficient n-octanol/water	Not soluble		
Granulometry	Not relevant		Titanium dioxide can be in very different forms from isolated nanoparticule to bulk material. Defining a single value is not relevant.

2 MANUFACTURE AND USES

2.1 Manufacture

Titanium dioxide is manufactured from mineral ores or from iron titanate or titanium slag. It is a solid, crystalline, white, odourless inorganic substance in the multiple morphologies of nano or non nanoparticles as primary particle size that will aggregate and agglomerate. It can also be engineered as nanosheets, nanotubes and nanofibres. The tonnage band registered in EU is 1,000,000 - 10,000,000 tons per annum.

2.2 Identified uses

Titanium dioxide is a pigment and an opacifying agent. Its other important properties are resistance to chemical attack, thermal stability, resistance to UV degradation (UV blocker) and photocatalysis potential.

Titanium dioxide is very widely used in industrial/professional settings and is included in numerous products and articles used by industrials, professionals and consumers. All existing process categories (PROC), environmental release categories (ERC), product categories (PC) and articles categories (AC) are claimed in the Reach registration dossier. Products/articles in which titanium dioxide is incorporated are numerous and include paints, varnishes, inks, coatings, plastics, rubbers, papers, plasters, adhesives, coated fabrics and textiles, glassware, ceramics, electroceramics, electronic components, catalysts, welding fluxes, welding rods, floor coverings, roofing granules, food additives (E 171), pharmaceuticals, cosmetics, dental impressions, etc. Due to its photocatalytic properties, when the size of the particle is reduced to the nanoscale in one or more dimensions, nano titanium dioxide is also used for water and surfaces treatment.

The uses of TiO_2 depend on its properties that are determined by the crystallinity, the size, the shape and surface chemistry of the TiO_2 particle.

No uses are reported as advised against in the Reach registration dossier.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Carcinogenicity

The following reported studies were all publications based on a bibliographic research carried out on all forms of TiO_2 (ended on August 2015). In addition, the information from the registration dossier on EC 236-675-5 published on ECHA website has been considered (date: 01/08/2015).

Table 4.1-01: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference Reliability
Oral route			
Fischer 344 rats and B6C3F1 mice (males and females) 0, 25 000, 50 000 ppm 103 weeks in diet (corresp. to 1250-2500 mg/kg bw/day in rats and 3750-7500 mg/kg bw/day with OECD conversion factors) Before guideline, no GLP status	Not carcinogenic by oral route. No firm conclusion in rats after reviewing by the Data Evaluation/Risk Assessment Subgroup of the Clearinghouse on Environmental Carcinogens.	TiO_2 anatase, purity ≥ 98%, size unspecified Tested material not fully characterized (at least size lacking) and very high tested doses	NCI, 1979 R2
Fischer 344 rats (males and females) 0, 1.0, 2.0, 5.0 % up to 130 weeks in diet (corresp. to 500, 1000, 2500 mg/kg bw/day with OECD conversion factors) Similar to guideline, no GLP status	Not carcinogenic by oral route.	TiO_2-coated mica (flat platelets, longest dimension, 10-35 µm; 28% titanium dioxide; 72% mica) Tested material not fully characterized (at least crystallinity and purity lacking) and high tested doses	Bernard, 1990 R2
Inhalation route			
Crl:CD rats (males and females) Exposure by inhalation whole body: 0, 10, 50 or 250 mg/m^3, 6 h/day, 5 days/week for 2 years Similar to guideline, no GLP status	↑ bronchioalveolar adenoma in ♀ and ♂ and squamous lesions (mostly keratin cysts) in ♀ at 250 mg/m^3. Impairment of clearance function, pulmonary inflammation and cell proliferative responses from 50 mg/m^3.	TiO_2 (purity 99.0%), rutile particles; MMD = 1.5-1.7 µm	Lee, 1985 R2
Female Wistar rats [Crl:(WI)BR] and NMRI mice Whole body exposure by inhalation: 18h/d, 5 days/week: 7.2 mg/m^3 for the first 4 months, then 14.8 mg/m^3 for 4 months followed by 9.4 mg/m^3 for 16 months for rats and 5.5 months for mice. Not guideline, no GLP status	↑ benign keratinizing cystic squamous cell tumours, squamous-cell carcinomas, bronchioalveolar adenomas and adenocarcinomas in rats. Not carcinogenic in mice. ↑ mortality and ↓ body weight in both species. Impairment of clearance function, bronchioalveolar hyperplasia and interstitial fibrosis in rats.	TiO_2, 15-40 nm, P25 (≈ 80% anatase and ≈ 20% rutile) Purity lacking. One concentration varying during the experiment, only females tested.	Heinrich, 1995 R3
F-344 rats (males and females) Whole body exposure by inhalation, 6h/day, 5 days/week to 5 mg/m^3 TiO_2 (respirable concentration of 3.87 mg/m^3) for 24 months Not guideline, no GLP status	Not carcinogenic by inhalation. Inflammatory reaction with bronchoalveolar hyperplasia.	TiO_2, type Bayertitan T, 99.5 % rutile, MMAD = 1.1 µm Purity lacking. One low concentration tested.	Muhle, 1989 R3

SD rats (males and females) 0 or 15.95 mg/m^3 by inhalation for 12 weeks, 6h/day, 5d/week Not guideline, no GLP status	Not carcinogenic by inhalation. Inflammatory reaction.	TiO_2, "standard size" with 99.9% < 0.5 µm Tested material not fully characterized (at least size and crystallinity lacking); one low concentration tested and short exposure duration.	Thyssen, 1978 R3
Instillation route			
Hras 128 transgenic female rats DHPN (initiation) for 2 weeks. Then, 250 µg/ml or 500 µg/ml TiO_2 once every 2 weeks from the end of the week 4 to week 16 by instillation. Not guideline, no GLP status	Promotor effect observed: ↑ multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung at all doses, and the multiplicity of mammary adenocarcinomas at 500 µg/ml. No carcinogenic without pre-treatment with DHPN.	TiO_2 non coated, rutile, 20 nm Purity lacking. Little experience with this model. No positive control included. Only females tested.	Xu, 2010 R3
F344/DuCrl Crj male rats DHPN (initiation) for 2 weeks, then 0.5 mg/rat TiO_2 once in week 4 by instillation. Not guideline, no GLP status	Not promotor potential by instillation. No lung lesion without pretreatment with DHPN.	Micro-TiO_2, rutile form, < 5 µm Nano-TiO_2, 80 nm (no clear crystalline identification) Many parameters did not match with standard protocol for carcinogenesis assessment; no valid positive control; only males tested.	Yokohira, 2009 R3
SPF Wistar female rats TiO_2 P25: 5x3mg, 5x6 mg or 10x6 mg by instillations TiO_2 P805: 15x0.5 mg or 30 x0.5 mg by instillation Micro TiO_2: 10x6 mg or 20x6 mg by instillation Animals sacrificed after 30 months. Not guideline, no GLP status	↑ benign tumours (adenomas and epitheliomas) and malignant tumours (adenocarcinomas and squamous cell carcinomas) with nano and micro TiO_2 at all tested doses. Higher number of tumours with nano-TiO_2 compared to fine TiO_2.	Nano-TiO_2 P25, majority anatase, 25 nm Nano-TiO_2 P805 (P25 coated with trimethoxyoctyl-silane), 21 nm Micro-TiO_2 anatase, 0.2 µm Purity lacking. Only females tested.	Pott, 2005 R2
Dermal route			
CD1(ICR) female mice DMBA (initiation) one time. One week after: 5, 10 and 20 mg/animal TiO_2 twice weekly for 19 weeks by dermal route. Two-stage skin carcinogenesis Japanese guideline 3.2; GLP compliant	No promotor potential by dermal route.	TiO_2 coated: 79.2%, spindle shape, long axis of 50-100 nm, short axis of 10-20 nm TiO_2 non coated: 96.0%, spindle shape, long axis of 50-100 nm, short axis of 10-20 nm No information on crystallinity. Positive control valid; only females tested.	Furukawa, 2011 R2

Male transgenic Hras 128 rats and wild-type SD rats DMBA (initiation) one time. Two weeks later: 50 or 100 mg TiO_2 twice a week until week 40 by dermal route. Two-stage skin carcinogenesis Not guideline, no GLP status	No promotor potential by dermal route.	TiO_2 non coated, rutile, 20 nm. Little experience with this model. No positive control; only males tested. High tumour activity with DMBA alone in Has 128 rats.	Sagawa, 2012 R3
Female CD1 mice DMBA (initiation) one time. Two weeks later: 10 or 20 mg TiO_2 twice a week until week 52 by dermal route. Two-stage skin carcinogenesis Not guideline, no GLP status	No promotor potential by dermal route.	TiO_2 non coated, rutile, 20 nm Positive control valid; only females tested.	Sagawa, 2012 R3
Female transgenic rasH2 mice and wild type CB6F1 mice DMBA (initiation) one time. Two weeks later: 10 or 20 mg TiO_2, 5 times per week until week 8 for transgenic mice and week 40 for wild-type mice by dermal route. Two-stage skin carcinogenesis Not guideline, no GLP status	No promotor potential by dermal route.	TiO_2 coated with silicone, 35 nm No positive control; only females tested. High tumour activity in the initiated rasH2 mice.	Sagawa, 2012 R3
Hras 128 rats and wild-type rats (males and females) UVB (initiation) twice weekly for 10 weeks, then 50 mg TiO_2 twice weekly until week 52 by dermal route. Two-stage skin carcinogenesis Not guideline, no GLP status	No promotor potential by dermal route.	TiO_2 non coated, rutile, 20 nm No positive control. Little experience with this model	Xu, 2011 R3

4.1.1 Non-human information

4.1.1.1 Carcinogenicity: oral

Fischer 344 rats and B6C3F1 mice (50/sex/group) were administered TiO_2 (3 lots of anatase Unitane® 0-220, purity ≥ 98%, size unspecified) in the diet at 0, 25,000 or 50,000 ppm for 103 weeks and then observed for one additional week (NCI, 1979). According to the conversion factors provided by the OECD (2002), the tested doses correspond to 1250 mg/kg bw/day and 2500 mg/kg bw/day in rats and to 3750 mg/kg bw/day and 7500 mg/kg bw/day in mice; doses higher than what is generally recommended in the OECD guideline. Surviving animals were killed at 104 weeks. The tested doses were selected from a subchronic studies performed in rats and mice at doses up to 100,000 ppm. There was no mortality, no effect on body gain and no gross or microscopic pathology at the highest tested dose. The maximum tested dose of 50,000 ppm in the carcinogenicity study was chosen as the maximum amount allowed for use in chronic bioassays in the Carcinogenicity Testing Program.

Administration of TiO_2 had no appreciable effect on the mean body weights of rats and mice. With the exception of white faeces observed in male and female rats and mice, there was no other clinical sign that was considered treatment-related. Survival of rats and male mice at the end of the bioassay was not affected by the tested substance; in female mice, a dose-related trend in decreased survival was noted. Sufficient numbers of dosed and control rats and mice of each sex were at risk for development of late-appearing tumours.

In the female rats, C-cell adenomas or carcinomas of the thyroid occurred at incidences that were dose related (P = 0.013), but were not high enough (P = 0.043 for direct comparison of the high-dose group with the control group) to meet the level of P = 0.025 required by the Bonferroni criterion (controls 1/48, low dose 0/47, high dose 6/44). Thus, these tumours of the thyroid were not considered to be related to the administration of the test chemical. Also in female rats, endometrial stromal polyps of the endometrium/uterus occurred at higher incidences in the dosed groups than in controls, but the incidences were not dose-related and were not high enough (P = 0.045 for direct comparison of the low-dose group with the control group) to meet the requirements of the Bonferroni criterion (controls 7/50, low dose 15/50, high dose 10/50). In male and female mice, no tumours occurred in dosed groups at incidences that were significantly higher than those of the corresponding control groups. It was concluded that under the conditions of this bioassay, TiO_2 was not carcinogenic by the oral route for Fischer 344 rats or B6C3F1 mice.

This study was reviewed by the Data Evaluation/Risk Assessment Subgroup of the Clearinghouse on Environmental Carcinogens in 1978. The primary reviewer considered that the evidence was insufficient to conclude that TiO_2 was not carcinogenic in female rats based on the increased incidence in C-cell adenomas and carcinomas of the thyroid. This reviewer recommended to modify the above conclusion and suggested that TiO_2 should be retested. In contrast, the second reviewer considered the study adequate and concluded that TiO_2 would not appear to pose a carcinogenic risk to humans. The following revised conclusion was agreed: "*it was concluded that, under the conditions of this bioassay, TiO_2 was not carcinogenic by the oral route of exposure for B6C3F1 mice, but that no firm conclusion can be reached about the possible carcinogenicity of this compound to Fischer 344 rats, at this time*". There was no objection to the recommendation that TiO_2 be considered for retest.

Another study was summarized in the IARC monograph, volume 93. Fischer 344 rats (60/sex/group) fed diets containing 0, 1.0, 2.0 or 5.0% TiO_2-coated mica (flat platelets, longest dimension, 10-35 µm; 28% TiO_2; 72% mica) for up to 130 weeks. According to the conversion factors provided by the OECD (2002), the tested doses correspond to 500, 1000 and 2500 mg/kg bw/day, with the highest dose higher than what is generally recommended in the OECD guideline. Low survival rates were noted, in particular for the females exposed to 1.0% TiO_2-coated mica (only 12/50). Reduction of body weight was observed but was reversible at termination of the study. The only treatment-related clinical sign was silver-colored feces. A significant increase in the overall incidence of adrenal medullary hyperplasia was found in the high dose males, without any progression. There was also a marginally elevated overall incidence of mononuclear cell leukemia in

this group, judged to be of no biological significance. The authors concluded that there was no evidence of a carcinogenic effect (Bernard, 1990).

4.1.1.2 Carcinogenicity: inhalation

Crl:CD rats (100/sex/group) were exposed whole body to TiO_2 (purity 99.0%), <u>rutile particles</u> with a spherical configuration, by inhalation at concentrations of 0, 10, 50 or 250 mg/m^3, 6 hr/day, 5 days/week for 2 years (Lee et al. 1985a; 1985b; 1986; Trochimowicz, 1988). Five males and five females from each group were killed after 3 and 6 months of exposure, and subsequently, 10 males and 10 females were killed after 1 year of exposure. All rats sacrificed by design, found dead, or sacrificed in extremis were submitted for gross and microscopic evaluation. All remaining rats were killed at the end of 2 years of exposure.

Table 4.1.1.2-01. Chamber concentrations and particle size distributions of TiO_2

Chamber concentrations[a] (mg/m^3)	MMD[b] (µm)	Respirable fraction[c] (%)
10.6 ± 2.1	1.5	78.2
50.3 ± 8.8	1.7	88.6
250.1 ± 24.7	1.6	84.3

[a] Mean ± Standard deviation (gravimetric determination)
[b] Mass median diameter (MMD), average of at least six determinations
[c] The average percentage of TiO_2 particles with MMD less than 13 µm

There were no abnormal clinical signs, body weight changes, excess of morbidity or mortality in any exposed group when compared to control group. There were no significant compound-related pathological lesions other than in the respiratory organs and the thoracic lymph nodes.

Lung weights at 10 mg/m^3 were comparable to those of the control group, but at 50 mg/m^3, lung weights (relative and absolute) were statistically significantly increased from 6 months throughout 2 years exposure. At 250 mg/m^3, the lungs showed a marked increase in weight from 3 months of exposure and were more than twice the weight of control lungs after 1 and 2 years exposure.

Macroscopically, accumulation of white foci was seen in the lungs of rats exposed to TiO_2 at all concentrations. White foci were observed at 3 months of exposure and increased in number and/or size from 6 months through 2 years of exposure. At the highest concentration, the lungs were markedly voluminous and failed to collapse. The tracheobronchial lymph nodes were markedly enlarged with a concentration and exposure time relationship.

TiO_2 retention in lung at 24 months of exposure was 3.1 % (26.5 mg per dried lung) at 10 mg/m^3, 9.6% (124 mg per dried lung) at 50 mg/m^3 and 28 % (665 mg per dried lung) at 250 mg/m^3. The lung clearance mechanism appeared to be overwhelmed by 12 months of exposure to 250 mg/m^3 and TiO_2 was accumulated markedly throughout 2 years of exposure. There was no significant difference in dust clearance between 10 and 50 mg/m^3 groups.

Incidence of main non-neoplastic lesions in the nasal cavity and trachea are summarized in the Table 4.1.1.2-02.

No tumours were observed. A significant increase in the incidence of rhinitis, tracheitis and pneumonia was found in all groups of exposed rats. The severity of the lesions was dose dependent and was minimal at 10 mg/m^3. The anterior nasal cavity often revealed acute and chronic inflammation with squamous cell metaplasia.

Table 4.1.1.2-02. Incidence of main non-neoplastic lesions in the nasal cavity and trachea

	Control		10 mg/m^3		50 mg/m^3		250 mg/m^3	
	(♂)	(♀)	(♂)	(♀)	(♂)	(♀)	(♂)	(♀)
Nasal cavity (number of rats examined)	79	76	71	74	73	74	76	73
Rhinitis, anterior	25	18	57	36	48	34	70	63
Rhinitis, posterior	13	3	13	10	3	1	14	18
Squamous metaplasia, anterior	8	7	26	14	20	21	44	40
Squamous metaplasia, posterior	-	1	-	-	-	1	1	2
Trachea (number of rats examined)	79	77	68	74	74	69	77	65
Tracheitis	2	1	52	34	53	37	61	28

Incidences of main neoplastic and non-neoplastic lesions in the lung were summarized in the Table 4.1.1.2-03.

Exposure to TiO_2 produced impairment of alveolar macrophage clearance functions, sustained persistence pulmonary inflammation and enhanced cell proliferative responses. The first manifestation of the pulmonary response to an overloaded lung clearance mechanism was an *accumulation of foamy dust cells* at 50 mg/m^3 after 1 year of exposure and at 250 mg/m^3 after 6 months exposure. *Alveolar proteinosis (lipoproteinosis)* also appeared to be an important marker indicating an overloaded lung clearance. It seemed to occur because of failure of lung clearance due to overwhelmed alveolar macrophages, since they were overloaded with fine TiO_2 particles and excessive alveolar surfactant derived from hyperplastic type II pneumocytes. *Cholesterol granulomas* were also developed at 50 and 250 mg/m^3 after 1 year exposure and were related to massive accumulation of foamy dust cells in the alveoli. The disintegrated foamy dust cell could release lysosomal enzymes to provoke a granulomatous tissue response. However, cholesterol granulomas appear to be species-specific tissue responses to excessive dust exposure since they are relatively rare and not associated with dust exposure in man. *Type II pneumocyte hyperplasia* was observed at all tested concentrations. This effect is known as a reversible adaptive and reparative tissue response to damaged type I pneumocytes. *Alveolar bronchiolarization* found at 50 and 250 mg/m^3 was characterized by epithelialization of ciliated columnar cells and mucous cells in some alveoli adjacent to the terminal bronchioles. This lesion appears to be another adaptive tissue response to dust

accumulation by extension of the mucoescalator capacity to the alveolar walls from adjacent terminal bronchioles for rapid removal of particles via the airways.

Concerning the neoplastic lesions, an increase of bronchiolalveolar adenoma and squamous cell carcinoma occurred at 250 mg/m^3. Bronchioalveolar adenoma originated from areas of alveoli showing marked hyperplasia of type II pneumocytes with dust cell aggregates. The squamous cell carcinomas were characterized by a dermoid, cyst-like appearance with a cystic space filled with laminated keratin material. They were developed from the squamous metaplasia in the alveoli showing bronchiolarization adjacent to the alveolar ducts. In most cases, they were extremely difficult to differentiate from keratinized squamous metaplasia. In the publication, the authors classified this pulmonary lesion as a cystic keratinizing squamous cell carcinoma even in the absence of a biological behaviour indicating malignancy. There were no signs of metastasis to regional lymph nodes or other organs.

A microscopic review of the proliferative squamous lesions observed in this study was published in 2006 (Warheit et al. 2006). These lesions were evaluated by four pathologists using current diagnostic criteria. Two of the lesions were diagnosed as squamous metaplasia, one as a poorly keratinizing squamous cell carcinoma and the remaining lesions as non-neoplastic pulmonary keratin cysts.

Table 4.1.1.2-03. Incidences of main neoplastic and non-neoplastic lesions in the lung

	Control		10 mg/m^3		50 mg/m^3		250 mg/m^3	
	(♂)	(♀)	(♂)	(♀)	(♂)	(♀)	(♂)	(♀)
Lung (number of rats examined)	79	77	71	75	75	74	77	74
Aggregates, foamy alveolar macrophage	14	8	19	15	53	70	76	74
Alveolar cell hyperplasia, TiO_2 deposition	-	-	67	72	75	74	77	74
Alveolar proteinosis	-	-	-	-	38	45	75	71
Bronchiolarization, alveoli	1	1	-	3	24	57	63	73
Broncho/bronchiolar pneumonia	1	1	7	11	8	10	7	5
Cholesterol granuloma	7	2	9	6	56	53	75	71
Collagenized fibrosis	11	3	7	4	49	41	76	73
Pleurisy	4	2	7	7	28	26	55	66
Anaplastic carcinoma, large cell	-	-	1	-	-	-	-	-
Bronchioalveolar adenoma	2	-	1	-	1	-	**12**	**13**
Squamous cell carcinoma	-	-	-	1	-	-	1	**13**
Classification of squamous lesions after re-evaluation (Warheit et al. 2006)								
Squamous metaplasia	-	-	-	-	-	-	-	2
Pulmonary keratin cyst	-	-	-	1	-	-	1	11

Squamous cell carcinoma	-	-	-	-	-	-	-	1

Female Wistar rats [Crl:(WI)BR] and NMRI mice were exposed whole body to aerosol of TiO_2 (P25, CAS no. 13463-67-7, primary particle size 15-40 nm, ≈ 80% anatase and ≈ 20% rutile) (Heinrich, 1995). Rats were exposed for up to 24 months (intermediate sacrifice 6 and 12 months) and mice for 13.5 months 18h/d, 5 days/week. Rats and mice were kept together in the same TiO_2 exposure atmospheres. The mean particle mass exposure concentrations were 7.2 mg/m^3 for the first 4 months, followed by 14.8 mg/m^3 for 4 months and 9.4 mg/m^3 for 16 months for rats and 5.5 months for mice. The reason for changing the exposure concentrations of TiO_2 was to obtain a similar particle lung load in rats exposed to high diesel soot and carbon black particle concentrations, substances also tested in this study. The cumulative particle exposure, calculated by multiplying the mean particle mass exposure concentration by the actual exposure time per day, corresponded to 88.1 g/m^3 x h for rats and 51.5 g/m^3 x h for mice. Following the exposure period, the animals were removed from the inhalation chambers and kept under clean air conditions for an additional 6 months for rats and 9.5 months for mice. The total experimental test lasted 30 months for rats and 23 months for mice.

The aerosol was generated by a dry dispersion technique using a screw feeder and a pressurized air dispersion nozzle. The median aerodynamic diameter (MMAD) of aggregates/ agglomerates was about 1.5 µm. In order to increase the deposition efficiency of the test aerosol in the deep lung, the particle size distribution was shifted toward smaller particles in the submicrometer regime by removing the coarse particles using a cyclone. The MMAD and the geometric standard deviation of the particles in the exposure chambers measured every month was 0.80 (1.80) µm. The specific surface area of the particles determined by the BET method was 48 (± 2.0) m^2/g.

The following table shows the number of animals used for the different biological tests performed in the study.

Table 4.1.1.2-04. Number of animals used and investigations performed

	Rats		Mice	
Investigations	Clean air control	TiO_2	Clean air control	TiO_2
Carcinogenicity	220	100	80	80
Histology (serial sacrifice)	80	80	40	40
DNA adducts (24 months)	14	14	-	-
Particle mass/lung (serial sacrifice)	66	66	40	40
Alveolar lung clearance	28	28	-	-

After 24 months, the mortality in rats was 60 % in the TiO_2 group compared to 42 % in the control group. At the end of the 130-week experimental time (exposure time and clean air period), the mean lifetime of the rats exposed to TiO_2 was significantly shortened compared to the control (90% mortality in the TiO_2 group versus 85 % in the control group). In mice, the mortality rate was 33% in the exposed group compared to 10 % in the clean air control group 13.5 months after the start of

exposure. A mortality rate of 50 % was reached after 17 months compared to 20% in the control group.

The body weight of the exposed rats was significantly lower from day 400 compared to the control and at the end of the 2-year exposure, the body weight was 365 g in exposed animals compared to 417 g in controls. In mice, the body weight was also significantly lower (5-7%) compared to the clean air control group after 8 months up to 17 months. During the last months, there was no significant difference in body weight between the control and exposed groups.

In rats and mice, the exposure to TiO_2 led to a substantial increase in lung wet weight, progressing with study duration. In mice, a slight decrease in lung wet weight was found in the TiO_2 group during the recovery phase.
In rats, alveolar lung clearance was already significantly compromised after inhalation of TiO_2 after 3 months of exposure (half-times of pulmonary clearance = 208 days in TiO_2 group versus 61 days in control group). After 18 months of exposure and 3 months of recovery time without particle exposure, no reversibility of the alveolar lung clearance damage could be detected (half-times of pulmonary clearance = 368 days in TiO_2 group after 3-month recovery period versus 357 days just after 18 months of exposure without recovery ; 93 days in control group). Alveolar lung clearance was not examined in mice.

After 6 months of exposure, slight bronchioalveolar hyperplasia and very slight to slight interstitial fibrosis were found in the lung of sacrificed rats. After 2 years of exposure, 99/100 rats showed bronchioalveolar hyperplasia and slight to moderate interstitial fibrosis was observed in the lungs of all rats. The presence of non-neoplastic findings in mice was not reported in the publication.

There were no lung tumours in the 20 satellite rats exposed to TiO_2 after 6 and 12 months. Lung tumours were found in 5/20 rats sacrificed after 18 months of exposure. After an exposure time of 24 months followed by 6 months of clean air, lung tumour rate was 32% in rats exposed to TiO_2. Among these animals, 8 showed 2 tumours in their lungs. Mostly benign keratinizing cystic squamous cell tumours and some squamous-cell carcinomas were found. Bronchioalveolar adenomas and adenocarcinomas were also observed at a high frequency. Only one lung tumour (adenocarcinoma) was found in 217 control rats. Tumour incidence in rats is summarised in Table 4.1.1.2-05 below.

In mice, the only types of lung tumours observed were adenomas and adenocarcinomas. The percentage of adenomas/adenocarcinomas was 11.3%/2.5% in TiO_2 group and 25%/15.4% in the control group. The lung tumour rate in the TiO_2 group (13.8 %) was lower than in the control group (30%) but not significantly different. Other effects than carcinogenic lesions in mice are poorly reported, therefore it is difficult to conclude on mice results in this study.

Table 4.1.1.2-05. Lung tumours in serial sacrifice groups of rats exposed to TiO_2 (between 7.2 to 14.8 mg/m^3) for 6, 12, 18 and 24 months and in rats after an experimental time of 30 months (24 months of exposure and 6 months of recovery)

Exposure period/ type of tumour	Clean air control	TiO_2

6 months	0/21	0/20
12 months	0/21	0/20
18 months		
Benign squamous-cell tumour[a]	0/18	2/20
Adenocarcinoma	0/18	2/20
Squamous-cell carcinoma[b]	0/18	3/20
Number of rats with tumour	0/18	5/20*
24 months		
Benign squamous-cell tumour	0/10	2/9
Adenocarcinoma	0/10	1/9
Squamous-cell carcinoma[c]	0/10	2/9
Number of rats with tumour	0/10	4/9*
30 months		
Benign squamous-cell tumour	0/217	20/100
Squamous-cell carcinoma	0/217	3/100
Adenoma	0/217	4/100
Adenocarcinoma	1/217	13/100*
Hemangioma	0/217	0/100
Number of rats with tumour[d]	1/217	32/100 (19/100)[e]

***** Significant at $p \leq 0.05$ (Fisher's exact test)
[a] Benign keratinizing cystic squamous-cell tumour
[b] Sometimes together with adenocarcinoma and benign-cell tumour
[c] Sometimes together with benign squamous-cell tumour
[d] Some animals had two lung tumours
[e] Count without benign keratinizing cystic squamous-cell tumours given in parentheses

A chronic inhalation study of a test toner was conducting using TiO_2 (type Bayertitan T) as negative control for fibrogenicity (Muhle, 1989, 1991, 1995). By chemical analysis, the material was 99.5 % rutile TiO_2. The MMAD was about 1.1 µm, with a geometric standard deviation of 1.6 and the respirable fraction was 78%. Males and females F-344 rats (50/sex/group) were exposed whole body 6h/day, 5 days/week to 5 mg/m^3 TiO_2 (corresponding to a respirable concentration of 3.87 mg/m^3) for 24 months using a dry aerosol dispersion technique. The animals were kept without further exposure for an additional 1.5-month observation period.

Exposure to TiO_2 did not cause overt signs of toxicity. No influence of treatment was found on food consumption, body weight development, clinical appearance, clinical chemistry values and mean survival.

No changes in lung weight were reported in the TiO_2 group although TiO_2 accumulated progressively in the lungs. The mean quantity retained in the lungs of rats at 24 months was 2.72 mg/lung. Inflammatory reactions induced by inhalation of TiO_2 were characterized in the bronchoalveolar lavage by minor changes at 15 months of exposure (such as decreased macrophages and increase of polymorphonuclear leukocytes and lymphocytes). The levels of cytoplasmic and lysosomal enzymes and total protein in the lavage fluid were comparable to those of air-only controls.

At microscopical examination, the extent of particle-laden macrophages increased with exposure time in the lung. A small but statistically insignificant incidence of fibrosis was seen in the TiO_2 group. Bronchoalveolar hyperplasia of the alveolar type, characterized by Type II pneumocytes was a rare finding in the control group and was observed in 9% of the rats exposed to TiO_2. The incidence of primary lung tumours was comparable among the TiO_2 and the air-only controls, and was consistent with historical background values. Two adenomas and one adenocarcinoma were observed in the air-only control group while one tumour of each type was detected in the TiO_2 control group. Only one concentration, relatively low, was tested in this study leading to no carcinogenic lesions. However, the fibrosis and bronchoalveolar hyperplasia observed can be considered as precursor lesions of carcinogenicity.

In a last publication (Thyssen, 1978), male and female Sprague-Dawley rats were exposed to TiO_2 as negative control for assessing the inhalation toxicity of polyurethane foam dust. Animals were exposed to 0 or 15.95 mg/m^3 of TiO_2 ("standard size" with 99.9% < 0.5 µm) for 12 weeks, 6h/day, 5d/week. Animals surviving 140 weeks were sacrificed.

After 140 weeks, 44/50 males and 45/50 female died in the treated group in comparison to 39/50 males and 45/50 females in the air alone group. The average lifespan was between 113 and 120 weeks in the TiO_2 group and 114-116 weeks in the air alone group.

A number of neoplasms were observed in the respiratory tract in both groups (4 in treated/ 2 in controls). One case of adenoma and squamous cell papilloma in the larynx, one squamous cell papilloma in the trachea and one case of lung adenoma were observed in the treated TiO_2 group. In comparison, one squamous cell papilloma in the trachea and one case of lung adenoma were reported in the air alone group. Other neoplasms seen in the lung were metastases from tumours from other sites. In addition, respiratory segments revealed mild to severe inflammatory reactions. Therefore, no treatment-related carcinogenic effect following inhalation of TiO_2 was observed in this study on the respiratory tract. It should be noted that this study was performed with only one concentration of an unspecified titanium dioxide for a relatively short exposure duration.

The following studies were performed by intra-tracheal route. They are presented as supportive data for carcinogenic effects of TiO_2 after respiratory exposure.

Xu et al (2010) conducted a study to detect carcinogenic activity of nanoscale TiO_2 administered by an intrapulmonary spraying (IPS) - initiation-promotion protocol in rat lung. TiO_2 was a rutile type, mean diameter 20 nm, without coating. Hras 128 transgenic female rats, which are known to have the same carcinogen susceptibility phenotype in the lung as wild-type rats but are highly susceptible to mammary tumor induction, were treated by N-bis(2-hydroxypropyl)nitrosamine (DHPN) in drinking water for 2 weeks to initiate carcinogenesis. Two weeks later, the rats were divided into 4 groups: DHPN alone (group 1), DHPN followed by 250 µg/ml TiO_2 (group 2), DHPN followed by 500 µg/ml TiO_2 (group 3) and 500 µg/ml TiO_2 without DHPN (group 4). No positive control was included. For this, TiO_2 was suspended in saline, autoclaved and sonicated for 20 minutes just before use; then the suspension was intratracheally administered to animals under isoflurane

anesthesia using a Microsprayer connected to a 1 mL syringue; the nozzle of the sprayer was inserted into the trachea through the larynx and a total of 0.5 mL suspension was sprayed into the lungs synchronizing with spontaneous respiratory inhalation. The preparations were administered by IPS once every 2 weeks from the end of the week 4 to week 16 (total of seven times). The total amount of TiO_2 administered was 0, 0.875, 1.75 and 1.75 mg/rat, for groups 1, 2, 3 and 4, respectively. Three days after the last treatment, animals were killed and brain, lung, liver, spleen, kidney, mammary gland, ovaries, uterus and neck lymph nodes were examined.

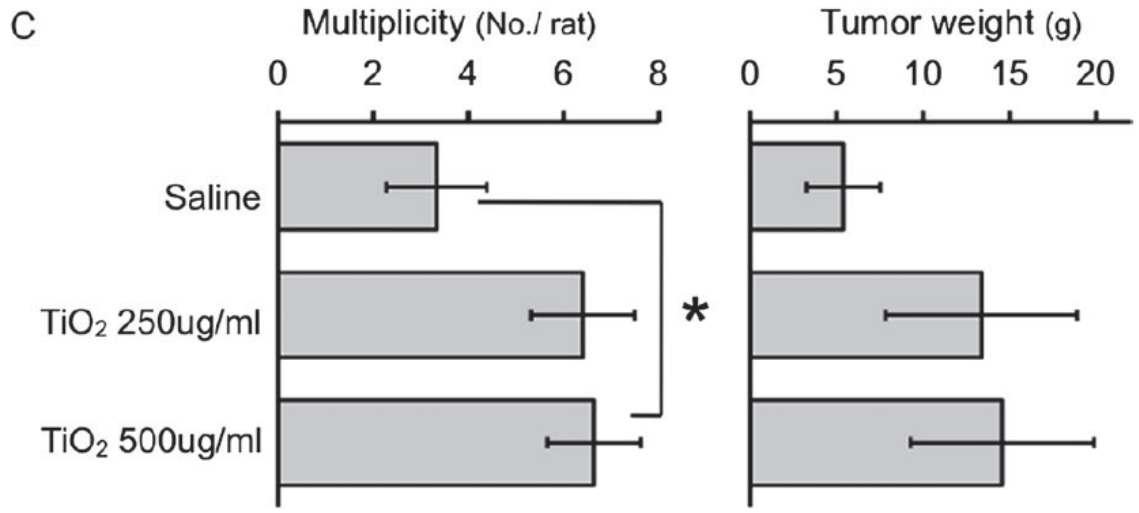
TiO_2 treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas. Alveolar proliferative lesions were not observed in rats receiving TiO_2 treatment without prior DHPN treatment, although slight inflammatory lesions were noted. According to the authors, this could be due to the weak carcinogenic potential and short duration of exposure.

Table 4.1.1.2-06. Effect of TiO_2 on incidence and multiplicity of DHPN-induced alveolar hyperplasia and adenoma of the lung

Treatment	No of rats	Alveolar hyperplasia		Lung adenoma	
		Incidence (%)	Multiplicity (no./cm^2)# #	Incidence (%)	Multiplicity (no./cm^2) #
Saline	9	9 (100)	5.91±1.19	0	0
TiO_2 250 µg/ml	10	10 (100)	7.36±0.97*	1 (10)	0.10±0.10
TiO_2 500 µg/ml	11	11 (100)	11.05±0.87**	4 (36)	0.46±0.21*

* P < 0.05; ** P < 0.001 versus saline control
# P < 0.05; ## P < 0.001 in trend test (Spearman's rank correlation test)

Figure 4.1.1-2-01. Effect of TiO_2 on multiplicity of adenocarcinomas in the mammary gland and on the size of mammary tumors



TiO_2 was distributed primarily to the lung, but minor amounts of TiO_2 were also found in other organs. Various sizes of TiO_2 aggregates were observed in alveolar macrophages. Of 452 particle aggregates examined, 362 (80.1%) were nanosize, i.e., < 100 nm. Overall, the average size was 84.9 nm and the median size was 44.4 nm.

To investigate the underlying mechanism of its carcinogenic effects, a suspension of 500 µg/ml of TiO_2 was administered to wild-type SD rats by IPS five times over 9 days. The total amount of TiO_2 administered was 1.25 mg/rat. Microscopic observation showed scattered inflammatory lesions with infiltration of numerous macrophages mixed with a few neutrophils and lymphocytes. Alveolar proliferative lesions were not observed. Morphologically, TiO_2 particles were observed as yellowish, polygonal bodies in the cytoplasm of cells. These cells are morphologically distinct from neutrophils and strongly positive for CD68, indicating that the TiO_2 engulfing cells were macrophages. TiO_2 aggregates of various sizes were found in macrophages, and aggregates larger than a macrophage were surrounded by multiple macrophages. Of 2571 particle aggregates examined, 1970 (76.6%) were < 100 nm and five particles were > 4000 nm in size. Overall, the average size was 107.4 nm and the median size was 48.1 nm. TiO_2 treatment significantly increased 8-OH-dG, superoxide dismutase (SOD) activity and macrophage inflammatory protein 1α (MIP1α) expression in the lung which is a member of the CC chemokine family and is primarily associated with cell adhesion and migration, proliferation and survival of myeloma cells.

The authors concluded that TiO_2 had lung carcinogenic activity. They suggest the following mechanism: phagocytosis of TiO_2 particles by alveolar macrophages resulting in ROS production and DNA damage and increasing MIP1α. MIP1α in turn was able to enhance proliferation of lung epithelium cells. The authors also suggest that TiO_2 exposure can be a risk factor for mammary carcinogenesis in predisposed population, such as individuals with BRCA mutations. The hypothesis proposed in this publication is that MIP1α secreted by alveolar macrophages and transported via the circulatory system caused proliferation of mammary epithelial cells and thereby promoted mammary carcinogenesis. This result can be an indication that TiO_2 can have a promoting activity away from contact site, suggesting induction of indirect systemic effect. However, it should be noted that these results have to be taken with caution considering the limited experience with this model.

Yokohira et al. (2009) assessed the carcinogenic potential of instilled TiO_2. For this, groups of 5 or 15 F344/DuCrl Crj male rats each were intratracheally treated either with 0.5 mg/rat micro-TiO_2 (rutile form, diameter less than 5 µm) or nano-TiO_2 (particle diameter of 80 nm; no clear crystalline identification) in week 4 with or without a pre-treatment with an initiator, i.e., 0.1% of DHPN given orally in drinking water for the 2 first weeks. An untreated control group and a DHPN alone treated group were added. No positive controls were included. Instead, groups consisting on intra-tracheal administration of quartz, as typical lung toxicant agent, +/- DHPN pre-treatment were included. However, no influence of quartz administration was found on the development of lung tumour. All rats were sacrificed at week 30. Lungs, liver and kidneys were weighed and subjected to histopathological examination.

No significant changes were found in organ weights. No lung lesions were observed with TiO_2 micro or nano without pre-treatment. All the DHPN groups displayed hyperplasias, adenomas and adenocarcinomas in the lungs. There were no significant intergroup differences in the lung neoplastic lesions induced by DHPN, although the areas of neoplastic lesions induced by the nanoparticles of TiO_2 demonstrated a tendency to increase compared with the microparticles administration. However, the large variability of results for adenocarcinomas areas can explain that no significant intergroup

difference was found. Finally, it should be stressed that many experimental parameters did not match with the standard protocol for carcinogenesis assessment (e.g., treatment schedule with only 1 treatment; few number of animals /group; only few organ examined, no valid positive control…).

Table 4.1.1.2-07: Number of histopathological lesions per cm^2 of lung

Treatment	Instilled particles	No.	Hyperplasia	Adenoma	Adeno-carcinoma	Total	Adenoma + Adeno-carcinoma
DHPN	Micro-TiO_2	15	19.9 ± 5.6	2.5 ± 1.5	0.1 ± 0.3	22.5 ± 6.3	2.6 ± 1.5
DHPN	Nano-TiO_2	15	20.0 ± 4.7	2.6 ± 1.4	0.3 ± 0.5	22.9 ± 5.4	2.9 ± 1.5
DHPN	Untreated	15	17.9 ± 2.8	2.0 ± 1.2	0.2 ± 0.6	20.1 ± 3.4	2.2 ± 1.1

In instillation experiments carried out by Pott and Roller (2005), SPF Wistar female rats were exposed by repeated intra-tracheal instillation (from age 8-9 weeks) of TiO_2 over 19 different dusts. Animals received up to 30 instillations of two types of nano-TiO_2 and one type of fine TiO_2, administered at weekly intervals. The dusts were suspended by ultrasonification in phosphate buffered sodium chloride solution and Tween 80® was added to improve the homogeneity of the dosed suspensions. The study was terminated after 30 months.

Table 4.1.1.2-08: Characteristics of the TiO_2 dusts

Substance	Particle size, mean(µm)	Density (g/ml)	Specific surface area (BET) (m^2/g)
TiO_2 P25, hydrophilic, majority anatase	0.030/0.021 0.025[a]	3.8	52
TiO_2 P805[b], AL 90,003-2, hydrophobic[c]	0.021 (data of T805)	3.8	32.5
TiO_2 anatase AL 23,203-3 [1317-70-0], (hydrophilic)	0.2	3.9	9.9

[a]There are no clearly measured values or more than one piece of information. On the basis of the data available, the value with footnote a was assumed to be close to the correct value

[b]Titanium dioxide T 805 from Degussa was ordered from Sigma-Aldrich, but the supplier only offered an amount of at least 40 kg P 805. Neither Sigma-Aldrich nor Degussa answered at all clearly when questioned insistently as to the difference between T 805 and P 805. So, it is not proven that P 805 is identical with T 805 from Degussa. In the IARC monograph volume 93, it is assumed that T805 is similar to P805

[c]In the case of T 805, ultrafine TiO_2 with the specification P 25 is coated with trimethoxyoctyl-silane to change the particle surface from hydrophilic to hydrophobic.

An increase of benign tumours (adenomas and epitheliomas) and malignant tumours (adenocarcinomas and squamous cell carcinomas) was observed after treatment with both types of TiO_2; with a higher number of tumours with nano-TiO_2 (P25, hydrophilic form) by comparison with exposure to fine TiO_2. When a total of 60 mg of nano-TiO_2 per animal were administered (10 instillations × 6 mg), a tumour incidence of up to 70% was observed, compared with 30% in animals exposed to fine TiO_2 particles with the same experimental design. When animals were treated with a total of 15 mg (5 instillations ×

3 mg) or 30 mg of P25 nano-TiO_2 (5 instillations × 6 mg), 52% and 67% of the rats studied developed tumours, respectively.

For hydrophobic TiO_2, the initial plan of dosage contained repeated instillations of 6 mg, but the acute mortality after the first instillation (rats died within half an hour of instillation) called for a drastic reduction of the single doses to 0.5 mg. After further tests, 0.5 mg was instilled 15 or 30 times respectively. According to the authors, the pathophysiological mechanisms of the acute lethal effect can be discussed as follows: 1) the lipophilic surface mediates a fast distribution of the ultrafine particles in the surfactant layer on the alveolar walls; 2) the organic silicon compound dissolves from the particle surface, damages the surfactant and the membranes of pneumocytes and capillaries; 3) the alveolo-capillary membranes swell, which results in a capillary block and hemorrhage in the alveoli: dark red spots were macroscopically detected on the lung surface by autopsy and erythrocytes were seen in the alveoli. According to Warheit (2006), a confounding factor which had not been addressed or properly controlled was the potential toxicity of 1% Tween, which was added as detergent selectively to the T-085 sample but not to P25 sample, creating an additional variable in the study.

Table 4.1.1.2-09: Animal groups in the sequence of the experiment, doses instilled (mass and volume dose), estimated dust volume retained in the lungs for a longer period, rats at risk, survival times, lung tumour incidences macroscopically and microscopically, and tumour incidence per µl dust burden in the lung

Dust, size class	Dose instilled		Dust volume / lung (ml)	Rats at start / at risk[c]	Sur-vival 50%[d] (wks)	Lungs with tumour(s) (%)					Lungs (%) with metastases of other tumours	Tum./ lung of dust[h] (%/µl)
						Macroscopy		Microscopy (primary)[g]				
	No. of inst. x mg	Vol.[b] (µl)				Total[e]	Primary[f]	Benign	Malign	Total		
TiO_2, UF[a] P25	5 x 3 5 x 6 10 x 6	3.9 7.9 16	2.6 5.3 11	48/42 48/46 48/46	114 114 104	47.6 52.2 54.3	35.7 47.8 43.5	21.4 17.4 23.9	31 50 45.7	52.4 67.4 69.6	14.3 15.2 15.2	20.2 12.7 6.3
TiO_2, UF[a] P805	15 x 0.5[i] 30 x 0.5[i]	2.0 3.9	(toxic)	24/11 48/15	86 114	9.1 20.0	0.0 20.0	0.0 6.7	0.0 0.0	0.0 6.7	9.1 6.7	-
TiO_2, anatase F-sm[a]	10 x 6 20 x 6	15 31	10 21	48/44 48/44	108 113	22.7 36.4	22.7 36.4	15.9 38.6	13.6 25.0	29.5 63.6	11.4 2.3	3.0 3.0
No treatment	-	-	-	48/46	113	6.5	0	0	0	0	13.0	-

[a]UF = ultrafine; F-sm = small fine.

[b]Total volume calculated from mass instilled and density. A standard for a "non-overload situation" in rats was set at a lung burden of 1 µl dust per g wet weight of control lungs deduced from experiments with Fischer rats. At this level, the halftime of lung clearance is about doubled. The lung wet weight of the control rats (Fischer strain) is given as 1.5 g.

[c]Number of sufficiently examined rats which survived at least 26 weeks after first instillation.

[d]Period after first instillation in which 50 % of the animals died excluding rats which died immediately after anesthesia.

[e]Percentage of rats with any macroscopically diagnosed lung tumour regardless of existing tumours located at other sites which lead to the conclusion that the lung tumour detected might be a metastasis.

[f]Percentage of rats with lung tumour(s) which are probably not a metastasis of a tumour located at other sites; these lung tumours were classified as macroscopically primary lung tumours.

[g]Primary lung tumour types diagnosed: benign: adenoma, epithelioma; malignant: adenocarcinoma, squamous cell carcinoma. Lungs with one or more malignant tumours may additionally have benign tumours.

[h]Relation of percentage of rats with primary lung tumours to the dust volume dose in the lung as a measure of the carcinogenic potency in this experimental group,

[i]The doses had to be reduced because of unexpected acute toxicity.

A re-evaluation of the histopathological findings of this study established that 30 mg of instilled nano-TiO_2 induced tumours in 50% of the animals studied, whereas after instillation of a total of 60 mg of fine TiO_2, tumours were found in 21% of the animals studied. The findings were interpreted to mean that the higher incidence of tumours was a result of direct effects of epithelial translocation of the nanomaterials into the interstitium (Becker, 2011).

According to Mohr *et al*., 2006, in a study performed by Pott and Roller (2005), eleven dusts were tested separately in rats and were classified as respirable granular bio-durable particles (GBP) without known significant specific toxicity. These dusts included: Carbon black, Titanium dioxide, Al-oxide, Al-silicate, Kaolin, Diesel soot, toner, zirconium oxide.
In 579 (58%) lungs of 1002 rats which survived more than 26 weeks after the first instillation of GBP, at least one primary lung tumor type was observed, and in 306 (31%) at least two types. Three benign tumor types were diagnosed in the 579 tumor-bearing rats: bronchiolo-alveolar adenoma in 46%, cystic keratinizing epithelioma in 53%, and non-keratinizing epithelioma in 2.6% of the rats. Two of three malignant tumour types (bronchiolo-alveolar carcinoma and squamous cell carcinoma) occurred in 46% and 31% of the tumour-bearing rats, respectively, and adenosquamous carcinoma was diagnosed in 0.9%. Numerous lungs with a malignant tumor also showed one or more benign tumor types. The proportionate incidences of the four predominantly diagnosed tumour types were compared with three summarized experimental groups which were exposed either to carbon black (two size classes), to titanium dioxide (two size classes), or to the total of the other nine GBP. No significant difference was detected. The same results were found with the fibrogenicity of ultra-fine GBP (Bellmann *et al*. 2006).
Another essential outcome of the 19-dust study is that GBP volume in connection with particle size turned out to be the most adequate dose metric for the carcinogenicity of GBP. The 4 tested GBP-ultra-fine were about 2 times more effective than the "small" GBP-small-fine and 5- to 6 times more effective than the "large" GBP-large-fine, mean diameters 1.8 - 4 µm).

Other data were available from the IARC monograph volume 93.

No difference in the incidence of lung tumours (17/24 *versus* 19/22 controls) or tumour multiplicity (2.24 ± 1.35 *versus* 1.42 ± 0.77) was noted in 20 week-A/J female mice receiving a single *intratracheal instillation* of a suspension of 0.5 mg TiO_2 (> 99.9% pure; size unspecified) in saline or saline alone and maintained until 105 weeks of age (Koizumi, 1993). However, this study was performed at only one unique low dose.

No respiratory tract tumours were found in male and female Syrian golden hamsters receiving 0 or 3 mg TiO_2 (purity unspecified, particle size: 97% < 5 µm; 51% < 0.5 µm) in 0.2 ml saline once a week for 15 weeks and observed until spontaneous death (between 110-120 weeks for controls and 70-80 weeks for treated group). This decreased lifespan was not explained further in the IARC monograph. In comparison, two tracheal papillomas were found in untreated controls (Stenbäck, 1976). In a further study, Syrian golden hamsters received intratracheal instillations of 3 mg TiO_2 (purity unspecified, particle size: 97% < 5 µm; 51% < 0.5 µm) + benzo[*a*]pyrene or benzo[*a*]pyrene alone once a week for 15 weeks. Animals were observed until spontaneous death, occurring by 90-100 weeks for benzo[*a*]pyrene control group and 60-70 weeks for treated group. TiO_2 + benzo[*a*]pyrene induced tumours in the larynx (11/48 papillomas and 5/48 squamous-cell carcinomas), in the trachea (3/48 papillomas, 14/48 squamous cell carcinomas and 1/48 adenocarcinoma) and lung (1/48 adenoma, 1/48 adenocarcinoma, 15/48 squamous-cell carcinomas and 1/48 anaplastic carcinoma. Two papillomas occurred in the trachea of the benzo[*a*]pyrene control group (Stenbäck, 1976).

4.1.1.3 Carcinogenicity: other routes

The following data has been extracted from the IARC monograph volume 93.

After a single *subcutaneous injection* of saline or 30 mg of one of the 3 preparations of TiO_2 (> 99% pure (size unspecified in the IARC monograph), coated with antimony trioxide; > 95% pure, coated with aluminium oxide; or > 85% pure, coated with both compounds) in saline, Sprague-Dawley rats were observed until spontaneous death (136, 126, 146, 133 weeks in the control and the three TiO_2 groups, respectively). No tumour was observed at the site of injection in any group (Maltoni, 1982). The IARC noted the inadequate reporting of the study.

No difference in the incidence of local or distant tumours was observed in groups of Marsh-Buffalo mice receiving a single *intraperitoneal injection* of either saline or 25 mg TiO_2 (purity > 98%, size unspecified in the IARC monograph) in saline and observed until 18 months after treatment (Bischoff, 1982). In another study, Wistar rats received intraperitoneal injections of TiO_2 (P25) in saline solution: the first group received a total of 90 mg/animal in 5 weekly injections, the second group received a single injection of 5 mg/animal and the third group three injections of 2, 4 and 4 mg/animal. A concurrent control received a single injection of saline alone. Average lifespans were 120, 102, 130 and 120 weeks, respectively. No intra-abdominal tumour was reported in 47 and 32 rats that were examined in the second and third groups, 6/113 rats (5.3%) examined in the first group had sarcomas, mesotheliomas or carcinomas of the abdominal cavity. Two of 32 controls (6.3%) had abdominal tumours (type not specified). In a similar experiment with female Sprague-Dawley rats receiving a single intraperitoneal injection of 5 mg/animal TiO_2, 2/52 rats (3.8%) developed abdominal tumours (type not specified) (average lifespan of 99 weeks). However, no control group was available for comparison (Pott, 1987). The IARC noted the limited reporting of the study. In a last intraperitoneal study, Fischer rats received a single injection of several man-made mineral fibres, including TiO_2 (rutile) whiskers (fibre length about 2.5 µm; fibre diameter about 0.125 µm). The fibres were given at 5, 10 or 20 mg with 1mg of dust suspended in saline before injection. Two years after

administration, no peritoneal mesothelioma was induced by titanium dioxide whiskers (Adachi, 2001). The IARC noted the inadequate reporting of the study.

4.1.1.4 Carcinogenicity: dermal

A two-stage mouse skin carcinogenesis bioassay was performed to examine the promoter potential of coated and uncoated TiO_2 nanoparticles via dermal route (Furukawa, 2011). The study was performed with the GLP of the Japanese Ministry of Health and Welfare Ordinance No. 21 (1997) and in compliance with guideline for Carcinogenicity studies of Drugs 3.2 (in vivo additional tests for detection of carcinogenicity) of the Japanese Ministry of Health and Welfare (1999). The coated TiO_2 was characterized by a TiO_2 content of 79.2%, spindle shape with long axis of 50-100 nm and short axis of 10-20 nm. The uncoated TiO_2 was characterized by a TiO_2 content of 96.0%, spindle shape with long axis of 50-100 nm and short axis of 10-20 nm. No information on crystallinity was reported for these types of TiO_2. As initiation treatment, 7,12-dimethylbenz[a]anthracene (DMBA) [used as initiator] or vehicle alone (acetone) was applied to fur clipped back skin of CD1(ICR) female mice one time. Starting 1 week after the initiation treatment, TiO_2 (5, 10 and 20 mg/animal) in Pentalan 408 or 12-o-tetradecanoylphorbol 13-acetate (TPA; used as positive control promoter) were applied twice weekly for 19 weeks as post-initiation treatments. A further group received Pentalan 408 only as a vehicle control.

No changes in survival, general condition and body weight related to treatment were observed. On macroscopic observation, 1–2 nodules/group on the skin were observed in groups exposed to both coated and uncoated TiO_2 as well as the control group after DMBA initiation. The nodules were histopathologically diagnosed as squamous cell hyperplasia, sebaceous gland hyperplasia, squamous cell papilloma and keratoacanthoma. In contrast, the positive control group was effective, with 100% of the animals developing nodules. Other findings included the presence of foreign bodies (possibly TiO_2) on the surface of the mouse skin suggesting that significant amounts of TiO_2 did not penetrate the dermis. Furthermore, enlargement of mandibular and abdominal region lymph nodes, spleen and thymus was also observed but without dose-response relationship. The authors concluded that TiO_2-NP do not possess post-initiation potential for mouse skin carcinogenesis, mainly because of the lack of penetration.

A second two-stage skin carcinogenicity study was performed in rats and mice (Sagawa, 2012). The tested TiO_2 was a non-coated rutile form and a particle size of 20 nm. When diluted in Pentalan 408 solution, the mean length of TiO_2 was 4.97 ± 0.50 µm. The back skin of male Hras 128 rats and wild-type SD rats received a single topical application (painting) of 0.5 ml DMBA solution as initiation treatment. Two weeks later, the animals were divided into 3 groups: vehicle alone (Pentalan 408) (group 1); 50 mg TiO_2 in Pentalan 408 (group 2) or 100 mg TiO_2 in Pentalan 408 (group 3). Treatment was administered twice a week until week 40. TiO_2 was also applied on back skin of female CD1 mice, 2-week after a single application of DMBA, at 10 mg or 20 mg twice a week until week 52. A further group received DMBA as initiator and then TPA as positive control promoter.

No statistically significant differences were found in tumor incidence (squamous cell carcinomas and papillomas) or multiplicity between treated and control groups of Hras 128 rats, wild-type SD rats or CD1 mice.

Table 4.1.1.4-01. Effect of non-coated TiO_2 on skin carcinogenesis

Group	Treatment	No of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
Experiment with Hras128 rats								
1	DMBA + Pentalan 408	17	16 (94)	9.65±7.05	0	0	16 (94)	9.65±7.05
2	DMBA + 50 mg TiO_2	16	14 (88)	6.81±6.21	2 (13)	0.19±0.54	14 (88)	7.00±6.52
3	DMBA + 100 mg TiO_2	17	16 (94)	7.59±3.86	2 (12)	0.12±0.331	16 (94)	7.71±3.93
Experiment with wild-type SD rats								
1	DMBA + Pentalan 408	12	3 (25)	0.25±0.45	0	0	3 (25)	0.25±0.45
2	DMBA + 50 mg TiO_2	12	2 (17)	0.17±0.39	2 (17)	0.17±0.39	4 (33)	0.33±0.49
3	DMBA + 100 mg TiO_2	12	1 (8)	0.08±0.29	0	0	1 (8)	0.08±0.29
Experiment with wild-type CD1 mice								
1	DMBA + Pentalan 408	16	3 (19)	0.25±1.30	0	0	3 (19)	0.25±1.30
2	DMBA + 50 mg TiO_2	16	1 (6)	0.06±0.25	0	0	1 (6)	0.06±0.25
3	DMBA + 100 mg TiO_2	15	2 (13)	0.13±0.35	0	0	2 (13)	0.13±0.35
4	DMBA + TPA	15	13 (87)*	2.00±1.41*	2 (13)	0.13±0.35	13 (87)*	2.00±1.41*

SCP: squamous cell papillomas; SCC: squamous cell carcinoma
* Significantly different from group 1 (control) by Student's t-test ($p < 0.001$).

The authors concluded that TiO_2 did not cause skin tumour promotion in the skin carcinogenesis-sensitive Hras 128 rat model or in CD1 mice. This was probably due to the lack of penetration of the particles through the epidermis to the dermis where cytogenetic cells responsible for skin carcinogenesis reside. This study was reviewed by the SCCS in 2013 which concluded that since 94% of the Hras rats treated with DMBA alone developed tumours, the model is not adequate and no conclusion can be drawn from the study.

A further experiment was performed with a TiO_2 coated with silicone (mean particle diameter of 35 nm). This type of coating is used to prevent aggregate formation and to enhance dispersal. Female

rasH2 mice and wild type CB6F1 mice received single application of DMBA. Two weeks later, the animals were divided in 3 groups. Group 1 received vehicle only (silicone oil), group 2 received 10 mg of TiO_2 in silicone oil and group 3, 20 mg of TiO_2 in silicone oil. A further group received 20 mg of TiO_2 without pre-treatment with DMBA. Mice were painted 5 times per week. The rasH2 mice were killed after 8 weeks and the wild-type mice after 40 weeks. No positive control was included.

An increase in the number of tumours was found in mice initiated with DMBA but this was not significant. No conclusion can be drawn from this study according to SCCS opinion (2013) due to the lack of positive controls and very high tumour incidence in the "initiated" mice. In the group only treated with TiO_2, neither squamous cell papillomas nor squamous cell carcinoma were found.

Table 4.1.1.4-02. Effect of coated TiO_2 on skin carcinogenesis

Group	Treatment	No of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
Experiment with rasH2 mice								
1	DMBA + silicone	15	14 (93)	7.27±4.74	5 (33)	0.60±0.99	14 (93)	7.87±5.17
2	DMBA + 10 mg TiO_2	15	15 (100)	8.13±3.66	9 (60)	1.00±1.00	15 (100)	9.13±3.76
3	DMBA + 20 mg TiO_2	15	15 (100)	6.80±3.88	8 (53)	0.73±0.80	15 (100)	7.53±3.31
4	20 mg TiO_2	15	0	0	0	0	0	0
Experiment with wild-type CB6F1 mice								
1	DMBA + silicone	15	1 (7)	0.07±0.26	1 (7)	0.07±0.26	2 (13)	0.13±0.35
2	DMBA + 10 mg TiO_2	15	2 (13)	0.13±0.35	0	0	2 (13)	0.13±0.35
3	DMBA + 20 mg TiO_2	15	2 (13)	0.20±0.56	0	0	2 (13)	0.20±0.56
4	20 mg TiO_2	15	0	0	0	0	0	0

SCP: squamous cell papillomas; SCC: squamous cell carcinoma

A third study using the two-stage skin model was performed in rats with ultraviolet B radiation as initiation treatment (Xu, 2011). The tested TiO_2 was of rutile type, without coating and had a mean primary diameter of 20 nm. When diluted in Pentalan 408, the size of TiO_2 particles ranged from 10 nm to 300 µm. Hras 128 rats and wild-type rats were exposed to UVB (ultraviolet B) radiation on shaved back skin twice weekly for 10 weeks. Then, the shaved area was painted with 50 mg of TiO_2 in Pentalan 408 twice weekly until sacrifice planned on week 52. Female Hras 128 rats were sacrificed at week 16 because of early mammary tumor development. A further group received TiO_2 without UVB radiation pre-treatment. However, no negative (vehicle alone without UVB radiation) and positive control groups was included.

In male Hras 128 rats, skin papillomas developed from week 32 and the incidence was 1/8 in the groups treated with TiO_2 with and without UVB. No skin tumors were observed in female Hras 128 rats and wild-type rats of both sexes. Eye lid squamous cell papillomas were found in wild-type female rats exposed to UVB with or without TiO_2 with incidences of 12.5% and 14.3%, respectively. No statistically significant inter-group differences in incidence, multiplicity or weight were found. Mammary tumors, diagnosed as adenocarcinomas, were induced with high incidence in Hras128 rats of both sexes. Wild-type female rats also had a relatively high incidence of mammary tumors compared with historical controls of spontaneous mammary tumor development. No statistically significant inter-group differences in incidence, multiplicity or weight were observed. TiO_2 aggregates of various sizes were observed in the upper *stratum corneum* and in some hair follicles at the level of granular cell layer, but TiO_2 was not found in the underlying epidermis, dermis or subcutaneous tissue.

Table 4.1.1.4-03. Skin and mammary tumors in Hras128 and wild-type rats

Group	Treatment	No of rats	Skin tumor		Mammary tumor	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
Experiment with Hras128 rats						
Males						
1	UVB + TiO_2	8	1 (12.5)	0.13±0.35	4 (50)	0.50±0.53
2	UVB	8	0	0	3 (36)	0.38±0.51
3	TiO_2	8	1 (12.5)	0.13±0.35	4 (50)	0.50±0.53
Females						
1	UVB + TiO_2	6	0	0	5 (83)	1.67±1.37
2	UVB	5	0	0	2 (40)	0.60±0.89
3	TiO_2	6	0	0	6 (100)	1.33±0.52
Experiment with wild-type rats						
Males						
1	UVB + TiO_2	6	0	0	0	0
2	UVB	5	0	0	0	0
3	TiO_2	5	0	0	0	0
Females						
1	UVB + TiO_2	8	1 (12.5)	0.13±0.35	1 (12.5)	0.13±0.35
2	UVB	7	1 (14.3)	0.14±0.38	1 (14.3)	0.14±0.38
3	TiO_2	8	0	0	0	0

The authors concluded that the lack of skin carcinogenesis promotion activity was probably due to the lack of penetration of TiO_2 particles through the epidermis to the dermis, where skin tumours arise. This study was reviewed by the SCCS in 2013 which concluded that this model is not generally accepted for studying initiation and promotion of skin tumours. Since no positive control was included it is not possible to make any conclusion with regard to potential carcinogenic properties of TiO_2 from this study.

4.1.2 Human information

Case reports

Yamadori et al. (1986) reported a titanium dioxide pneumoconiosis accompanied by a papillary adenocarcinoma of the lung in a 53-year-old male who was engaged in packing TiO_2 for about 13 years and with a 40-year history of tobacco smoking. Titanium was diffusely deposited in the lung and was engulfed by macrophages in the interstitium and alveolar spaces. Slight fibrosis of the interstitium around bronchioles and vessels was noticed.

Other case reports were summarized in IARC monograph vol. 93 and NIOSH Current Intelligence Bulletin (CIB) 63. None of these case reports provided quantitative industrial hygiene information about workers' TiO_2 dust exposure. Deposits of titanium dioxide in lung tissue as well as in lymph nodes were found. Non-neoplastic respiratory effects were observed in workers, including decline in lung function, pleural disease with plaques and pleural thickening and mild fibrosis changes. More severe reactions were observed in a few cases. However, the workers in these studies were also exposed to asbestos and/or silica.

Case-control studies

The risk of inhabitants of Montreal developing lung cancer was studied in a case-referent study conducted between 1979 and 1985 (Boffetta et al., 2001). For the purpose of this analysis, 857 histopathologically confirmed cases of lung cancer in the male population (aged 35–70 years) were selected. The control groups consisted of 533 randomly selected healthy people and 533 people with cancer in organs other than the lung. The cases and referents were similar with respect to age and ethnicity. However, the cases smoked more than the referents and asbestos and benzo(a)pyrene was slightly more prevalent among the cases. Three main circumstances of exposure were considered: TiO_2 production, manufacture and use of TiO_2 containing products, mainly paints and metal arc welding. Exposure to TiO_2 or TiO_2 components was assessed on the basis of a questionnaire. Three concentration categories were defined: 0.05-1 mg/m^3, 1-10 mg/m^3 and over 10 mg/m^3. Thirty-three cases and 43 controls were classified as ever exposed to TiO_2. Results of unconditional logistic models were adjusted for age, socioeconomic status, ethnicity, respondent status (i.e. self or proxy), tobacco smoking, asbestos and exposure to benzo[a]pyrene. There was no indication of a correlation between lung cancer development and the frequency, level or duration of TiO_2 exposure. According to IARC monograph volume 93, the main limitations of this study are the reliance on self-reported occupational histories and expert opinion rather than measurement of exposure. A strength of this study was the availability of lifetime smoking histories and other covariates. Additional limitations reported by the NIOSH were the use of surrogate indices for exposure, the absence of particle size characterization and the non-statistically significant lung cancer OR (odd ratio) for exposure to TiO_2 fumes, which was based on a small group of subjects and most were also exposed to nickel and chromium.

Ramanakumar et al., 2008 described two case-control studies performed in Montréal. Interviews were conducted in 1979-1986 for the first study (857 cases, 533 population controls, 1,349 cancer controls) and in 1996-2001 for the second study (1,236 cases and 1,512 controls). Some results from the first study have already been described in the publication Boffetta (2001), however, the publication of Ramanakumar (2008) described a new statistical approach and combined results of the

two studies. Forty percent of workers exposed to TiO_2 were painting industry workers, 19% were construction labourers and 17% were motor-body repairmen. The lifetime prevalence of exposure to TiO_2 was about 4%. Concentration levels were defined as low, medium and high. Lung cancer risk was analysed in relation to each exposure, adjusting for several potential confounders, including smoking. Although some odd ratios of lung cancers were above 1.0, none were statistically significantly increased. Subjects with occupational exposure to TiO_2 did not experience any detectable excess risk of lung cancer. Limitations are the same as reported for Boffetta, 2001.

A further hypothesis-generating case–control study in Montréal, Canada is described in the IARC monograph volume 93 (Siemiatycki, 1991). More than 4000 subjects were interviewed and included patients with 20 different types of cancer and a series of population controls. A panel of industrial hygienists reviewed each job history reported by study subjects and assessed exposure to 293 substances. Results on associations between TiO_2 and several sites of cancer were reported. Some indications of excess risk were found in relation to squamous-cell lung cancer (OR: 1.6; 90% CI, 0.9–3.0; 20 cases) and urinary bladder cancer (OR: 1.7; 90% CI, 1.1–2.6; 28 cases). No excesses were observed for any exposure to TiO_2 for all lung cancer combined (OR: 1.0; 90% CI, 0.7–1.5; 38 cases), for kidney cancer (OR: 1.1; 90% CI, 0.6–2.1; seven cases) or for cancer at several other sites other than the urinary bladder.

Cohort studies

In a cohort study with a nested case–control study, 1575 workers exposed to TiO_2 and employed for more than one year in two US factories were observed between 1956 and 1985 for cancer and chronic respiratory disease incidence and from 1935 to 1983 for mortality (Chen et al, 1988). TiO_2 exposure varied from 0 to more than 20 mg/m^3. Observed numbers of incident cases of cancer were compared with expected numbers based on company rates, and the observed numbers of deaths were compared with both company rates and rates in the USA. The observed number of all cancer cases was slightly higher than expected in the TiO_2-exposed cohort (38 observed, 32.6 expected). There were 8 lung cancer cases compared to 7.7 expected; this difference is not statistically significant. Cohort analysis suggested that the risks of developing lung cancer and other fatal respiratory diseases were not higher for TiO_2-exposed employees than for the referent groups. Nested case-control analysis found no statistically significant associations between TiO_2 exposure and risk of lung cancer, chronic respiratory disease and chest roentgenogram (X-ray) abnormalities. No cases of pulmonary fibrosis were observed among TiO_2-exposed employees. According to IARC monograph volume 93, it is noted that details of exposure to TiO_2 and other factors were not described, that cancer mortality and specific cancer sites were not reported in detail, that incident cases of cancer only in actively employed persons were used for both observed and company reference rates, and that the numbers of incident cases were compared only with company rates. Similar comments concerning the lack of details were made by the NIOSH (2011). It has also been noted that the presence of other chemicals and asbestos could have acted as confounders.

A retrospective cohort mortality study was conducted among 4,241 workers handling TiO_2 in four US companies (Fryzek et al., 2003). Participants were employed from January 1960 for at least 6 months and were observed until December 2000. The heaviest exposure to TiO_2 occurs in the

milling and packing areas where TiO_2 is finely processed by the micronizers and dust from the bags used for shipment is dispersed through the air during bagging by the packers. Most of the exposure measurements were area samples rather than personal samples. Geometric mean of the sampling data ranged from 1 to 6.1 mg/m^3, with exposure decreasing over time. The number of expected deaths was based on mortality rates by sex, age, race, time period and the state in which the plant was located. Cox proportional hazard models that adjusted for the effects of age, sex, geographical area and date of first employment were used to estimate relative risks of exposure to titanium dioxide (i.e. average intensity, duration and cumulative exposure) in medium- or high-exposure groups versus the lowest exposure group. The SMR (standardized mortality ratio) for all causes of death was significantly less than expected (SMR = 0.8; 95% CI = 0.8-0.9). The total observed number of lung cancers were within the expected range for TiO_2 exposed workers (SMR = 1.0; 95% CI = 0.8-1.3). A significantly elevated SMR for lung cancer (SMR = 1.5; 95% CI = 1.0-2.3) was found among short-term workers (worked 9 or fewer years) after 20 or more years of follow-up. However, SMRs for mortality from all causes and mortality due to lung cancer and non-malignant respiratory disease decreased with longer durations of employment. Additional analyses were performed in response to a suggestion that the RRs (risk ratios) may have been artificially low, especially in the highest category of cumulative exposure, because of statistical methods used. The new analyses yielded hazard ratio similar to those in the original analysis. According to IARC monograph volume 93, this cohort was relatively young (about half were born after 1940) making the duration of exposure to TiO_2 and the latency period for the development of lung cancer rather short. Moreover, the oldest company reports were not available for the authors to evaluate. Additional limitations reported by the NIOSH consisted in the lack of information about ultrafine exposure and the limited data on non-occupational factors (e.g. smoking).

A mortality follow-up study of 15,017 workers (employment started from 1927-69 and ended 1995-2001) was carried out in 11 European companies (from Finland, France, Germany, Italy, Norway and UK) manufacturing TiO_2 (Boffetta et al., 2004). The factories predominantly produced pigment-grade TiO_2 using sulfate and/or chloride processes. The follow-up ranged from 27 years in Italy to 47 years in the UK. The overall proportion of cohort members lost to follow-up was 3.3% and that to emigration was 0.7%. During the follow-up, cohort members accumulated 371,067 person-years of observation (with 95.5% of men) and 2652 members died. The yearly average estimated exposure to TiO_2 dust by factory varied from 1.0 to 0.1 mg/m^3; however, average levels ranged up to 5 mg/m^3 for individual occupational titles. The median estimated cumulative exposure to respirable TiO_2 dust in the cohort was 1.98 mg/m^3 year. Workers employed in the surface treatment area (drying, packing, blending) and mixed jobs had the highest estimated cumulative exposure. Exposure to other pollutants was generally low. The prevalence of smokers was higher among cohort members compared to the national population in Finland, Germany and Italy. The only cause of death with a statistically significant increased SMR was lung cancer (1.23; 95% CI 1.10-1.38) based on a fixed-effects statistical model. However, there was no relationship with exposure to TiO_2 considering duration of employment and concentration. The SMRs varied from 0.76 (95% CI 0.39–1.32) in Finland to 1.51 (95% CI, 1.26–1.79) in Germany. Because the heterogeneity between countries was of borderline significance (p-value=0.05), a random-effects model was also fitted and gave a SMR of 1.19 (95% CI 0.96–1.48). A positive, non-significant dose–response relationship was suggested between estimated cumulative exposure to TiO_2 dust and mortality from kidney cancer. No increase was found for this neoplasm in the SMR analysis: the SMRs for the three categories of estimated

cumulative exposure to TiO_2 dust (< 4 mg/m^3; 4-13.9 mg/m^3; ≥ 14 mg/m^3 – year) were 0.45 (95% CI, 0.12–1.16), 1.15 (95% CI, 0.31–2.89) and 1.18 (95% CI 0.37–2.67). According to IARC monograph volume 93, the strengths of this study are the large size, the high follow-up rate and the detailed exposure assessment. The availability of data on tobacco smoking, although limited to slightly more than one-third of the cohort, provided some reassurance that tobacco smoking was unlikely to be a confounder. Besides the lack of adjustment for smoking, other limitations are possible exposure misclassification, the exclusion of part of the early experience of the cohort from the analysis, which reduces the power of the study to detect an association, and the relatively recent beginning of operation of some of the factories that resulted in a follow-up period that was too short to allow the detection of an increase in risk for lung cancer.

4.1.3 Other relevant information

a) Acute toxicity studies

A total of 3 publications describing animal studies with acute intratracheal instillation were reported here since they bring some information on the impact of physico-chemical properties on the toxicity of TiO_2.

Four laboratories evaluated lung responses in C57BL/6 mice to engineering nanomaterials delivered by oropharyngeal aspiration, and three laboratories evaluated Sprague-Dawley or Fisher 344 rats following intratracheal instillation (Bonner, 2013). The nanomaterials tested included three forms of TiO_2 [anatase/rutile spheres (TiO_2-P25), anatase spheres (TiO_2-A), and anatase nanobelts (TiO_2-NBs)].

Table 4.1.3-01. Physicochemical characterization (taken from Xia, 2013)

Quality	Technique	TiO_2-P25	TiO_2-A	TiO_2-NBs
Size (nm)	TEM	~ 24	~ 28	L:7000; W:200; T:10
Size in H_2O (intensity-based) (nm ± SD)	DLS	209 ± 8 (PdI 0.065)	292 ± 70	2,897 ± 117
Phase and structure	XRD	81% anatase and 19% rutile	100% anatase	100% anatase
Shape/morphology	TEM	Spheroid	Spherical	Belt
Surface area (m^2/g)	BET	53	173	18
Zeta potential in H_2O at pH 6.0 (mV ± SD)	Zetasizer	–34.4 ± 1.6	–30.7 ± 0.8	–30.3 ± 2.8
Elemental analysis (weight percent)	ICP-MS	98.6	NA	NA

Abbreviations: L, length; NA, not available; PdI, polydispersity index; T, thickness; W, width; XRD, X-ray defraction.

The concentrations used were: 0, 10, 20 or 40 µg for the oropharyngeal aspiration and 0, 20, 70, 200 µg for intra-tracheal instillation. One day after treatment, bronchoalveolar lavage fluid was collected to determine differential cell counts, lactate dehydrogenase (LDH), and protein. Lungs were fixed for histopathology. Responses were also examined at 7 days after treatment. All types of titanium dioxide caused significant neutrophilia in mice at 1 day in three of four labs at the highest concentration. TiO_2-NB caused neutrophilia in rats at 1 day in two of three labs, and TiO_2-P25 and TiO_2-A had no significant effect in any of the labs. Inflammation induced by TiO_2 in mice and rats resolved by day 7. In conclusion, the different types of TiO_2 produced similar patterns of neutrophilia and pathology in rats (by intra-tracheal instillation) and mice (by oropharyngeal aspiration) after a single intra-tracheal instillation, despite some variability in the degree of neutrophilia.

The pulmonary toxicity of three commercially available forms of nano-TiO_2 was assessed in Crl:CD®(SD)IGS BR male rats (Warheit et al., 2007). The 3 nano-TiO_2 tested were P25, uf-1 and uf-2 and were compared with fine TiO_2 (R-100). Particles were administered diluted in PBS (phosphate-buffered saline) intra-tracheally once at 1 mg and 5 mg/kg bw. Following exposure, the lungs were evaluated for bronchoalveolar lavage (BAL) fluid inflammatory markers, cell proliferation, and by histopathology at post-instillation time points of 24 h, 1 week, 1 and 3 months.

Table 4.1.3-02. Summary of physicochemical properties of the tested TiO_2

	P25	uf-1	uf-2	fine TiO_2
Crystallinity	80/20 anatase/rutile	rutile	rutile	R-100, rutile
Composition	100% TiO_2	98% TiO_2 and 2% Al	88% TiO_2 core with SiO_2 (7%) and aluminium (5%) coating	99% TiO_2 and 1% alumina
Primary particle size (nm)	25	100	100	300
Median size in water (nm)	130	136	149	382
Median size in PBS (nm)	2692	2144	2891	2667
Surface area (m^2/g)	53	18	36	5.8
pH in deionized water	3.28	5.64	7.14	7.49
pH in PBS	6.70	6.78	6.78	6.75
Chemical reactivity (delta b)	23.8	10.1	1.2	0.4

In all cases, inflammation was observed, as evidenced by an increase of percent neutrophils in the BAL fluid. P25 caused more pronounced inflammatory with significant pulmonary inflammation and cytotoxic effects lasting through 1 month post-exposure. Only transient pulmonary inflammatory response was found with the other types of TiO_2. A significantly higher lung parenchymal proliferation index occurred in the P25 group at 5 mg/kg after 24 h and 3 months. No histopathological lung findings were observed with uf-1, uf-2 and fine TiO_2. Vigourous macrophages accumulation, concomitant with a sequestration of the aggregated macrophages within the alveolar regions of the lungs was found with P25.

Differences in responses to anatase/rutile TiO_2 particles *versus* the rutile uf-1 and uf-2 TiO_2 particles could be related to crystal structure, inherent pH of the particles, or surface chemical reactivity. According to the authors, based on these results, inhaled rutile ultrafine-TiO_2 particles are expected to have a low risk potential for producing adverse pulmonary health effects. Authors also concluded that the lung toxicity of anatase/rutile TiO_2 should not be viewed as representative for all ultrafine-TiO_2 particle-types. In conclusion, the findings point to the significance of surface modification (coating) and crystallinity (anatase/rutile) for toxic potential.

Chen et al. (2006) exposed adult male ICR mice to intratracheal single dose of 0.1 or 0.5 mg nanoTiO_2 in order to investigate pulmonary toxicity and its molecular pathogenesis. Lung tissues were collected at 3rd day, 1st week, and 2nd week for morphometric, microarray gene expression, and pathway analyses. The characteristics of the tested nanoTiO_2 were the following: rutile crystal phase, highly dispersed and hydrophilic fumed TiO_2 with a diameter of 19–21 nm, a specific surface area of 50 ± 15 m^2/g, and a purity of ± 99.5%. In order to avoid aggregation, the nano TiO_2 suspension was ultrasonicated before it was used. The authors demonstrated that nanoTiO_2 can

induce severe pulmonary inflammation and emphysema. NanoTiO_2 induced differential expression of hundreds of genes including activation of pathways involved in cell cycle, apoptosis, chemokines, and complement cascades. The results indicated that pulmonary emphysema is triggered by nano TiO_2 activation of macrophage, up-regulations of placenta growth factor (P1GF) and other inflammatory cytokines (CXCL1, CXCL5, and CCL3) that resulted in disruption of alveolar septa, type II pneumocyte hyperplasia, and epithelial cell apoptosis. No significant pathological changes were seen using the same dose of micro TiO_2 (180-250 nm) suggesting that nano-TiO_2 caused a significantly greater pulmonary inflammatory response.

b) Repeated-dose toxicity studies

Several studies from subacute to chronic exposure were performed with TiO_2. These studies were summarized in different published reviews (IARC, 2010; SCCS, 2014; NIOSH, 2011). The main common effect observed after TiO_2 exposure was an inflammation associated with pulmonary effects including lung epithelial cell injury, cholesterol granulomas and fibrosis. Some of the studies aimed to investigate the impact of physico-chemical properties of TiO_2 (particle size, coating, crystallinity…) on the toxicity, while other studies compared the effects of TiO_2 in several rodent species.

Among all these studies, 3 assays by inhalation were further described here since they bring information on inter-species differences, possible specific mechanisms of toxic action, reversibility and impact of physico-chemical properties on the toxicity of TiO_2.

In subchronic inhalation studies (Everitt et al, 2000; Bermudez et al, 2002, 2004; Hext et al, 2005), female CDF(F344)/CrlBR rats, B3C3F1/CrlBR mice and Lak:LVG(SYR)BR hamsters were treated with aerosol concentrations of 0.5, 2 or 10 mg/m^3 of nano-TiO_2 (P25, average primary particle size of 21 nm) or 10, 50 or 250 mg/m^3 of fine TiO_2 for 13 weeks. Groups of 25 animals for each species and time point were used in the study performed with ultrafine TiO_2. Sixty-five rats and mice and 73 hamsters were used in the study with fine TiO_2. Following the exposure period, animals were held for recovery periods of 4, 13, 26 or 52 weeks (46 weeks for fine-TiO_2-exposed hamster or 49 weeks for the nano-TiO_2-exposed hamsters). At each time point, burdens in the lung and lymph nodes and selected lung responses were examined. The responses studied were chosen to assess a variety of pulmonary parameters, including inflammation, cytotoxicity, lung cell proliferation and histopathological alterations.

Particle size analysis and chamber concentrations of fine (pigmentary) and ultrafine (nano) TiO_2 aerosol are given hereafter (Table 4.7.1.2-01). It can be noted that the aerosol generated for ultrafine TiO_2 was made up of particle aggregates.

Table 4.1.3-03. Summary of exposure conditions

Species	Fine TiO_2 (rutile)		Ultrafine (nano)-TiO_2 (P25)	
	Chamber concentrations (mg/m^3)	Mass median aerodynamic diameter (µm)	Chamber concentrations (mg/m3)	Mass median aerodynamic diameter (µm)
Hamster	9.9 ± 1.0 mg/m^3	1.36 ± 0.07	0.54 ± 0.06 mg/m^3	1.29 ± 0.30

	49.7 ± 4.0 mg/m^3 251.1 ± 17.3 mg/m^3		2.2 ± 0.1 mg/m^3 10.8 ± 1.0 mg/m^3	
Mouse	9.5 ± 1.2 mg/m^3 47.0 ± 4.6 mg/m^3 240.3 ± 20.0 mg/m^3	1.39 ± 0.04	0.52 ± 0.03 mg/m^3 2.1 ± 0.1 mg/m^3 10.5 ± 0.7 mg/m^3	1.45 ± 0.49
Rat	9.6 ±1.1 mg/m^3 47.7 ± 5.1 mg/m^3 239.1 ± 19.3 mg/m^3	1.44 ± 0.09	0.53 ± 0.03 mg/m^3 2.1 ± 0.1 mg/m^3 10.7 ± 0.6 mg/m^3	1.44 ± 0.0.57

During exposure phase, no significant mortalities occurred with fine TiO_2 although treatment-related deaths were noted in mice exposed to ultrafine TiO_2. In the post-exposure phase, morbidity and mortality was principally found in hamsters due to severe chronic renal disease in both studies with ultrafine and fine TiO_2.

Following the end of the exposure period, a depression in body weight was noted in all groups and all species exposed to both types of TiO_2 (4-5% in mice, 2-3% in rats and 5-11% in hamsters). A more marked body weight loss was noted in hamsters exposed to ultrafine TiO_2. Recovery occurred over the next three to four weeks in mice and rats but was slower in hamsters, with recovery within approximately 6 weeks.

Clear species differences in pulmonary clearance and lesions were observed.

In the study performed with fine TiO_2, the dose-related increase in TiO_2 lung burdens were higher in mice followed by rats and then hamsters after 13 weeks of exposure. At the end of the recovery period, rats and mice of the high-dose group retained approximately 75% of the initial burden whereas hamsters retained approximately 10%. The calculated particle retention half-time for the three dose levels was 100, 324 and 838 days in rats, 50, 417 and 621 days in mice and less than 110 days in hamsters. In the study performed with ultrafine TiO_2, rats and mice exhibited equivalent TiO_2 lung burdens whereas lung burdens in hamsters were approximately 2 to 5 fold lower than those of rats and mice after 13 weeks of exposure. At the end of the recovery period, rats of the high-dose group retained approximately 57% of the initial burden compared to approximately 46% for mice and approximately 3% for hamsters. The calculated particle retention half-time for the three dose levels was 63, 132 and 395 days in rats, 48, 40 and 319 days in mice and 33, 37 and 39 days in hamsters. Therefore, under the conditions of these studies, hamsters were better able to clear TiO_2 particles than were similarly exposed mice and rats.

Inflammation, as evidenced by increases in macrophage and neutrophil numbers and in soluble indices of inflammation (LDH and protein) in bronchoalveolar lavage fluid, was noted in all three species exposed to 50 and 250 mg/m^3 of fine TiO_2 and in rats and mice at 10 mg/m^3 of ultrafine TiO_2.

Pulmonary lesions were most severe in rats exposed to both types of TiO_2 with epithelial and fibroproliferative lesions which were progressive even following cessation of particle exposure and diminution of pulmonary inflammation. These effects consisted of alveolar hypertrophy and hyperplasia of type II epithelial cells surrounding aggregations of particle-laden macrophages of minimal to mild severity at the mid dose of fine and ultrafine TiO_2 and which became more severe at the highest concentration of 250 mg/m^3 for fine TiO_2 and 10 mg/m^3 for ultrafine TiO_2. Alveolar

metaplasia (bronchiolization) and septal fibrosis were also noted in rats of the high dose groups by 52 weeks post-exposure. After exposure to fine TiO_2, hamsters developed minimal alveolar type II cell hypertrophy and hyperplasia in both mid and high dose groups and only alveolar type II cell hypertrophy was found in mice. Epithelial, metaplastic and fibroproliferative changes were not noted in both mice and hamsters. In conclusion, rats were unique in the development of a progressive fibroproliferative lesion and alveolar epithelial metaplasia in response to a subchronic exposure to a high concentration of p-TiO_2 and uf-TiO_2.

In Baggs et al study (1997), male Fisher 344 rats were exposed whole body for 6 h/d, 5 days/week for 12 weeks to filtered air (negative control), pigment-grade TiO_2 (TiO_2-F, particle size 250 nm) at 22.3 mg/m^3, ultrafine TiO_2 (TiO_2-D, particle size 20 nm) at 23.5 mg/m^3 or cristobalite (positive control fibrogenic particle) at 1.3 mg/m^3. Groups of 3 or 4 animals were sacrificed at 6 and 12 months after the completion of exposure. After completion of the study, lung burdens were 5.22 ± 0.75 mg for TiO_2-D and 6.62 ± 1.22 mg for TiO_2-F. These values decreased to 3.14 ± 0.59 mg and 1.66 ± 0.76 mg 12 months after exposure of TiO_2-D or TiO_2-F, respectively. Interstitial fibrosis in the lung was found in TiO_2 groups at 6 months post-exposure with significant increase of septal collagen levels. Slightly more fibrosis was found in animals treated with nano-TiO_2 than with fine TiO_2, suggesting that ultrafine particles can have a greater biological activity than larger ones. One year post-exposure, the amount of interstitial fibrosis in TiO_2 groups was not significantly greater than in the negative control group. However, increased number of alveolar macrophages persisted, usually with retained particles. In comparison, moderate focal interstitial fibrosis and moderately severe focal alveolitis were observed 6 months after exposure to SiO_2. After 1 year, fibrosis decreased but was still present.

Warheit et al. 2005 assessed the pulmonary toxicity of inhaled or intra-tracheally instilled TiO_2 particle formulations with various surface treatments in male Crl:CD(SD)IGS BR rats. The pulmonary effects were compared with those of a non-surface TiO_2 ("base TiO_2") and control. In the first study, rats were exposed to TiO_2 formulation for 4 weeks at aerosol concentrations ranging from 1130-1300 mg/m^3 (MMAD = 1.3-1.8 µm) and lung tissues were evaluated by histopathology immediately after exposure, as well as at 2 weeks and 3, 6, and 12 months post-exposure. In the second study, rats were intra-tracheally instilled with nearly identical TiO_2 particles formulations at 2 and 10 mg/kg. The exposure period was followed by 24h, 1 week, 1 month and 3 month recovery period. BAL biomarkers and histopathology of lung tissues were assessed at the end of each recovery period.

Table 4.1.3-04. Composition of TiO_2 particle formulations tested

	Inhalation studies	Instillation studies
Base TiO_2	99% TiO_2 – 1% Al	99% TiO_2 – 1% Al
TiO_2-I	99% TiO_2 – 1% Al + organic	99% TiO_2 – 1% Al + organic
TiO_2-II	96% TiO_2 – 4% Al	96% TiO_2 – 4% Al
TiO_2-III	85% TiO_2–7% Al + 8% AMO	82% TiO_2 – 7% Al + 11% AMO
TiO_2-IV	92% TiO_2 –2% Al + 6% AMO	92% TiO_2 – 2% Al + 6% AMO
TiO_2-V	94% TiO_2–3% Al + 3% AMO	94% TiO_2 – 3% Al + 3% AMO

Al = alumina = Al_2O_3. AMO = amorphous silica – SiO_2. Organic, refers to triethanolamine. Base TiO_2, and TiO_2-I, -II, -IV, and –V formulations are identical between the two studies. TiO_2-III formulations differ slightly between the two studies. All TiO_2 particles were rutile type and particles size ranged from 290 nm (TiO_2-V) to 440 nm (TiO_2-III).

Although all formulation induced minor pulmonary inflammation with accumulation of TiO_2 particles, the results from these studies demonstrated that for both inhalation and instillation, only TiO_2 formulation with the largest components of both alumina and amorphous silica surface treatments produced mildly adverse pulmonary effects (with collagen deposition) when compared to the reference particles. The authors concluded that the surface treatments can influence the toxicity of TiO_2 particles in the lung and that the intra-tracheal installation-derived, pulmonary bioassay studies represent an effective preliminary screening tool for inhalation studies.

Comparing these studies, results demonstrate many similarities since the type of effects and the response of the different animal species are similar for nano and fine TiO_2. Furthermore, a comparison of the lung burdens, using surface area as dose-metrics, reveals that the lung burdens in animals exposed for 13 weeks to 10 mg/m^3 of ultrafine-TiO_2 or to 50 mg/m^3 of pigmentary TiO_2 were approximately the same for all three species.

4.1.4 Summary and discussion of carcinogenicity

Summary of carcinogenicity studies

Oral route:

Two studies assessed the carcinogenic potential of TiO_2 administered in diet at doses up to 50 000 ppm to rats and mice. Low level of characterization is available: in the first study (NCI, 1979), TiO_2 was characterized by an anatase form (unspecified size) and in the second study (Bernard, 1990), it was a fine TiO_2-coated mica of 10-35 µm (unspecified crystalline phase). From these studies, the overall conclusion is that TiO_2 is not carcinogenic by the oral route although no firm conclusion can be reached about the possible carcinogenicity of this compound to Fischer 344 based on an increase of adenoma/adenocarcinomas of the thyroid according to one reviewer of the NCI (1979) study. However, it should be noted that the doses were very high, often higher than that is recommended in the OECD guideline.

Some studies investigated the uptake and translation of TiO_2 ingested via oral route. When the most recent publications were considered, most of them concluded on a rather low systemic availability of TiO_2, although some accumulation was reported in different organs. Systemic uptake of TiO_2 would possibly occur via translocation through both the regular epithelium lining the ileum and through Peyer's patches as demonstrated by Brun (2014) with TiO_2 NP (anatase) in *ex vivo* and *in vivo* rodent models. Although the potential for absorption and accumulation seems to be likely dependent on the concentration and size of the TiO_2 particles (Jovanovic, 2015), only low to no accumulation of TiO_2 was found in organs after oral administration in experimental animals. For example, low increased total Ti tissue levels in spleen and ovaries with some alterations in thyroid, adrenal and ovaries were found in rats exposed for 5 day to anatase TiO_2 nanoparticles (primary size < 25 nm) at 1 and 2 mg/kg

bw/day (Tassinari, 2014). Nanogenotox WP7 report (*identification of target organs and biodistribution including ADME parameters*) concluded that oral administration of different nanoparticles of TiO_2 (anatase, rutile, anatase/rutile) results in a rather low uptake via gastrointestinal tract even after a 5-day oral administration, with very low levels in the liver and spleen. MacNicoll (2015) did not find any significant internal exposure after oral ingestion of both nano or larger particles of TiO_2, with administered TiO_2 found in the faeces of rats. Extremely low absorption was also reported by Cho (2013) when rats were exposed to nano-TiO_2 for 13 weeks. *In vivo* genotoxicity studies in rodents can also bring some information on systemic uptake of TiO_2 after oral administration. Sheng (2013), Gui (2013) and Nanogenotox WP6 report (2013) reported some accumulation of TiO_2 in different organs after oral exposure to nano-TiO_2 for up to 90 days (see Annex I). Similar results were obtained from human studies. In a study performed after an oral challenge, very little TiO_2 (< 0.1 %) was absorbed gastro-intestinally with no difference in absorption for any of the three particles tested (anatase of 15 nm, rutile of 70 nm and 1.8 µm) (Jones, 2015). A fraction of pharmaceutical/food grade anatase titanium dioxide has been shown to be absorbed systemically by human with normal gut permeability following a single ingestion of 100 mg, as reflected by the presence of particles in the blood (Pele, 2015).

In conclusion, no carcinogenic concern has been identified after oral exposure to TiO_2. Oral uptake of TiO_2 seems to be rather limited even if it cannot be excluded that some forms of TiO_2 could be better absorbed, in particular with specific coating and/or size. Considering the presented carcinogenic mode of action (see paragraph Carcinogenic mode of action) of TiO_2 requiring a sufficient accumulation of particles, the low absorption of different forms of TiO_2 reported in various kinetics studies might explain the negative carcinogenic outcome in the 2 studies available.

Dermal route:

Three two-stage skin carcinogenesis studies were performed to examine the promotor potential of TiO_2. In the first well-performed study, coated and uncoated nano-TiO_2 (unspecified crystalline phase) administered to CD1(ICR) female mice did not cause skin tumour promotion (Furukawa, 2011). Similar negative results were obtained from the two other studies of low quality comparing effects of TiO_2 in transgenic or wild-type rats and mice. In Sagawa (2012) study, a non-coated rutile nano-TiO_2 was administered to transgenic (Hras 128), wild-type SD rats and CD1 mice; and a nano-TiO_2 coated with silicone was administered to transgenic (rasH2) and wild type CB6F1 mice. In the Xu (2011) study, a non-coated rutile nano-TiO_2 was administered to transgenic (Hras 128) and wild-type rats. However, according to the SCCS opinion (2013), it is difficult to draw a firm conclusion from these two studies due to lack of positive control and since little experience with the rat model is currently available. Furthermore, in Sagawa (2012), there was a very high tumour activity in the "initiated transgenic mice".

Skin penetration of TiO_2 was assessed in these studies and showed that TiO_2 was in the *stratum corneum* but did not penetrate the dermis. Based on these observations, the authors suggested that the lack of skin tumour promotion of TiO_2 can be due to the lack of penetration of the particles through the dermis. This was supported by results from many *in vitro* and *in vivo* dermal penetration studies detailed in the SCCS opinion (2013). However, it remains somewhat uncertain if particles can

penetrate through damaged skin or during repeated or long term applications, since a number of studies have indicated that TiO_2 nanoparticles can enter the hair follicles and sweat glands.

In conclusion, no carcinogenic concern has been identified after dermal exposure to TiO_2. Dermal penetration of TiO_2 seems to be rather limited even if it cannot be excluded that some forms of TiO_2 could be better absorbed, in particular with specific coating and/or size. Considering the presented carcinogenic mode of action of TiO_2 (see paragraph Carcinogenic mode of action) requiring a sufficient accumulation of particles, the low absorption might explain the lack of systemic carcinogenic effect reported in the available studies.

Inhalation route - Human data:

Human data were available from case reports, case-control studies and cohort studies. A significantly elevated risk for lung cancer was observed in two of the three cohort studies. In the first study (Fryzek, 2003), elevated SMR was found in short-term workers (≤ 9 years) after 20 or more years of follow-up in US companies. However, it decreased with longer duration of employment. In the second study (Boffetta, 2004), the statistical significance of the increased SMR for lung cancer compared to the general population was not reached in all the European countries considered, showing heterogeneity of the observations between countries. For both studies, there was no evidence of relationship with concentration and duration of exposure to TiO_2. In the Boffetta (2004) study, although not significant, a dose-response relationship was suggested between exposure to TiO_2 and mortality from kidney cancer. The other cohorts did not report an increased risk of kidney cancer. Methodological limitations were noted for all studies. In addition data on primary particle size or size distribution of the TiO_2 particles were lacking. In this context, epidemiological data are considered inadequate.

Inhalation route - animal data:

Lung tumours were observed in rats following chronic inhalation of TiO_2 in 2 publications (Lee, 1985 and Heinrich, 1995) out of 4 studies. Among these studies, only one (Lee, 1985) has a protocol similar to guideline when considering the number of animals and dose levels, the route of exposure and the duration of the study. Other studies were only performed with one low concentration. Furthermore, Thyssen (1978) exposed rats only for 12 weeks.

In the first study (Lee, 1985), 12/77 males and 13/74 females presented bronchioalveolar adenoma in rats exposed to 250 mg/m^3 of fine TiO_2 (rutile form; MMD = 1.5-0.7 µm) for 2 years. Squamous cell lesions, classified as cystic keratinizing squamous cell carcinoma by the authors, were found in 1 male and 13 females at the same exposure concentration. A re-evaluation of the proliferative squamous lesions found in this study showed that over the 13 reported in females, only one was confirmed as squamous cell carcinoma (Warheit and Frame, 2006).

In the second study (Heinrich, 1995), 32/100 females rats showed lung tumours, consisting on benign keratinizing cystic squamous cell tumours, squamous-cell carcinomas and bronchioalveolar adenomas or adenocarcinoma after exposure to a cumulative particle exposure of 88.1 g/m^3 x h (or about 10 mg/m^3) of ultrafine TiO_2 (P25, 80%/20% anatase/rutile, 15-40 nm) for 24 months. No increase of lung tumour was found in female mice, but the 30% lung tumour prevalence in controls may have decrease the sensitivity for detecting carcinogenic effects in this study. Since diesel exhaust

was also tested in this study, it is possible to compare the carcinogenicity of TiO_2 to a substance presenting sufficient evidence for carcinogenicity in experimental animals (IARC, 2012). The incidence of tumours induced by nano-TiO_2 (32% after 88.1 g/m^3 x h of TiO_2) in rats was approximately similar to that induced by diesel exhaust (22% after 61.7 g/m^3 x h).

No increase of lung tumours was reported in two other inhalation studies performed in rats with TiO_2, type Bayertitan T, 99.5 % rutile (Muhle, 1989, 1991, 1995) or with TiO_2, "standard size" with 99.9% < 0.5 µm (Thyssen, 1978). However, the Muhle study was performed at a concentration lower than those associated with lung tumour in the 2 above studies. The Thyssen study was only performed for 12 weeks, a duration not sufficient to adequately assess any carcinogenicity potential.

Supportive information can be obtained from intra-tracheal studies. Among these studies, two assessed the promotor potential of TiO_2 (Xu, 2010 and Yokohira, 2009) and one assessed the occurrence of tumours, 30 months after a repeated administration of TiO_2 (Pott, 2005). Although instillation is not a physiological route for human exposure and even if differences in terms of dose rate, particle distribution or clearance were noted compared to inhalation, similar types of lung tumours (benign adenomas and epitheliomas, adenocarcinomas and squamous cell carcinomas) were observed after instillations of TiO_2 (fine anatase or ultrafine P25) in female rats (Pott, 2005). The incidence of tumours was ≥ 50% for ultrafine TiO_2 (from 15 mg) and ≥ 20% for fine TiO_2 (from 60 mg). In comparison, diesel soot at 15 and 30 mg and quartz at 5 mg, both known as carcinogenic, induced a tumour incidence of 26%, 40% and 66%, respectively. Xu (2010) also reported a carcinogenic promotor potential of nano-TiO_2 (rutile type, 20 nm) in transgenic Hras 128 female rats initiated with DHPN. The incidence of lung adenomas was 10% after a total dose of 0.875 mg and 36% after a total dose of 1.75 mg, with a significant statistically increased multiplicity at the highest dose. In this study, an increase of multiplicity of mammary adenocarcinomas was also reported, suggesting some promoting activity of TiO_2 away from the contact site in predisposed animals. However, the results from Xu (2010) study need to be taken with caution considering the little experience with this model. In contrast, no promotor potential was reported in the Yokohira et al. publication (2009). However, this study is not judged reliable as many experimental parameters did not match with the standard protocol for carcinogenesis assessment (e.g., treatment schedule with only 1 treatment; few numbers of animals/group; biological parameters actually measured…).

In conclusion, although no definitive conclusion can be drawn about the carcinogenic effect after inhalation of TiO_2 based on human data, lung tumours were reported in one inhalation study and one intra-tracheal study of acceptable quality. Carcinogenic potential was also reported in two further (inhalation or intra-tracheal) studies of lower reliability but of adequate relevance.

Role of physicochemical properties of TiO_2 (size, crystalline phase, coating) on carcinogenicity

Since TiO_2 compositions vary in crystalline phase, morphology and surface chemistry (and all combinations thereof), the impact of variability of these characteristics on the hazard profile has to be considered.

Impact of the size: nanoform versus microform of TiO_2

TiO_2 can be non-nano (bulk) and nano sized. In the registration dossier submitted for the substance identified as "titanium dioxide" for EC no 236-675-5, some data are provided on 4 different titanium dioxide samples as MMAD + Geometric standard deviation. However, the MSCA-FR noted that the data included would not cover all the possible morphologies that is stated in the registration dossier as being within the scope of the registered substance.

The impact of the size on carcinogenic potential of TiO_2 was assessed based on the data presented in this proposal as the registration dossier did not include (data allowing) discussion of the impact of this parameter on the hazard profile.

For reasons that are not yet fully understood, the phagocytic clearance of nanoparticles is less efficient than clearance of fine particles of the same material (Ferin, 1992; Oberdörster, 1994). This lower efficiency could be related to agglomeration of nanoparticles, which is more likely to lead to volumetric overload (Pauluhn, 2009). Additionally the contribution of direct cytotoxic effects – resulting from the greater surface area and therefore higher reactivity – cannot be ruled out (Borm, 2004; Sager, 2008). Thus, a higher effect of nanoparticles in comparison to fine particles can be expected in the lung.

Increased incidences of lung tumours were found in studies performed with both fine and ultrafine TiO_2, with some indications of a higher carcinogenic potential of ultrafine TiO_2. Indeed, increased lung tumours were found at lower concentration with TiO_2-NP: at a mean concentration of about 10 mg/m^3 of ultrafine P25 TiO_2 (Heinrich, 1995) and at 250 mg/m^3 for fine TiO_2 (Lee, 1985) for 24 months in rats. However, the higher duration of the post-exposure period in the Heinrich study (6 months versus one week in the Lee study) may have increased the likelihood of detecting lung tumours in the ultrafine TiO_2-exposed rats. Furthermore, differences in the exposure duration per day (6h in the Lee study versus 18h in the Heinrich study) were noted and can have an impact on the retained particle lung burden. Finally, it can be noted that the "mg/m^3" metrics is might not be the best metrics for nanoparticles and thus a comparison of the concentrations at which the tumours occurred can be not appropriate to conclude on a higher toxicity of one form over the other.

The higher carcinogenicity of TiO_2-NP was also reported where administerd by the intra-tracheal route, since lung tumours were found at a higher incidence in the group treated with ultrafine TiO_2 (tumour incidence of about 70 % after nano-TiO_2 P25 exposure compared to about 30% after fine TiO_2 anatase exposure at a total dose of 60 mg) (Pott, 2005). In term of types of tumours, these studies do not specifically support a conclusion of nano-specific tumour lesions since similar tumours were reported with both fine and ultrafine TiO_2 (bronchioalveolar adenomas/adenocarcinomas and squamous cell carcinomas).

In conclusion, a higher carcinogenic potential of ultrafine TiO_2 can be suggested from these studies, but cannot be confirmed because confounding factors such as route of exposure, concentrations, exposure duration and post-exposure follow-up are present in the studies available.

Based on these studies, IARC (2010) classified TiO_2 as possibly carcinogenic to humans (Group 2B) without differentiation between ultrafine and fine TiO_2 particles. However, based on the same studies,

the NIOSH (2011) concludes that although ultrafine TiO_2 should be considered a potential occupational carcinogen, there are insufficient data at this time to classify fine TiO_2 as a potential occupational carcinogen since effects were observed at concentration (250 mg/m^3) that was significantly higher than currently accepted inhalation toxicology practice. However, they noted that when TiO_2 is expressed as particle surface area dose, both fine and ultrafine TiO_2 fit the same dose-response curve.

Additional information can be provided by single or repeated-dose toxicity studies comparing effect of fine and ultrafine TiO_2 on lung. Although pathological changes (severe pulmonary inflammation and emphysema) were found in lung after a single intra-tracheal administration of nano-rutile; similar effects were not observed with the same dose of micro-TiO_2 (Chen, 2006). In the Baggs et al. study (1997), male Fisher 344 rats developed slightly more fibrosis with nano-TiO_2 (20 nm) than animals that had inhaled fine TiO_2 particles (250 nm) after a 3-month inhalation exposure. This supports that ultrafine TiO_2 particles may have a greater biological activity than larger ones. In the contrary, according to Bermudez et al (2002, 2004) and Hext et al. (2005), the type of effects and the response of the different tested animal species are similar for both nano and fine TiO_2. In this study, lung effects occurred after inhalation exposure from 10 mg/m^3 with ultrafine TiO_2 (P25, 21 nm) and from 50 mg/m^3 with fine TiO_2. However, the lung burden at 10 mg/m^3 of nano-TiO_2 was comparable with the burden at 50 mg/m^3 of fine TiO_2, when surface area was used as dose-metrics.

Moreno-horn&Gebel, 2014 reviewed the evidence for systemic toxicity for granular biodurable nanomaterials and conclude that there was no evidence that toxicological properties of nanomaterial differs from their micromaterial counterparts. They did not conclude on the possible long-term systemic effects of these particles.

In summary, even if several studies tend to demonstrate that the nano-form is more “reactive” (biologically active) than the micro-form, none was able to clearly correlate the hazard to specific forms or categories. In addition, carcinogenic effects were reported for nano and micro-forms. Classifying all the titanium dioxide particle sizes for carcinogenicity is therefore justified.

Impact of the crystallinity: rutile, anatase or mix anatase/rutile

TiO_2 exists under different crystal phases, such as rutile, anatase and brookite, which might have an impact on toxicological properties. Although no carcinogenicity data is available on brookite form, studies were performed with rutile, anatase or a mix of the anatase/rutile forms.

Carcinogenic effects were found with nano-P25 TiO_2 (80/20% anatase/rutile) after inhalation (Heinrich, 1995) and intra-tracheal instillations (Pott, 2005), with micro-rutile TiO_2 after inhalation (Lee, 1985) and with micro-anatase TiO_2 after intra-tracheal instillation (Pott, 2005). Nano-rutile TiO_2 also showed a promoter effect after administration by intra-tracheal instillation in a study of questionable validity (Xu, 2010). Nano-rutile was negative in a carcinogenicity study by inhalation at low concentration (Muhle, 1989). None of the studies assessed the toxicity of TiO_2 of different crystallinity but with a similar size; thus no clear conclusion can be made on the impact of the crystallinity alone on carcinogenic potential of TiO_2. Based on these results, it rather seems that the

crystalline form has no significant impact on the carcinogenicity potential of TiO_2 since carcinogenic effect was observed with anatase, mix anatase/rutile and rutile forms.

However, some studies showed that crystallinity can have a significant impact on toxic potential with anatase form being more reactive than rutile in terms of inflammation. Warheit et al (2007) compared pulmonary toxicity after a single intra-tracheal administration of different forms of TiO_2: P25 (80/20% anatase rutile) and three forms of rutile (non-coated nano-rutile, coated nano-rutile and fine rutile). P25 caused more pronounced inflammation than the rutile forms. According to the authors, based on these results, inhaled rutile ultrafine-TiO_2 particles are expected to have a low risk potential for producing adverse pulmonary health effects. They also concluded that the lung toxicity of anatase/rutile TiO_2 should not be viewed as representative for all ultrafine-TiO_2 particle-types. In contrast, when lung responses to P25 and to two anatase forms of TiO_2 (spheres and nanobelt) were compared after intra-tracheal instillation, all forms produced similar pattern of neutrophilia and pathology in rats and mice (Bonner, 2013). *In vitro* studies, as summarized in the NIOSH report (2011), report that crystal structure influences particle surface ROS generation.

In conclusion, although some *in vitro* or *in vivo* acute exposure to TiO_2 suggests an impact of the crystallinity on inflammation responses, the available data on rutile and anatase do not allow drawing strong conclusion on which crystallinity is the most toxic and to which extent. In contrast, in chronic studies, no difference between crystalline forms was found in term of carcinogenic potential. Classifying all the crystalline forms for carcinogenicity is therefore justified.

Impact of the coating

TiO_2 can be modified by using various coatings to enhance or maintain its properties.With the exception of some composition of titanium dioxide used as a food additive, all commercially produced titanium dioxide (micro or nanosize) is coated by a variety organic or inorganic coating materials. The coating includes hydrophilic, hydrophobic and amphiphilic materials. The most common coatings are composed of oxyhydrates and oxides of aluminium and silicone. Oxides and oxyhydrates of zirconium, tin, zinc, phosphorous, cerium and boron are also used. The stability of the coating may differ between the different coating materials. If new data are available on specific coated material of titanium dioxide, that demonstrate that this type of specific test material does not behave as titanium dioxide in the scope of this dossier, the entry may be revised later *via* a proposal to exclude specific forms.

The impact of coating on carcinogenic potential is difficult to assess since only one study was performed with a nano-coated (P805) TiO_2 (P25 coated with trimethoxyoctyl-silane) by intra-tracheal route (Pott and Roller, 2005). High acute toxicity was observed with the coated TiO_2 leading to a reduction of the initial dosage plan. Only one benign tumour (6.7%) was found with 30 instillations of 0.5 mg of P805 compared to about 50% with 5 instillations of 3 mg of P25. However, interpretation of the results is difficult since only few animals survived at the end of the experiment. Furthermore, no direct comparison can be made from this study considering the different dosage protocol.

Supporting information can be provided by acute and repeated dose toxicity studies performed with different forms of coated titanium dioxide.

In 2001, Oberdörster reported that 500µg hydrophobic and silanized ultrafine TiO_2 did no show toxicity and a much lower pulmonary inflammation was induced in comparison to the hydrophilic uncoated TiO_2 in rat lung.

In the study of Höhr et al. (2002), acute inflammatory responses and cell damage were investigated 16h after instillation of surface modified (hydrophilic and hydrophobic) fine and ultrafine TiO_2 particles at equivalent doses in rats. The authors observed that for the same surface area, the inflammatory response in female rats to uncoated TiO_2 covered with surface hydroxyl groups was similar to that of TiO_2 particles with surface OCH_3-groups (hydrophobic). The authors concluded that the surface area rather than hydrophobic surface determines acute pulmonary inflammation by both fine and ultrafine Titanium dioxide.

Warheit et al., 2003 assessed and compared the acute lung toxicity of intratracheally instilled hydrophobic in comparison to hydrophilic surface-coated titanium dioxide (TiO_2) particles. To conduct toxicity comparisons, the surface coatings of base pigment-grade TiO_2 particles were made hydrophobic by application of triethoxyoctylsilane (OTES), a commercial product used in plastics applications. Rats were intratracheally instilled with 2 or 10 mg/kg of the following TiO_2 particle-types: (1) base (hydrophilic) TiO_2 particles; (2) TiO_2 with OTES surface coating; (3) base TiO_2 with Tween 80; or (4) OTES TiO_2 with Tween 80. Saline instilled rats served as controls. Following exposures, the lungs of rats were assessed using bronchoalveolar lavage (BAL) biomarkers and histopathology of lung tissue at 24 hours, 1 week, 1 month, and 3 months post exposure. The results demonstrated that only the base, high-dose (10 mg/kg) pigment-grade TiO_2 particles and those with particle-types containing Tween 80 produced a transient pulmonary inflammatory response, and this was reversible within 1 week post-exposure. Based on the abstract it is not specified how the inflammatory response was measured. The authors conclude that the OTES hydrophobic coating on the pigment-grade TiO_2 particle does not cause significant pulmonary toxicity.

As described below, the surface coating of TiO2 with aluminum oxide and/or silica has been shown to produce higher pulmonary inflammation (PMNs in BALF) than the uncoated TiO2 at 24 h in SD rats administered a large dose of 10 mg/kg (Warheit, 2005)

Warheit et al. (2007) showed that P25 (anatase/rutile) induced a higher inflammatory response than three forms of TiO_2 with an aluminium coating after a single intra-tracheally administration. In contrast, fine coated TiO_2 (with alumina or amorphous silica) produced higher inflammation than uncoated TiO_2 after an intra-tracheal administration or after inhalation exposure for 30 days (Warheit, 2007).

Rossi et al., 2010 studied five different types of TiO_2 particles. The TiO_2 materials were rutile in microsize, rutile/anatase in nanosize, nanosize anatase, nanosize anatase/brookite and silica coated nano-sized rutile. Nanosize SiO_2 particles were also tested in the study. BALB/c mice were exposed by whole body inhalation to the particles (8 mice/group) for either 2 hours, 2 hours on four consecutive days for four weeks at 10 mg/m^3. In addition, effects of *in vitro* exposure of human macrophages and fibroblasts (MRC- 9) to the different particles were assessed. SiO_2-coated rutile

TiO_2 nanoparticles ($cnTiO_2$) was the only sample tested that elicited clear increase in pulmonary neutrophilia as determined by neutrophils infiltration to bronchoalveolar lavage. Uncoated rutile and anatase as well as nanosize SiO_2 did not induce significant inflammation. Inhalation exposure to nanosized SiO_2, used as a model for the coating material did not induce pulmonary inflammation. In order to explore the mechanism of pulmonary neutrophilia induce by $cnTiO_2$, murine and human macrophages were exposed *in vitro* to $cnTiO_2$. Significant induction of TNF-α and neutrophil attracting chemokines was observed. Stimulation of human fibroblasts with $cnTiO_2$-activated macrophage supernatant induced high expression of neutrophil attracting chemokines, CXCL1 and CXCL8. Interestingly, the level of lung inflammation could not be explained by the surface area of the particles, their primary or agglomerate particle size or radical formation capacity, but was rather explained by the surface coating. The authors concluded that the level of lung inflammation could not be explained by the surface area of the particles, their primary or agglomearate particle size, or free radical formation capacity but rather by surface coating.

Mature female mice (*n* = 9/controls, 8/treated) were exposed for 1 hour per day for 11 consecutive days to 42.4 ± 2.9 (SEM) mg/m^3 $nanoTiO_2$ particles (Halappanavar et al., 2011). Physicochemical characteristics of the $nanoTiO_2$ consisted of rutile TiO2-based material, nanosized particles, modified with amounts of zirconium, silicon, aluminum, and coated with polyalcohol. The aim of the study was to assess inflammatory response to $nanoTiO_2$ exposure in mouse lungs. Pulmonary response was assessed using DNA microarrays and pathway specific PCR arrays related to pulmonary inflammation from bronchial lavage. The bronchoalveolar lavage fluid analysis was published by Hougaard et al. (2010). The percentage of neutrophils was significantly increased in the nano-tiO_2 group compare to control. However the number of macrophage was significantly decreased. The authors observed changes in the expression of the genes associated with proinflammatory, immune response and complement cascade-related genes with concomitant changes in microRNAs that persist for up to five days after exposure.

In the study of Leppänen, 2015, irritation and inflammation potential of commercially available silica-coated TiO_2 engineered nanomaterials (10_40 nm, rutile) were studied. The thorough characterization of the particles has been described by Rossi et al., 2010 and the very same silica-coated TiO_2 particles were used in the study. Single exposure (30 min) at mass concentrations 5, 10, 20 and 30 mg/m^3, and repeated exposure (altogether 16 h, 1 h/day, 4 days/week for 4 weeks) was performed in male and female mice respectively. Mass concentration of 30 mg/m^3 to silica-coated TiO2 induced first phase of pulmonary irritation (P1), which was seen as rapid, shallow breathing. During repeated exposures, pulmonary irritation evolved into more intense pulmonary irritation: inflammatory cells infiltrated in peribronchial and perivascular areas of the lungs, neutrophils were found in BAL fluids, and the number of CD3 and CD4 positive T cells increased significantly. In line with these results, pulmonary mRNA expression of chemokines CXCL1, CXCL5 and CXCL9 was enhanced. Also expression of mRNA levels of proinflammatory cytokines TNF-α and IL-6 were elevated after repeated exposures. Also sensory irritation was observed at the beginning of both single and repeated exposure periods, and the effect intensified during repeated exposures. Airflow limitation started to develop during repeated exposures. The authors concludes that taken together, these results indicated that silica-coated TiO_2 induce pulmonary and sensory irritation after single and repeated exposure, and airflow limitation and pulmonary inflammation after repeated exposure.

In the study of Landsiedel et al., 2014, a standard short-term inhalation study was applied for hazard assessment of 13 metal oxide nanomaterials and micron-scale zinc oxide. Rats were exposed to test material aerosols (ranging from 0.5 to 50 mg/m^3) for five consecutive days with 14- or 21-day post-exposure observation. In this study, the form of TiO_2 was rutile (with minimally anatase), as a fiber, in nanosize, with surface coating (Ti: 16; O: 63; C: 9; Al: 7; Si: 5; Na: <1; dimethicone/ Methicone copolymer as surface coating). Bronchoalveolar lavage fluid (BALF) and histopathological sections of the entire respiratory tract were examined. Pulmonary deposition and clearance and test material translocation into extra-pulmonary organs were assessed. Eight nanomaterials did not elicit pulmonary effects. Five materials (coated nano-TiO_2, coated nano ZnO and micro ZnO, nano-CeO_2 and Al-doped nano-CeO_2) evoked concentration-dependent transient pulmonary inflammation. Overall, coated nano-TiO_2 caused mild pulmonary inflammation that was not fully reversible with a NOAEC of 0.5 mg/m^3. In this study, the materials were ranked by increasing toxic potency into 3 grades:

-lower toxic potency: $BaSO_4$; SiO_2. acrylate (by local NOAEC); SiO_2.PEG; SiO_2.phosphate; SiO2.amino; nano-ZrO_2; ZrO_{2e}.TODA; ZrO_2.acrylate;

-medium toxic potency: SiO_2.naked;

-higher toxic potency: coated nano-TiO2; nano-CeO_2; Al-doped nano-CeO_2; micron scale ZnO; coated nano-ZnO.

According to the authors, the study revealed the type of effects of 13 nanomaterials, and micron-scale ZnO, information on their toxic potency, and the location and reversibility of effects.

The impact of surface coating and the ROS mechanism in lung toxicity of TiO_2 has been reviewed in the publication of Wang et al., 2014. The authors concluded that size, shape, crystal phase and surface coating should be appropriately characterized when evaluating the potential biological effects of nanoparticles.

In the study of Farcal et al., 2015, six representative oxide nanomaterials provided by the EC-JRC Nanomaterials Repository were tested in nine laboratories. The in *vitro* toxicity of the nanomaterials was evaluated in 12 cellular models representing 6 different target organs/systems (immune system, respiratory system, gastrointestinal system, reproductive organs, kidney and embryonic tissues). The toxicity assessment was conducted using 10 different assays for cytotoxicity, embryotoxicity, epithelial integrity, cytokine secretion and oxidative stress. Thorough physico-chemical characterization was performed for all tested nanomaterials. Commercially relevant nanomaterials with different physico-chemical properties were selected: two pure rutile nanosize TiO_2 with different surface chemistry – hydrophilic (NM-103) and hydrophobic (NM-104) both coated with Al, two forms of ZnO – uncoated (NM-110) and coated with triethoxycapryl silane (NM-111) and two SiO_2 produced by two different manufacturing techniques – precipitated (NM-200) and pyrogenic (NM-203). The reactivity, solubility and biodurability of TiO_2 was tested in different media. Dissolution studies showed that TiO_2 is almost insoluble, whereas Al impurities, which may originate from external coating or from the NM themselves, were partially soluble. In addition, Si impurities were also detected. The authors conclude that the Al coatings may be unstable under *in vitro* conditions. The authors concluded that the results could not establish a consistent difference between the hazardous properties of the titanium dioxide NM-103 (hydrophilic) and NM-104 (hydrophobic) in

any of the cell models adopted; cell specific toxicity effects of all NMs were observed; With regard to ZnO, coating of ZnO may influence the toxic however, contradictory results were obtained.

The hydrophilic/hydrophobic properties of a variety of commercial TiO_2 as nanoparticles, to be employed as inorganic filters in sunscreen lotions, were investigated both as such (dry powders) and dispersed in aqueous media (Bolis, 2012). The possible *in vitro* neuro-toxicological effect on dorsal root ganglion (DRG) cells upon exposure to TiO_2, as a function of crystal phase, surface area and coating was investigated in the study. All investigated materials, with the only exception of the uncoated rutile, were found to induce apoptosis on DRG cells; the inorganic/organic surface coating did not protect against the TiO_2-induced apoptosis. The risk profile for DRG cells, which varied for the uncoated samples in the same sequence as the photo-catalytic activity of the different polymorphs: anatase-rutile>anatase>>rutile, was not correlated with the surface hydrophilicity of the uncoated/coated specimens. Aggregates/agglomerates hydrodynamic diameter was comprised in the ~200-400 nm range, compatible with the internalization within DRG cells.

It is also reported in the SCCS report (2014) that appropriate coating of nanomaterial to quench surface photocatalytic activity can reduce the likelihood of generation of reactive oxygen species. Since oxidative stress is involved in the carcinogenic potential of TiO_2, it could be expected that this response can be modulated by some coatings at an unknown level.

The data presented above show that coating can impact the toxicity of TiO_2 and that the inflammation response can differ between different forms although a clear pattern cannot be drawn from the existing data. Carcinogenicity was observed with both anatase and rutile titanium dioxide. Between these two crystal phases, ROS generation and pulmonary inflammation response differs. Indeed, the quantitative aspects of the inflammatory response that are sufficient to cause a high probability of lung tumor development are not known. Therefore, it is impossible to identify a threshold of inflammation below which carcinogenicity would not occur. It is also impossible to distinguish which coating, if any, will induce inflammation below this threshold.

Moreover, based on the data generated/collected in the registration dossier and in compliance with the Annex VII-XI information requirements, that all entities they consider as “titanium dioxide” are hazard equivalent, can be registered as one substance and have the same classification. They also considered that the impact of surface treatment on titanium dioxide particles irrespective of the specific surface area or the type of chemical treatment undertaken does not impact the properties relevant for hazard. Again taking this statement at face value, it implies that they have concluded that the hazard profile of titanium dioxide in any phase or phase combination, non-surface treated and surface treated for all specific surface areas are equivalent.

FR-MSCA therefore considers that coating is not a parameter to consider for classification.

Impact of the shape

TiO_2 can be in all possible crystal phases and their combinations, such as spheres, nanorods, nanowires, nanotubes, thin films or nanoporous structures.

From carcinogenicity studies by the respiratory route, it seems that all were carried out with the spherical form of TiO_2, even it was not clearly characterized in all the publications (Muhle, 1989; Thyssen, 1978; Xu, 2010; Yokohira, 2009). Therefore, the impact of shape on carcinogenicity cannot be clearly assessed from these studies.

Very few toxicological data, such as short or medium *in vivo* studies, are available with non-spherical TiO_2. Similar patterns of neutrophilia and pathology were found with two types of spheres (including P25) and one nanobelt after a single intra-tracheal or oropharyngeal aspiration in rodents (Bonner, 2013). A further study (Warheit (2006)) summarized in the NIOSH report showed a reversible increase in the percentage of polymorphonuclear leukocytes with two types of nanoscale TiO_2 rods, nanoscale TiO_2 dots and microscale rutile after an intra-tracheal administration in rats.

Shapes of TiO_2 could be divided in two main types, spherical and elongated-like shapes. It might be hypothesized that elongated-like shapes would have a similar behaviour to fibres. It is generally recognised that the main difference of carcinogenic mode of action between fibres and granular particles is that fibres can translocate to the pleura to induce malignant mesotheliomas while this mode of action is not reported with granular spherical particles. However, both fibres and granular particles induced lung tumours with a similar mode of action consisting in a persistent inflammation due to an incomplete phagocytosis and a release of reactive oxygen and nitrogen species.

In conclusion, it seems that both spherical and non-spherical particles have the potential to induce lung tumours, secondary to a persistent inflammation. Non-spherical particles, translocating to the pleura, could induce additional tumours in this tissue.

It has been concluded based on the data generated/collected in the registration dossier andin compliance with the Annex VII-XI information requirements, that all entities they consider as “titanium dioxide” are hazard equivalent, can be registered as one substance and have the same classification. Taking this statement at face value, it implies that no combinations of phase, particle size and surface chemistry are considered to impact on properties relevant for the hazard profile and that all combinations of phase, particle size and surface chemistry can therefore be considered equivalent.

In summary, all forms of TiO_2 are susceptible to induce lung tumours, secondary to oxidative stress and chronic inflammation. Biopersistence and poor solubility are believed to be the most important factors in this toxicity. These parameters appear to be applicable to TiO_2 whatever its other physico-chemical properties. In this context, no separate evaluation has been done for this CLH report and the classification proposal covers all commercialized titanium dioxide in all phases and phase combinations; Particles in all sizes/morphologies .

<u>Carcinogenic mode of action</u>

- **Inflammation and oxidative stress**

Particle toxicology currently favors secondary genotoxicity as the major mechanism underlying tumor formation of TiO_2, *i.e.* indirect oxidative stress and chronic inflammation processes. Indeed,

pulmonary inflammation was reported in several studies from single to chronic exposures. As a defense mechanism following TiO_2 inhalation, macrophages and neutrophils are recruited to clear the foreign material. Biopersistence and poor solubility of the particle are believed to be important factors in the efficiency of the clearance. Indeed, biopersistent particles of poor solubility, such as TiO_2, cannot be fully phagocytosed by the macrophages and thus accumulate in the lungs. In case of prolonged exposure, clearance capacity is thus overloaded leading to lung tissue damage and epithelial cell proliferation. Oxidative stress is considered as the underlying mechanism of proliferative responses to TiO_2: ROS are released by inflammatory cells and/or by particle reactive surfaces whether spontaneously or when interacting when cellular components; oxidants can thereafter damage lung epithelial tissue and also induce genetic damages. Some data suggest that the extent of inflammation varies with certain forms, with higher toxicity with nano versus fine forms and anatase versus rutile forms (Chen, 2006; Baggs, 1997; Warheit, 2007, Warheit, 2005). However, at this time, it is considered that there is not enough data to identify the most toxic form and those that are not. More important, even if different severities in term of inflammation and ROS release can be expected depending on the physico-chemical properties, it seems that all forms have a potential for lung carcinogenicity.

- **Genotoxicity**

Positive results were obtained in several *in vivo* and *in vitro* genotoxicity studies (genotoxicity data are summarised in Annex I).

Available studies greatly differ in terms of quality and in order to make a reliable assessment of the results, the following parameters were taken into account: sufficient characterization of the tested material, use of known or validated protocols reported with sufficient level of details, inclusion of negative and positive controls and evidence of uptake or cytotoxicity in case of negative results.

Considering these criteria: only 1/6 experiments (Comet assay) among the reliable *in vivo* studies was positive. Among the *in vitro* studies, 93/170 experiments (16/34 micronucleus tests, 76/125 Comet tests and 1/4 Chromosomal Aberrations tests) were positive. An experiment has been defined by one form of TiO_2 and a specific protocol (ex. cells, media, exposure-duration, standard or modified protocol…). Physico-chemical properties seems to have an impact on the response but the data provided cannot distinguish which specific characteristics might lead to such effect. Furthermore, the different test conditions used (cells/organs examined, route and duration of exposure, method of dispersion…) do not permit an easy comparison of the studies and a firm conclusion of the impact of such protocol among others. It was also noted that in many cases, the statistical test used was inappropriate and the interaction with the system was not correctly addressed.

From all these studies, a mechanism of action can be hypothesized even if a clear conclusion cannot be reached. Genotoxicity of TiO_2 is rather due to oxidative lesions, as observed by increase of oxidative DNA lesions measured whether directly (8-oxo-dG) or indirectly (using modified-Comet assays). Evidence for induction of oxidative stress was also observed by decrease of intracellular antioxidant defenses (such as SOD, GSH-Px), increase of lipid peroxidation, production of ROS or alteration of genes expression involved in stress responses from transcriptomic analyses. In addition, some accumulation of particles in nucleus cells was reported in few publications. Thus, even if the presence of particles in the nucleus, with quantitative data, are rarely evaluated in the publication, a primary genotoxic mechanism by direct particle interaction with DNA cannot be totally ruled out.

- **Mechanism of toxicity of biodurable granular particles**

Many inorganic particles equally insoluble and biopersistent are associated with increased risk of lung cancer. Particles (fine or ultrafine) are thought to impact on genotoxicity and cell proliferation by their abilities to generate reactive oxygen species. Several reviews have been published in which this carcinogenic mode of action has been described (Stone et al., 2007; Donaldson and Tran 2002; Driscoll et al. 1996, 1997; Green 2000; Knaapen et al. 1999, 2004, 2006; Lehnert 1993; Oberdörster 1988, 1994, 1995; Oberdörster et al. 1994 a, 2005, 2007; Nel et al., 2006; Vallyathan, 1998; ILSI, 2000, NIOSH, 2011). The term biodurable granular particules (GBP), low toxicity dusts, poorly soluble particles or poorly soluble low toxicity particles (PSLT) are used for these types of substances.

In the report of NIOSH (2011) on occupational exposure of titanium dioxide, pulmonary response of PSLT is discussed. The dose-response relationships for both the inflammation and tumorigenicity associated with TiO_2 exposure are consistent with those for other PSLT. Based on this evidence, NIOSH conluded that the adverse effects produced by TiO_2 exposure in the lungs are likely not substance-specific, but may be due to a nonchemical-specific effect of PSLT particles in the lungs at sufficiently high particle surface area epoxsures. PSLT particles included titanium dioxide, toner, diesel exhaust particulate, carbon black, and to a lesser extend talc, coal dust and barium sulphate.

Roller (2009) considers that the EU criteria (67/548/EEC) for Carcinogenicity category 2 (ie Carc.1B based on regulation (EC) 1272/2008) appear to be fulfilled for bio-durable nanoparticles, including TiO_2, based on a clear positive evidence for the carcinogenicity of nano-GBP[1] in one species, together with supporting evidence such as genotoxicity data and structural relationship with substances that are regarded as carcinogens or for which data from epidemiological studies suggest an association.

German MAK commission has proposed recommendations for general threshold limit value and for carcinogen classification of granular bio-durable particles (GBP), which include titanium dioxide.

The following toxic effects and mechanism of action is proposed by the German MAK commission for the bio-durable particles: “Following inhalation, biopersistent granular dusts may accumulate in the lungs and cause impairment of lung clearance. Animal studies revealed inflammatory reactions, fibrosis and tumours in the lungs after repeated inhalation and intratracheal instillation of biopersistent granular dusts. Genotoxicity studies *in vitro* led to mainly negative results. In *in vivo* studies carried out with intratracheal instillation, titanium dioxide and carbon black induced mutations in pulmonary epithelial cells only at concentrations that also caused significant inflammatory reactions and epithelial hyperplasia in the lungs.”

A number of conclusions were drawn related to the establishment of cancer classification:

- GBP cause lung cancer in rats due to chronic inflammation as a result of dust overload in the alveolar region of the lung;
- If clearance mechanisms are not overwhelmed and, thus, inflammation is prevented, lung cancer risk will not be increased.
- The lung overload effect observed in rat inhalation studies is relevant for human risk assessment. Thus, a HEC (human equivalent concentration) exists that relates to the NOAEC, the maximum concentration that avoids lung overload in rats.

[1] GBP: respirable granular bio-durable particles without known significant specific toxicity

- All GBP are carcinogenic to humans with a threshold effect.

Interspecies comparison

Interspecies variations in experimental animals

Clear species differences in pulmonary clearance and lung lesions were observed after inhalation exposure to TiO_2 for 13 weeks (Bermudez et al., 2002, 2004). Although qualitatively similar early lung response was observed in rats, mice and hamster, pulmonary lesions were more severe and occurred at a lower concentration in rats and, only this species developed progressive fibro proliferative lesions and alveolar epithelial metaplasia.

In mice, impaired pulmonary clearance and inflammation with proliferative epithelial changes were reported, without metaplasia or fibrosis (Bermudez et al., 2002, 2004). This can be explained by the increase of antioxidant (glutathione) levels in lung tissue during particle exposure found in mice but not in rats which can suggest that mice are less sensitive to oxidative damage (Oberdörster, 1995). Although no carcinogenicity study was performed with the same strain of mice (B3C3F1/CrlBR) used in the Bermudez (2002) study, Heinrich (1995) did not report any lung tumour in NMRI mice. However, the high background tumour response in the control group might have limited the ability to detect any carcinogenic effects in this study. Finally, a comparison of the sensitivity of different strains of mice to TiO_2 was not possible since there is no information on non-neoplastic lesion and pulmonary clearance in the carcinogenicity study.

In hamsters, only little lung adverse effect was observed in the Bermudez (2002) study. This can be related to a well effective lung clearance system represented by a markedly lower retention half-time compared to that in rats and mice. Furthermore, hamsters have antioxidant protection mechanisms different from rats and humans (Driscoll et al., 2002), suggesting that this species is not adequate for testing particulate substances which may elicit inflammatory oxidative damage.

Finally, although no lung tumour was found in mice and hamsters, they are known to give false negatives to a greater extent than rats in bioassays for some particulates that have been classified by IARC as human carcinogens (limited or sufficient evidence), including crystalline silica and nickel subsulfide. The lung tumour response to other known human particulate carcinogens (such as tobacco smoke, asbestos, diesel exhaust…) is significantly less in mice than in rats. Therefore, the risk of several known human particulate carcinogens would be underestimated by using dose-response data & hazard properties from rodent models other than rats.

Extrapolation to humans

The relevance of rat model predicting human response to inhaled particles is the subject of controversial discussion. A comparison of lung tumor types in rats and humans and the relevance of rat model in risk assessment are well described by the NIOSH (2011):

First concerning comparison of human and rat lung structure, some differences in the small airways were presented (e.g. lack of well-defined respiratory bronchioles in rats); however, this region of the lungs is the primary site of particle deposition in both species, and particles that deposit in this region

can translocate into the *interstitium* where they can elicit inflammatory and fibrotic response. Furthermore, humans and rats display some consistency in response to dust exposure: inflammatory reaction with fibrosis at high concentrations. Some variability in terms of severity of response can nevertheless be found between humans and rats. For example, centriacinar fibrotic response was more severe in humans exposed to silica or coal dust compared to rats exposed to the same dust; on the contrary, rats showed more severe intra-alveolar acute inflammation, lipoproteinosis and alveolar epithelial hyperplasia response than humans when they were chronically exposed to silica, talc or coal dust. However, quantitative comparison between rats and humans is not possible since exposure duration and concentrations and confounding factors were often poorly reported in human studies.

Lung tumours observed after TiO_2 inhalation in rats occurred in an overload context. Lung overload consists on a failure of the lung clearance associated with increase of particle lung burden and possible translocation to the interstitium and lymph nodes. The observed altered particle accumulation/retention and chronic inflammation can indicate that the maximum tolerated dose has been exceeded. Although lung overload was observed after TiO_2 inhalation in rats and mice, this was not found in hamsters. Furthermore, different patterns of particle retention have been observed in rats, monkeys and humans when exposed to coal dust or diesel exhaust particles, with higher volume percentage in the alveolar lumen in rats and in the interstitium in monkeys and humans. In addition, particle deposition in humans is not uniform with hot-spots, containing high deposition of particles, at bifurcations in the terminal airways. Interestingly, the localization of human lung tumours in this region is rather high. This can suggest that local overload may occur in humans at concentrations lower than those inducing a generalized overload in the lung. Case studies in workers exposed to TiO_2 showed that TiO_2 persist in the lungs with extensive pulmonary deposition even after workplace exposure to TiO_2 had ceased. Furthermore, it has been shown that lung clearance of particles is slower in humans than in rats, by approximately an order of magnitude and some humans, in particular workers, may be exposed to concentrations resulting in doses that would overload particle clearance. For example, lung overload condition had been clearly reached in coal workers exposed to high concentrations of airborne particles; this finding being thus consistent with what has been found in experimental animals (Oberdörster, 1995). In addition, lung overload after TiO_2 inhalation is characterized among other by lipoproteinosis, fibrosis and metaplasia in rats. Although these effects were not observed in mice and hamsters (Bermudez (2002) study), these lesions have been reported in humans exposed to TiO_2. In conclusion, it appears that lung retention and chronic pulmonary inflammation are more consistent with the findings in rats than in mice and hamsters. Thus, the overload concept seems to be also relevant for humans, and in particular for workers exposed to high dust exposure.

Controversy exists over the biological significance of cystic keratinizing squamous cell tumours, which developed in response to chronic inhalation of diverse particulate materials, and their relevance to humans. In fact, this type of lesion appears to be a unique rat tumour occurring under exaggerated exposure conditions, with a possible trend to gender specificity, since it is found at a higher incidence in females. These lesions have not been reported in the literature in mice or hamsters exposed to dust under similar conditions and have not usually been seen in humans. A workshop with different pathologists took place in USA in 1992 in order to obtain a consensus on a suitable descriptive diagnostic term for cystic keratinizing pulmonary lesions by TiO_2. Participants all agreed that the

lesions were not malignant neoplasms. Although most considered this lesion as not neoplastic, 3/13 considered it as probably benign tumour. The workshop members preferred the term "proliferative keratin cyst" (Carlton, 1994). In contrast, Kittel et al (1993) concluded that keratinizing cystic squamous cell lesions of the lung (review of 691 cases from 6 studies) are true neoplasms and that the growth pattern of these cystic lesions is inconsistent with that of a simple cyst. In a further international workshop in Germany, there was an agreement that the cystic keratinizing lesions were looked upon a family of related morphological changes ranging from squamous metaplasia with marked keratinization through pulmonary keratinizing cysts to cystic keratinizing epithelioma and finally pulmonary squamous cell carcinoma (Boorman, 1996). This opinion was further supported by Rittinghausen et al (1997) who concluded that cystic keratinizing epitheliomas are not necessarily an endpoint of development, but may progress to (cystic keratinizing) squamous cell carcinomas. In summary, at this time, the relevance of these tumors to man remain unclear. However, other types of tumours (adenomas, adenocarcinomas, squamous cell carcinomas) found in rats exposed to TiO_2 do occur in humans. Indeed, in humans, the major cell types of lung cancer worldwide are adenocarcinoma and squamous cell carcinomas (also seen in rats) and small- and large-cell anaplastic carcinomas (not seen in rats). If smoking-related tumour types were eliminated, then the major lung tumour types in humans would be adenocarcinomas and bronchioalveolar carcinomas, which correspond closely to the types of lung tumours occurring in rodents after TiO_2 exposure.

Although not fully understood, the hypothesized carcinogenic mode of action of TiO_2 seems to be mainly due to secondary genotoxicity, i.e. indirect oxidative stress and chronic inflammation. In humans, chronic inflammation has also been associated with non-neoplastic lung diseases in workers with dusty jobs and can increase the risk of lung cancers. Furthermore, a direct genotoxic mechanism, with a direct interaction between DNA and TiO_2, cannot be ruled out in particular for nanoscale TiO_2, since particles were observed in cells, including nucleus. Therefore, there is no reason to consider that such mechanisms of action are not relevant to humans.

Expert advisory panels have concluded that chronic inhalation studies in rats are the most appropriate tests for predicting the inhalation hazard and risk of fibers to humans. In absence of mechanistic data to the contrary, the rat model is adequate to identify potential carcinogenic hazards of poorly soluble particles to humans, such as TiO_2.

Assessment by scientific and regulatory bodies

The toxicological profile, and in particular carcinogenic potential, of TiO_2 was reviewed by several scientific and regulatory bodies.

In 2006, *the IARC (International Agency for Research on Cancer)* evaluated carcinogenic risks to humans related to TiO_2 exposure (monograph published in 2010). The IARC assessment was based on epidemiological studies (3 epidemiological cohort studies and one population-based case–control study from North America and western Europe) and on experimental carcinogenicity studies in rats, mice and hamsters by different routes of exposure (oral, inhalation, intratracheal, subcutaneous and intraperitoneal administrations). Briefly, following IARC, human carcinogenicity data do not suggest an association between occupational exposure to TiO_2 and risk for cancer. All the studies had methodological limitations and misclassification of exposure could not be ruled out. None of the

studies was designed to assess the impact of particle size (fine or ultrafine) or the potential effect of the coating compounds on the risk of lung cancer. Regarding animal carcinogenicity data, the incidence of benign and malignant lung tumours was increased in female rats in one inhalation study while in another inhalation study, the incidence of benign lung tumours was increased in the high-dose groups of male and female rats. Cystic keratinizing lesions that were diagnosed as squamous-cell carcinomas but re-evaluated as non-neoplastic pulmonary keratinizing cysts were also observed in the high-dose groups of female rats. Furthermore, intratracheally instilled female rats showed an increased incidence of both benign and malignant lung tumours following treatment with two types of TiO_2. In contrast, tumour incidence was not increased in intratracheally instilled hamsters and female mice, and two inhalation studies (one in male and female rats and one in female mice) gave negative results. Moreover, oral, subcutaneous and intraperitoneal administrations did not produce a significant increase in the frequency of any type of tumour in mice or rats. The IARC concluded that TiO_2 should be classified as possibly carcinogenic to humans (Group 2B). The classification results from the fact that, although there is a clear indication of carcinogenic potential in animal tests, the epidemiological data situation is inadequate. It should be noted that the IARC classification does not differentiate between ultrafine particles (nano-TiO_2) and fine TiO_2 particles.

In 2008, *the German MAK Commission* for the Investigation of Health Hazards of Chemical Compounds in the Work Area provisionally classified TiO_2 as a Category 3A carcinogenic substance i.e. a carcinogenic mode of action is known, but there is insufficient data to establish a maximum workplace concentration value because a benchmark dose or a NOAEC could not be derived from the existing animal experiments. However, the current MAK classification procedure does not take ultrafine particles (i.e. nanoparticles) into account in its assessment (Becker et al., 2011). The proposed mechanism of action for tumour formation is a primarily non-genotoxic mechanism consisting on pulmonary inflammation characterized by the increased infiltration of macrophages, granulocytes and, to a limited extent, lymphocytes. The phagocytes absorb titanium dioxide particles and try to degrade the particles with reactive oxygen and nitrogen species. The intensive production and release of these species damages the genomic DNA of the immediately adjacent cells, including the DNA of Type II alveolar epithelial cells, precursor cells in lung tumours. The accumulation of genetic changes results in alveolar hyperplasia and metaplasia of type II cells which are precursor stages of lung tumours.

In *2009, Tsuda* published a mini-review of carcinogenic potential of engineered nanomaterials and concluded that nanoparticles, including TiO_2, are clearly potentially toxic/carcinogenic to humans based on the increased lung tumours found in female rats. Direct production of ROS by TiO_2 or production of ROS by macrophages to destroy the foreign material in the inflammation is proposed as a possible mechanism of action. The same year, as summaries below, *Roller (2009)* considers that the EU criteria (67/548/EEC) for Carcinogenicity category 2 appear to be fulfilled for bio-durable nanoparticles, including TiO_2, based on a clear positive evidence for the carcinogenicity of nano-GBP in one species, together with supporting evidence such as genotoxicity data and structural relationship with substances that are regarded as carcinogens or for which data from epidemiological studies suggest an association.

A critical review by a working group of the German Federal Environment Agency and the German Federal Institute for Risk Assessment on the carcinogenic potential of nanomaterials, including TiO_2, has been summarized by *Becker et al (2011)*. It was concluded that inhalation studies in rats point a possible carcinogenic potential of nano-TiO_2 at high concentration but epidemiological studies are inconclusive. The hypothesized mode of action behind tumour formation favours secondary genotoxicity i.e. oxidative stress and chronic inflammation processes. However, a primary genotoxic mechanism by direct particle interaction with DNA cannot be ruled out. The small size of the nanoparticles and their ability to reach intracellular structures including the nucleus, point to this possibility. Concerning interspecies comparison, extrapolation of results from inhalation and instillation studies in rats to humans is still subject of controversial discussion. Indeed, it appears that overload concept holds true for rats and to a lesser extent for mice, but not for hamsters. Hamsters have antioxidant protection mechanisms different from rats and humans and this physiological characteristic does not favour the use of hamsters for testing particulate substances which may elicit inflammatory oxidative damage. Finally, for regulatory purposes, data from the most sensitive animal species will be used for hazard assessment, provided no adequate argument for making an exception to this rule exists.

In 2011, *the National Institute for Occupational Safety and Health (NIOSH)* reviewed the animals and human data relevant to assessing carcinogenicity of TiO_2. TiO_2 particles of fine and ultrafine sizes show a consistent dose-response relationship for adverse pulmonary responses in rats, including persistent pulmonary inflammation and lung tumours, when dose is expressed as particle surface area. NIOSH concluded that TiO_2 is not a direct-acting carcinogen, but acts through a secondary genotoxicity mechanism. The toxicity may not be material-specific but appear to be due to a generic effect of poorly soluble, low-toxicity particles in the lungs at sufficiently high exposure. It was concluded that there are insufficient data at this time to classify fine TiO_2 as a potential occupational carcinogen since the tumorigenic dose (250 mg/m^3) was significantly higher than currently accepted inhalation toxicology practice. Although data on the cancer hazard for fine TiO_2 are insufficient, the tumour-response data are consistent with that observed for ultrafine TiO_2 when converted to a particle surface area metric. Thus to be cautious, NIOSH used all of the animal tumour response data when conducting dose-response modelling and determining separate RELs for ultrafine and fine TiO_2. Finally, NIOSH is concerned about the potential carcinogenicity of ultrafine and engineered nanoscale TiO_2 if workers are exposed at the current mass-based exposure limits for respirable or total mass fractions of TiO_2.

A review of toxicological data on TiO_2 nanoparticles was published in 2013 by *Shi et al* that reaches similar conclusion as described above with carcinogenic effect in animals not confirmed by epidemiological studies. Although the mechanism is not well understood, both genetic and non-genetic factors elicited by TiO_2-NP in cells may predispose to carcinogenicity. In summary, it was concluded that there is still much remaining to elucidate. For this, a better characterization of tested materials in future studies, long-term animal studies, toxicokinetics studies and further investigations on molecular mechanisms underlying cancer occurrence are needed.

In 2014, the *Scientific Committee on Consumer Safety (SCCS)* published a revised opinion on TiO_2 (nano form). Concerning genotoxicity, the SCCS considers that the current evidence in relation to

potential genotoxicity of TiO_2 nanomaterials is not conclusive since some TiO_2 nanoparticles have been shown to be able to damage DNA and should be considered genotoxic but negative results have also been reported. For carcinogenicity, they concluded that TiO_2 particles have shown to lead to carcinogenic effects after inhalation based on a two-stage rat lung carcinogenicity study showing a promotor activity of non-coated TiO_2 after intra-pulmonary spraying. Based on these results, the SCCS does not recommend the use of nano-TiO_2 in sprayable applications.

In conclusion, scientific and regulatory bodies have also considered TiO_2 as a possible carcinogen to human when inhaled.

4.1.5 Comparison with criteria

For this CLH report, data on TiO_2, whatever its morphologies, crystal phase and surface treatment, were taken into account. Based on the analysed dataset, it is concluded that criteria for classification as Carc. 1B – H350i for TiO_2 by inhalation are fulfilled.

A substance is classified in Category 1A for carcinogenicity if the substance is known to have carcinogenic potential for humans, mainly based on human evidence.

Available human data on the effects of titanium dioxide are rare, exposure was generally indirect, with possible co-exposure to other nanoparticles. The studies were not conclusive and had weaknesses. Human data are therefore insufficient to classify titanium dioxide as Carc. 1A.

Category 1B is applicable to substances presumed to have carcinogenic potential for humans, based largely on animal evidence.

No carcinogenic effect was reported after oral administration of TiO_2, even if no firm conclusion can be made for rats in one of the two available studies. After dermal administration, TiO_2 has no promoter potential in mice. Limited absorption was reported, although some studies indicated that TiO_2 can accumulate slightly in organs after oral administration and can enter the hair follicles and sweat glands after dermal application. Furthermore, it cannot be excluded that some forms of TiO_2 could be better absorbed, in particular with specific coating and/or size. However, considering the hypothesized carcinogenic mode of action of TiO_2 requiring a sufficient accumulation of particles, the low absorption of the TiO_2 might explain the lack of systemic carcinogenic effect reported in the available studies by oral and dermal routes. Would a specific form of TiO_2 be so easily absorbed via dermal or oral route that it would significantly accumulates, its carcinogenic potential via these routes should be questioned.

Although no definitive conclusion can be drawn about the carcinogenic effect after inhalation of TiO_2 based on human data, lung tumours were reported in 2 inhalation studies in animals, with fine rutile TiO_2 (Lee, 1985) and nano anatase/rutile P25 TiO_2 (Heinrich, 1995), respectively. In the Lee (1985) study, performed with a protocol similar to OECD guideline, increase of bronchioalveolar adenoma was reported in both sexes. In the Heinrich (1995) study, the tumours consisted in bronchioalveolar adenoma, bronchioalveolar adenocarcinoma, cystic keratinizing squamous cell tumours and

squamous cell carcinoma. This study is of lower quality since it was performed in females only and with a unique concentration level varying during the experiment. However, since the effects are consistent with those of the other studies, they are considered relevant. Indeed, similar types of lung tumours were reported by Pott (2005) after intra-tracheal administration of fine anatase TiO_2 and nano anatase/rutile P25 TiO_2. A further study (Xu, 2010) reported a carcinogenic promoter potential (increased multiplicity of lung adenomas and mammary adenocarcinomas) of nano-TiO_2 (rutile type, 20 nm) administrated by IPS in transgenic Hras 128 female rats initiated with DHPN. However, this effect needs to be taken with caution since there is only little experience with this model.
The IARC Working Group concluded that there was **sufficient evidence that TiO_2 is carcinogenic in experimental animals based on a similar dataset (except Xu (2010)) (IARC, 2006).** It should be noted that, although it cannot be directly transposable, there is a strong link between CLP and the IARC classification criteria since the definition of sufficient and limited evidence are part of the CLP criteria (guidance on the Application of the CLP criteria (version 4.1 – June 2015)).

Benign and malignant lung tumours were reported in different studies. The malignant responses were observed in a single species and a single sex (Heinrich, 1995; Pott, 2005; Xu, 2010). Indeed, bronchioalveolar adenocarcinoma and squamous cell carcinoma were only observed in female rats. Nevertheless, it should be noted that only females were tested in Heinrich (1995), Pott (2005) and Xu (2010) studies. In contrast, only benign tumours (bronchioalveolar adenomas) were found in both sexes in Lee (1985) study when considering the re-evaluation by Warheit (2006). However, considering the type of lung tumours reported and the hypothesized mode of action, a sex-specificity is not expected. Furthermore, although difference in sensitivity to oxidative damage and/or in clearance efficient may explain species differences, it is noted that only one study (Heinrich, 1995) assessed carcinogenic effect of TiO_2 (nano anatase/rutile P25) in mice but the high background tumour response in the control group might have limited the ability to detect any carcinogenic effects in this study.

Relevance of these tumours to humans needs to be assessed in order to conclude on the need for classification. First, lung tumours observed after TiO_2 inhalation in rats occurred in an overload context, which could suggest that the maximum tolerated dose has been exceeded. Although inter-species variability was found in particle retention, the overload concept seems to be relevant for humans (in particular for workers exposed to high dust exposure) since it appears that lung retention and chronic pulmonary inflammation in humans are consistent with the findings in rats.

Controversy exists over the biological significance of cystic keratinizing squamous cell tumour because this type of lesion appears to be a unique rat tumour occurring under exaggerated exposure conditions and has not usually been seen in humans. Several workshops have discussed the definition of cystic keratinizing pulmonary lesions, which were in the end seen as a family of related morphological changes ranging from squamous metaplasia with marked keratinization through pulmonary keratinizing cysts to cystic keratinizing epithelioma and finally pulmonary squamous cell carcinoma. In conclusion, although at this time, the relevance of keratinizing cystic tumour to humans remains unclear; other types of tumours (bronchioalveolar adenomas or adenocarcinomas and squamous cell carcinomas) found in rats exposed to TiO_2 do occur in humans.

Finally, the hypothesized carcinogenic mode of action of TiO_2 seems to be mainly due to secondary genotoxicity based on inflammation and induction of oxidative lesions reported in repeated-dose toxicity studies and/or in genotoxicity studies. However, a direct genotoxic mechanism, with interaction between DNA and TiO_2, cannot be ruled out since particles were found to accumulate in cell nuclei. Therefore, there is no sufficient justification not to consider the carcinogenic effects and the underlying mode of action as not relevant to humans.

In summary, no carcinogenic concern was reported by both oral and dermal routes but there is sufficient evidence of carcinogenicity in experimental animals after inhalation. Indeed, a causal relationship has been established between TiO_2 and the increase of malignant lung tumours in female rats and benign lung tumours in males and female rats in 2 inhalation and 2 instillation studies.

It is proposed to classify TiO_2 specifically by inhalation: Carc Cat 1B- H350i. The specification of the route of exposure was based on the following considerations:

- Only local tumours were reported after inhalation exposure;
- No carcinogenic concern after oral and dermal administrations was identified for the tested forms;
- A low absorption is expected by oral and dermal routes. Since the hypothesized mode of action of TiO_2 is mainly due to inflammatory processes, it is considered that sufficient concentration of particles in a tissue is required to reach a failure of clearance mechanisms and thus proliferative lesions. Although it cannot be excluded that some forms of TiO_2 could be better absorbed than others, no significant accumulation of TiO_2 is expected. Therefore, it is considered that exposure to TiO_2 by oral or dermal routes would not lead to sufficient accumulation of particles to induce a carcinogenic effect.

TiO_2 was not proposed to be placed in Category 2 since malignant tumours were reported in more than one experiment of adequate quality. These malignant findings are only found in rats, the unique tested species. It is also recognised that other rodent species would be less sensitive for the hypothesized mode of action leading to an underestimation of carcinogenicity.

SinceTiO_2 exists under several forms (characterized by morphology, crystallinity, surface treatment), the impact of the physico-chemical properties on carcinogenic potential was assessed. Nevertheless, the data available (exclusively from scientific literature) is limited to only few forms (different crystal forms and morphologies, non surface-treated). From some studies, it can be suggested that the nano-form is more “reactive” (biologically active) than the micro-form since carcinogenic effects appears at a lower concentrations with nano-forms. However, the mass concentration used may not be the best metrics for nanoparticles and a direct comparison of concentrations at which the tumours occurred may not be appropriate to conclude that ultrafine TiO_2 is more toxic than fine TiO_2. Furthermore, since the types of tumours reported after inhalation or instillation of fine and ultrafine TiO_2 were the same (bronchioalveolar adenomas/adenocarcinomas and squamous cell carcinomas), it supports the conclusion that carcinogenicity is not nano-specific. Concerning crystallinity, it cannot be concluded that these properties have a significant impact on the carcinogenicity potential of TiO_2 since carcinogenic effects were reported with TiO_2 of rutile, mix anatase/rutile and anatase phases. Regarding impact of the coating, only one intra-tracheal study assessed carcinogenicity effect of a

coated-TiO_2. In this study, the administration of the substance led to a high acute toxicity and only few animals survived at the end of the experiment. Thus, the results are not easily interpretable. However, acute and repeated dose toxicity studies showed that different coated-TiO_2 induced inflammatory response after respiratory exposure. Considering that the hypothesized carcinogenic mode of action of TiO_2 seems to be mainly due to secondary genotoxicity based on inflammation and induction of oxidative lesions, it can be hypothesis that coated forms also have a potential to produce lung tumours by this way. Finally, TiO_2 can be formulated under different shapes, in particular spherical and non-spherical particles. No carcinogenicity study is available on non-spherical TiO_2 but it can be hypothesized that these forms can have a similar carcinogenic behaviour as fibres. It is generally recognised that the main difference of carcinogenic mode of action between fibres and granular particles is that fibres can translocate to the pleura to induce malignant mesotheliomas although it was not reported with granular particles. However, both fibres and granular particles induced lung tumours with a similar mode of action consisting in a persistent inflammation due to an incomplete phagocytosis and a release of reactive oxygen and nitrogen species. In conclusion, no significant impact of size, crystallinity, coating and shape on carcinogenicity can be identified from the available studies. In contrast, it is believed that the biopersistence and poor solubility of TiO_2 is rather more relevant than the other physico-chemical parameters to explain carcinogenic potential of TiO_2. In this context, no separate evaluation has been done for the carcinogenicity endpoint and the classification proposal covers all the existing forms of TiO_2.

It is also known that there are other substances with this specific property of behaving like GBP which result in the same type of lung effects with this mode of action. Biopersitent granular dusts elicited neoplasms in rats are considered relevant for humans.

Different specific considerations are required in order to conclude on the level of concern and the classification category, such as the occurrence of benign and/or malignant tumours in one or several sites, response in one of both sexes of one or more species, mode of action and relevance to humans.

Even if only some compositions without treatment of titanium dioxide have been tested for carcinogenicity, a classification as Carcinogen Category 2 for the other crystal forms, morphologies and surface treatment might underestimate the hazard since the proposed mode of action is mediated by inflammation is also considered relevant to all the forms including in the scope of the dossier.

4.1.6 Conclusions on classification and labelling

TiO_2 should be considered as being potentially carcinogenic to humans when inhaled and thus be classified Carc. Cat 1B – H350i. This classification applied for both fine particles and nanomaterials of TiO_2 without being able of any distinction in terms of morphology, crystal phase, and surface treatment.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

In the CLH report submitted by France, the carcinogenicity of TiO2 was assessed using studies available in the public literature and in the registration dossier. The dossier submitter (DS) indicated that all the publications used in the assessment were based on a bibliographic search carried out on all forms of TiO2 and that in addition, the information from the registration dossier (on EC 236-675-5) published on the ECHA website was considered. Only one study (Furukawa, 2011), a dermal tumour promoter study, was described as GLP and guideline compliant. One study via the oral route (Bernard, 1990) and one study via the inhalation route (Lee *et al*., 1985) were described by the DS as being "similar to guideline". Tumour promoting potential was investigated in two of the studies conducted via the instillation route (following pre-treatment with N-bis(2-hydroxypropyl)nitrosamine (DHPN)) and in all the studies conducted via the dermal route (following pre-treatment with 7,12-dimethylbenz[a]anthracene (DMBA) or UVB). In addition, the DS has drawn on data published by IARC, in particular, the monographs addressing titanium dioxide from 2006 and 2010.

In one study conducted via the oral route (NCI, 1979) both rats and mice were used and the other (Bernard, 1990) was conducted only with rats. In both studies the highest dose was 50 000 ppm in the diet, equivalent to 2500 mg/kg bw/d in rats and 7500 mg/kg bw/day in mice. None of the tumours seen were considered by the DS to be treatment-related.

In the multiple promoter studies conducted via the dermal route, no evidence of tumour promotion was seen.

In the studies conducted via inhalation or intra-tracheal administration of TiO2 in rats and/or mice, lung tumours were reported in rats in an overload context. Overload was defined by an impairment of normal pulmonary clearance due to high accumulation of particles.

Benign lung tumours (bronchioalveolar adenomas) were observed in both sexes in rats in the inhalation study by Lee *et al*. (1985) in which micro-sized rutile TiO2 was tested. In the inhalation study conducted in female rats and mice by Heinrich *et al*. (1995), nano-sized anatase/rutile TiO2 induced malignant tumours (squamous cell carcinomas and bronchioalveolar adenocarcinomas) in female rats only. Lesions described as "cystic keratinizing tumours" were also observed in female rats in Heinrich *et al*. (1995), but the relevance to humans was considered unclear. The Heinrich *et al*. (1995) study was of lower quality since it was performed in females only and the concentration level varied during the experiment. However, since the effects were consistent with those of the other studies, they were considered relevant.

After intra-tracheal administration of nano-TiO2 P25 (majority anatase), nano-TiO2 P805 (P25 coated with trimethoxyoctyl-silane, 21 nm) or micro-TiO2 anatase, an increased incidence of benign tumours (adenomas and epitheliomas) and malignant tumours (adenocarcinomas and squamous cell carcinomas) were reported by Pott and Roller (2005). A further study (Xu, 2010) reported that TiO2 treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas. Alveolar proliferative lesions were not observed in rats receiving TiO2 treatment without prior DHPN treatment, although slight inflammatory lesions were noted. As

there was only little experience with this model, the DS considered that this effect needed to beconsidered with caution.

Concerning the mode of action, the DS considered that the main mechanism to explain the effects induced by TiO2, in common with effects seen with other substances, was inflammation and an indirect genotoxic effect through ROS production arising from the biopersistence and insolubility of all forms of TiO2 particles. However, the DS could also not exclude a direct interaction with DNA, since TiO2 was found in the cell nucleus in various *in vitro* and *in vivo* studies. The DS also noted that some substance characteristics (in particular shape and coating) might also lead to more potent carcinogenicity or to other specific lesions via a specific mode of action.

The DS noted that data from humans do not suggest an association between occupational exposure to TiO2 and risk for cancer. However, all these studies have methodological limitations and the reported levels of exposure are debatable.

The DS justified classification as Carc. Cat 1B – H350i for TiO2 on the basis that there was an increase in the incidence in both malignant and benign lung tumours in one species and these were reported in two studies by inhalation and two studies by instillation after exposure to TiO2. Although malignant tumours were observed only in single sex, it was noted that only females were tested in the studies reporting malignant tumours (Heinrich *et al*., 1995; Pott and Roller, 2005 and Xu, 2010). In Lee *et al*. (1985), both sexes were tested but only benign tumours (bronchioalveolar adenomas) were found (in both sexes). However, considering the type of lung tumours reported and the hypothesized mode of action, sex-specificity was not expected by the DS. Regarding the observed species difference in Heinrich *et al*. (1995), the DS considered that there may be a species difference in sensitivity to oxidative damage and/or in clearance efficiency, but there was only one study assessing carcinogenic effect of TiO2 (nano anatase/rutile P25) in mice and the high background tumour response in the control group might have limited the ability to detect any carcinogenic effects in this study. Regarding the human relevance of the observed tumours, the DS noted that although in studies with other (non-TiO2) inhaled particles inter-species variability was found in particle retention, lung overload can also occur in humans, in particular in workers exposed to high dust concentrations. Furthermore, the DS concluded that lung retention and chronic pulmonary inflammation occurring in humans were consistent with the findings in rats. According to the DS, a direct genotoxic mechanism was also possible. Altogether, the DS concluded that there was not sufficient justification to consider the carcinogenic effects as not relevant to humans.

The DS concluded that since the data provided cannot distinguish whether a specific form of TiO2 is linked to its toxic effect, this classification should be applied to "titanium dioxide in all phases and phase combinations; particles in all sizes/morphologies".

Classification was proposed only for the inhalation route, because only local tumours were found after respiratory exposure. Insufficient evidence of carcinogenicity was identified by the oral and dermal routes, allowing them to be excluded. The DS noted that the negative results in different carcinogenicity studies might be explained by limited absorption, since the hypothesized mode of action requires sufficient accumulation of particles to induce inflammation and proliferative lesions.

Comments received during public consultation

514 comments were received during the public consultation on the CLH report. Comments were from 5 MSCA, 38 individuals and the remainder from organisations. The analysis by the DS of the issues addressed in the comments is contained in the annex to the RCOM.

Similar comments appeared more than once under different headings/endpoints in the RCOM and the DS noted that 132 comments referred to comments from Titanium Dioxide Manufacturers Association (TDMA), Titanium Dioxide Industry Consortium (TDIC) or Verbrand Der Chemischen Industrie (VCI).

Comments from three MSCA addressed substance identity. Two MSCA expressed the view that classification of the different TiO2s should be considered separately. One of these MSCA acknowledged that the lack of information on particle characteristics (size, shape, crystallinity, surface area, coating, purity, etc) used in the different studies limited the value of the data and conclusions. One MSCA pointed out that titanium dioxide may also appear in fibrous form and that such fibres would be assumed to have an asbestos-like action and therefore the carcinogenicity of rigid biodurable WHO fibres is orders of magnitude higher than for granular material. This MSCA also noted that the classification should apply to respirable particles and that use of different coatings or dopants could affect the properties of the material.

All MSCA agreed that no classification was warranted for the dermal and oral routes. Four MSCA broadly agreed with the classification proposed. Two of the MSCA suggested further discussion on the arguments addressing differences in potency between the different forms of TiO2 and the implications of tumours being secondary to chronic inflammation and oxidative stress and overload conditions.

One MSCA considered that due to uncertainties arising from the experimental conditions (test material characterisation, high concentrations, relevance to humans arising from species differences) the criteria for classification as Carc 1B; H350i were not met, but Carc. 2 should be considered.

Extensive comment on the proposal was provided by industry, which were unanimously in favour of no classification and addressed specific points made in the CLH report individually. In particular, industry questioned the need to classify for hazards arising from the physical state of the substance, and did not consider that there was concern for humans based on toxicokinetic reasons (lung burden and overload) and toxicodynamic reasons (overload concept, species differences, negative epidemiology).

Extensive commentary was also provided on the specific studies used to support the proposal. In particular, the high concentrations at which findings of lung tumours were described in the study by Lee *et al*. (250 mg/m³) which "clearly exceeded the maximum tolerable dose (MTD) and therefore it would be inappropriate to be considered as a positive tumour response". Lung tissue data, a NIOSH document and applicability of relevant OECD guidelines/ guidance and ECHA guidance were provided as support. Industry argued that particle-overload related lung tumours in rats were not relevant for human risk assessment following chronic inhalation exposures. Discussion of the identification of the lesions (whether some of these were "proliferative keratin cysts") by various groups of pathologists was also provided.

Industry also considered that intratracheal instillation studies utilised excessive doses or non-standard routes of administration "and should therefore not be considered for classification

purposes". Fibrosis and bronchioalveolar hyperplasia were also not considered to be precursors of a carcinogenic response.

A number of MSCA as well as industry provided references to additional publications considered relevant to the proposal. Some of these were addressed in detail in the DS response to the comments. In responding to the comments received during public consultation, in addition to specific responses to some comments, an Annex to the RCOM was prepared in which general comments were provided. The DS also presented a revised proposal for the substance identity in their response.

Specific comments received during public consultation have been considered in detail by RAC under "Assessment and comparison with the classification criteria" (below).

Assessment and comparison with the classification criteria

Introduction

Titanium dioxide refers to a group of inorganic chemicals that have the molecular formula TiO2 and whose structural formula depends on the orientation and ordering of the bonds between the titanium and oxygen atoms. The structural formula includes highly ordered arrangements (crystalline) or highly disordered arrangements (amorphous). For the crystalline TiO2, anatase, rutile, brokelite and monoclinic TiO2(B) are the most common structures available.

In addition to its crystal structure, TiO2 can also be characterised by additional physico-chemical properties i.e. (a) size of primary particles, (b) shape/morphology of primary particles and (c) surface chemistry modified by coating of TiO2 particles. Surface coating is the intentional modification of the particle surface chemistry with a different chemical.

The substance identity reported in the REACH registration dossier covers all TiO2 in all crystal structures, morphologies and surface chemistries. The substance identity included as part of the TiO2 registration dossier under REACH and the identity of TiO2 used to generate experimental (eco-)toxicological data may not be fully equivalent in the absence of specific information.

A harmonised classification for mutagenicity was not proposed because according to the dossier submitter "the existing data show too many discrepancies that cannot be explained with the current state of the science" (CLH report). The dossier submitter nevertheless proposed to use the genotoxicity data as supporting evidence for the assessment of carcinogenicity. The CLH report therefore proposes to classify TiO2 with regard to its potential carcinogenicity properties only.

In addition to assessing the reliability, relevance and adequacy of the available experimental data, an additional complexity in the assessment relates to the TiO2 material that has been tested. In particular, granular or spherical nano- and micro-sized particles of TiO2 are considered to share their toxicological properties with substances commonly described as "poorly soluble low toxicity particles" (PSLT particles).

The CLH report specifically refers to the description and evaluation of TiO2-related carcinogenicity data. There are additional references to carcinogenicity data for PSLT particles. However, the PSLT data referred to in the CLH report are selective and are mainly used as supportive evidence for mode-of-action considerations. Because the scope of the CLH proposal is limited to TiO2, there might be relevant PSLT particle data which are not covered in this

opinion document. RACs opinion concerns the carcinogenicity of TiO2, and it is not the intention to provide an opinion on the classification of PSLT particles in general, and in particular on their potential carcinogenic properties by inhalation.

Toxicological testing was mainly performed on TiO2 with the rutile or anatase crystal structures, or combinations thereof. However, the description of tested TiO2 is not explicit concerning the shape of the primary particles; based on the available evidence RAC assumes that the tested TiO2 materials are more or less spherical or granular, but do not show a fibrous morphology. The key experimental studies available (repeated dose toxicity and carcinogenicity studies) were performed with nano-sized anatase/rutile particles (15 to 40 nm; Heinrich *et al*., 1995) and micro-sized rutile particles (200 to 300 nm; Lee *et al.,* 1985; Hext *et al*. 2005).

The size distribution of primary particles and the resulting aerosols need to be distinguished: notably nano-sized primary particles tend to agglomerate, thus resulting in aerosol size distributions similar to micro-sized primary particles. Therefore, RAC assumes that aerosols with similar MMADs (mass median aerodynamic diameters), irrespective of the size distribution of primary particles, will result in similar distribution and deposition in the relevant regions of the respiratory tract in the rat when experimentally tested according to OECD test guidelines.

There is no detailed reporting of the type of coating modifying the surface chemistry of tested TiO2 material. According to Warheit *et al*. (2015), virtually all of the key TiO2 inhalation studies have been conducted with "standard reference" particle types with few if any surface treatments.

In brief summary, the available information indicates that: the key carcinogenicity inhalation studies were performed with respirable, non-fibrous, nano- or micro-sized primary anatase/rutile or rutile particles with few if any surface treatments.

Description and assessment of key toxicity data for TiO2

Oral carcinogenicity studies

Fischer 344 rats and B6C3F1 mice (50/sex/group) were administered TiO2 (anatase, size unspecified) in the diet for 103 weeks (before guideline, no GLP status). The top dose corresponded to 2500 mg/kg/d in rats, and 7500 mg/kg/d in mice. The low dose tested in both species was 50% of the high dose level.

Surviving animals were killed at 104 weeks. There was no appreciable effect on the mean body weights of rats and mice. There were no other clinical signs that were considered treatment-related. Survival of rats and male mice were not affected; in female mice a dose-related trend in decreased survival was noted. No treatment-related tumours were reported in male or female mice.

In female rats, the incidence of C-cell adenomas or carcinomas of the thyroid was noted (controls 1/48, low dose 0/47, high dose 6/44). Also in female rats, endometrial stromal polyps of the uterus were seen (controls 7/50, low dose 15/50, high dose 10/50). There was no statistical significance for the endometrial stromal polyps; for the thyroid tumours a trend test was considered positive, while a statistical test for pairwise-comparison was considered negative (statistical tests not specified). The CLH report did not report on tumour rates in male rats. NTP concluded that: "… *under the conditions of this bioassay, TiO2 was not carcinogenic*

by the oral route of exposure for B6C3F1 mice, but that no firm conclusion can be reached about the possible carcinogenicity of the compound to Fischer 344 rats, at this time".

There was a second oral carcinogenicity study with a substance mainly composed of 72% mica (a mineral silicate) coated with TiO2 (28%), forming flat platelets with the longest dimension measuring 10 to 35 µm. Fischer 344 rats were tested for up to 130 weeks (diet study, corresponding to a maximum dose of 2500 mg/kg bw/d). Low survival rates (not dose-dependent) were noted; a transient reduction in body weight was observed; the only treatment related clinical sign was silver-coloured faeces. In high dose males, a marginally elevated overall incidence of mononuclear cell leukaemia was reported, but this was judged by the authors to be of no biological significance. The CLH report notes that the authors of the study concluded that there was no evidence of a carcinogenic effect.

RAC recognises that the characterisation of the test substances was limited: in the first study, the size of primary particles was not given (the crystal structure was anatase). The second substance tested was not pure TiO2: the particles consisted of a core of silicates coated with TiO2.

The CLH report referred to various oral absorption studies with nano- and micro-sized TiO2. Reporting of the extent of oral uptake was mainly semi-quantitative; specific percentages of oral uptake generally were not reported. Oral uptake was described as "rather low", "only low to no accumulation", "rather low uptake", "extremely low", "very little". The same conclusion was reported by EFSA (2016): (1) "The absorption of orally administered TiO2 particles (micro- and nanosized) in the gastrointestinal tract is negligible, estimated at most as 0.02-0.1% of the administered dose"; and (2) "No difference is observed in the absorption, distribution and excretion of orally administered micro- and nanosized TiO2 particles."

The dossier submitter concluded that a carcinogenicity concern after oral exposure to TiO2 had not been identified.

<u>Dermal carcinogenicity studies</u>

Guideline-compliant dermal carcinogenicity studies with TiO2 were not available. The dossier submitter reported on the results of five studies with a two-stage skin carcinogenesis testing protocol. Both wild-type and transgenic strains of rats and mice were tested. In all the studies, the substance DMBA was used as a carcinogenic initiator.

In all study reports it was concluded that TiO2 showed no promotor potential following dermal exposure. In some of the two-stage carcinogenesis studies, TiO2 was tested without applying the initiating agent. In these study groups there was no indication of dermal carcinogenicity potential of TiO2.

The dossier submitter concluded that there is no concern as to dermal carcinogenicity of TiO2.

<u>Inhalation carcinogenicity studies</u>

The CLH report summarised two publications reporting an increased incidence of lung tumours in rats following chronic inhalation of TiO2 (Lee *et al*., 1985 and Heinrich *et al*., 1995). In addition, 2 negative rat inhalation studies were summarised (Muhle, 1989 and Thyssen, 1978).

Diagnostic terms for cystic keratinizing lesions in the rat lung

Exposure to high air-borne concentrations of TiO2 particles and to other PSLT particles partly resulted in lung lesions generally described as keratinizing cysts of different morphological

stages. Over the last three decades, a confusing variety of diagnostic terms have been applied to these lesions, mainly due to evolving diagnostic methods and scientific understanding of the aetiology, biological relevance and development of these lesions. Two international workshops gathering toxicological pathologists (Boorman *et al*. 1996) addressed the morphology of these lesions to reach a consensus on suitable descriptive diagnostic terms and possibly the biological relevance of these specific rat lesions for humans. At these workshops it was agreed that these cystic keratinizing lesions can be placed in 4 groups, based on their stage of development, as follows (Boorman *et al*. 1996):

1) squamous metaplasia with marked keratinization (non-neoplastic lesion)
2) pulmonary keratinizing cysts (not yet considered as neoplasms)
3) cystic keratinizing epithelioma (benign tumours)
4) cystic keratinizing pulmonary squamous cell carcinoma (malignant tumours).

The cystic keratinizing lesions tend to occur late in these experimental rat studies, rarely before 20 months of exposure, and are generally found in female rats exposed to PSLT particles by inhalation; these lesions are uncommon in male rats.

Lung tumours diagnosed in Lee *et al*. (1985) were re-evaluated based on the revised diagnostic terms by Warheit and Frame (2006). In comments received during public consultation it was claimed that evaluation of cystic keratinizing lung lesions in the Heinrich *et al*. (1995) study did not consider the revised diagnostic terms. RAC notes that the pathological diagnosis of lung tumours in the study of Heinrich *et al*. (1995) was also adapted according to the revised diagnostic terms (Rittinghausen *et al*. 1997). Thus for both rat inhalation studies, the reporting of the cystic keratinizing lung lesions complies with the revised classification scheme and terminology.

Inhalation studies reporting no tumours

- *Muhle (1989) (TiO2)*

In the Muhle (1989) study, rutile TiO2 (MMAD about 1.1 µm) was tested. Male and female F344 rats (50/sex/group) were exposed for 6h/day, 5 days/week to 5 mg TiO2/m³ for 24 months. The animals were kept without further exposure for an additional 1.5-month observation period. Minimal bronchoalveolar hyperplasia and fibrotic reactions, but no TiO2-related carcinogenic lesions were reported for this relatively low TiO2 exposure level.

- *Thyssen (1978) (TiO2)*

In the rat inhalation study of Thyssen (1978), an unspecified form of TiO2 was tested for the relatively short exposure duration of 12 weeks (the total study duration was 140 weeks). Animals were exposed to 16 mg/m³ (6h/day, 5 days/week). No treatment-related carcinogenic lesions were observed.

Assessment of studies reporting tumours

- *Lee et al. 1985 (TiO2)*

In Lee *et al*. (1985) male and female CD rats were exposed to rutile TiO2 (purity 99.0%) with an MMAD of 1.5-1.7 µm. The test material had a spherical configuration. Although the size of the primary particles was not reported, it can be however assumed that primary particles of the rutile TiO2 were in the range of 200 to 300 nm (Hext *et al*. 2005). Animals were exposed for 6 hours/day and 5 days/week for up to 2 years. Animals were killed and examined at the

end of the 2-year exposure period. Three concentrations were tested: control, 10, 50 and 250 mg/m³. The test protocol of Lee *et al*. (1985) was considered comparable to current OECD guidelines by the dossier submitter (reliability 2).

No abnormal clinical signs, body weight changes or excess mortality in any exposed group were reported. Pathological lesions were restricted to the respiratory tract including the thoracic lymph nodes. Starting at the low dose level of 10 mg/m³ there was a concentration-dependent observation of white foci at the lung surface. Lung weights at 10 mg/m³ were comparable to those of the control group; at 50 mg/m³ there was a significant increase in lung weights; at 250 mg/m³ the lung weights were more than twice the weight of control lungs (male rats: 3.25 vs 7.84 g, female rats: 2.35 vs 7.21 g). The TiO2 lung burden at the end of the study was 26.5 mg/lung at 10 mg/m³, 124 mg/lung at 50 mg/m³ and 665 mg/lung at 250 mg/m³ (Lee *et al*. 1986).

The main non-neoplastic and neoplastic lesions in the lung are summarized as follows: at 10 mg/m³ the alveoli showed slight cell hyperplasia, at 50 mg/m³ there was the additional observation of alveolar proteinosis, bronchiolarization of alveoli and fibrosis. No increase in lung tumours was observed at 10 or 50 mg/m³. At 250 mg/m³ the incidence and severity of non-neoplastic lesions increased.

Table: Neoplastic lesions in CD rats with revised diagnostic criteria for cystic keratinizing lesions (Lee et al. 1985, Warheit and Frame, 2006)

	Control	**10 mg/m³**	**50 mg/m³**	**250 mg/m³**
Bronchio-alveolar adenoma (no adenocarcinoma)	2/79 (m) 0/77 (f)	1/71 (m) 0/75 (f)	1/75 (m) 0/74 (f)	12/77 (m) 13/74 (f)
Squamous metaplasia (non-neoplastic)	0/79 (m) 0/77 (f)			2/74 (f)
Pulmonary keratin cyst (non-neoplastic)	0/79 (m) 0/77 (f)	1/75 (f)		1/77 (m) 11/74 (f)
Squamous cell carcinoma (poorly keratinizing)	0/79 (m) 0/77 (f)			1/74 (f)

At the highest concentration, an increased incidence of bronchio-alveolar adenoma but no adenocarcinoma was observed. Essentially all of the cystic keratinizing lung lesions at the high dose level were re-diagnosed as non-neoplastic pulmonary keratin cysts (Warheit and Frame, 2006). Important criteria for the diagnosis of cystic keratinizing epithelioma (a benign neoplasm) were consistently not met according to the authors. In one female rat, a poorly keratinizing squamous cell carcinoma was diagnosed. Before revision and harmonisation of the pathological diagnostic terms had occurred, the non-neoplastic pulmonary keratin cysts were reported as cystic keratinizing squamous cell carcinoma (Lee *et al*. 1985).

Inhalation of high concentrations of TiO2 (four-week exposure, pigment-grade rutile type) resulted in impaired pulmonary clearance mechanisms. Particle retention half-times of 68, 110 and 330 days were reported for the 5, 50 and 250 mg/m³ groups respectively (Warheit *et al*.

1997). Based on subchronic inhalation of pigmentary TiO2 lung retention half-times of 324 and 838 days (exposure levels of 50 and 250 mg/m³) were calculated (Bermudez *et al*. 2002).

- *Heinrich et al. 1995 (TiO2)*

In Heinrich *et al*. (1995), female Wistar rats and female NMRI mice were tested.

Ultrafine TiO2 (P25, Degussa, classical nano-sized TiO2 test material; here without further characterisation of purity) with a primary particle size of 15-40 nm and a composition of ~80% anatase and ~20% rutile was used to generate a test atmosphere with an MMAD of 0.8 µm (GSD of 1.80).

Some exposure conditions in this study were more stringent than in Lee *et al* (1985): the exposure schedule was 18 hours/day and 5 days/week. Exposure was 24 months for rats and 13.5 months for mice; surviving animals were only killed at the end of an additional recovery period of 6 months (rats) or 9.5 months (mice). Only female Wistar rats were exposed both to the control atmosphere and to the single exposure level of 10 mg/m³ (between 7.2 and 14.8 mg/m³).

The test protocol of Heinrich *et al* (1985) was considered by the DS to be of lower reliability (Klimisch reliability score of 3); according to the DS, this low reliability score was specifically based on the lack of a full characterisation of the substance tested and based on the exposure protocol (only one concentration, varying during the experiment, only females treated). In the opinion of RAC, the published results of the Heinrich *et al*. (1995) study are considered to be sufficiently reliable, relevant and adequate for the assessment of the carcinogenic potential of TiO2: the main characteristics of substance identity were reported (P_{25} from Degussa, ~80% anatase and ~20% rutile, primary particle size 15-40 nm) and the variations in the exposure concentrations (7.2 mg/m³ for the first 4 months, followed by 14.8 mg/m³ for 4 months and 9.4 mg/m³ for an additional 16 months) are not considered to compromise the basic results in female rats tested. In an external validation of the study (based on OHAT methodology) the authors acknowledged a "high external and internal validity lending an overall high level of confidence in the available evidence" (Thompson *et al*. 2016).

- Rats

After 24 months of exposure, there was 60% mortality compared to 42% mortality in controls. At termination the mean body weight of the exposed animals was 365 g compared to a body weight of 417 g of the control rats. TiO2 exposure led to an about 5-fold increase in lung wet weight (from about 1 g in controls to about 5 g in the test group after 24 months of exposure; no relevant lung wet weight changes in controls). The retained particle lung burden was ~ 40 mg TiO2/lung. The overall lung clearance (measured by radioactively labeled test aerosols) was significantly reduced in the test group:

Table: Half-times of TiO2 lung clearance in Heinrich et al. (1995)

Duration of exposure	**Control**	**Test group**
3 months of exposure	61 days	208 days
12 months of exposure	72 days	403 days
18 months of exposure	93 days	357 days
18 months and 3 month of recovery	93 days	368 days

Nearly all test group rats showed broncho-alveolar hyperplasia and developed slight to moderate interstitial fibrosis in the lungs. These non-neoplastic lesions were already reported after 6 month of exposure. There were no lung tumours in 20 satellite rats exposed to TiO2 after 6 and 12 months of exposure.

Two histological types of rat lung tumours were observed: there was a 13/100 incidence of adenocarcinoma in test animals (versus 1/217 in controls). The original findings of cystic keratinizing lesions were re-evaluated based on the already mentioned revised diagnostic criteria and classification: following this reevaluation, the incidence in female rats with cystic keratinizing epitheliomas (considered to be benign tumours) was 16/100, the incidence of squamous cell carcinomas was 4/100 (including 3/100 cystic keratinizing squamous cell carcinoma; Rittinghausen *et al* 1997).

Table: Key lung neoplastic lesions in female Wistar rats with revised diagnostic criteria for cystic keratinizing lesions (Heinrich et al. 1995, Rittinghausen et al. 1997)

Neoplastic lesions	**Control**	**Female Wistar rats** **10 mg/m³**
Adenoma	0/217	4/100
Adenocarcinoma	1/217	**13**/100 ($p<0.05$)
Cystic keratinizing epitheliomas (benign tumours)	no corresponding lesions reported	16/100
Squamous cell carcinomas	no corresponding lesions reported	4/100 (including 3 animals with cystic keratinizing squamous cell carcinoma)

- Mice

In female NMRI mice lung tumour rates in the test group (13.8%, adenomas and adenocarcinomas) were not significantly different from the tumour rate of the control animals (30%). Treatment was however for a much shorter period (13.5 months) than for the female rats.

Comparison of key carcinogenicity studies by inhalation (TiO2)

Because of the specific importance of these two key carcinogenicity studies (Lee *et al*, 1985 and Heinrich *et al*, 1995) for classification of TiO2, their main results are further contrasted in tabular form (see the table below). The following points were noted by RAC:

Both fine and ultrafine TiO2 were tested for carcinogenicity. The ultrafine (or nano-scale) TiO2 had a primary particle size of 15-40 nm. The primary particle size of fine rutile particles tested was 200 to 300 nm. Irrespective of the different sizes of the primary particles tested, the MMADs were rather similar: fine rutile particles had an MMAD of 1.5-1.7 µm; ultrafine TiO2 had an MMAD of 0.8 µm.

Although there was a 2-year exposure period in both studies, the animals exposed to fine rutile particles were examined directly following this exposure period, while the animals

exposed to ultrafine TiO2 particles were examined following the additional exposure-free period of 6 months.

Exposure levels associated with neoplastic findings are rather high: lung clearance half-times in both studies estimated to be about 1 year (Warheit et al. 1997) indicate a rather substantial slowing down of physiological lung clearance rates. Under these overload conditions, two different histological types of lung tumours developed. In the Lee *et al*. (1985) study, benign tumours (bronchio-alveolar adenoma and one poorly keratinizing squamous cell carcinoma) were observed. In the Heinrich *et al*. (1995) study both adenocarcinomas and (cystic keratinizing) squamous cell carcinomas (in addition to cystic keratinizing epitheliomas) were observed.

Table: Comparison of results of the two key inhalation carcinogenicity studies

	Heinrich *et al.* (1995)	**Lee *et al.* (1985)**
Species tested	Female Wistar rat	Male and female CD rat
Substance identity	80% anatase and 20% rutile	Rutile
Primary particles	15 to 40 nm	200 to 300 nm
MMAD	0.8 µm	1.5 -1.7 µm
Exposure schedule	18 h/day, 5 days/week	6h/day, 5 days/week
Duration of exposure	24 months	24 months
Time-point of final section	30 months	24 months
Concentration	~10 mg/m³	10, 50 and 250 mg/m³
TiO2 lung burden after 24 months	~ 40 mg/lung	26.5 mg/lung (10 mg/m³) 124 mg/lung (50 mg/m³) 665 mg/lung (250 mg/m³)
Lung clearance half-times	61-93 days in controls 368 days after recovery period	330 days at 250 mg/m³ (Warheit *et al*. 1997)
Neoplastic and non-neoplastic findings:	**At 10 mg/m³:**	**At 250 mg/m³:**
Bronchio-alveolar adenoma	4/100 (0/217) f	12/77 (2/79) m 13/74 (0/77) f
Adenocarcinoma	13/100 (1/217) f	-
Squamous metaplasia	-	2/74 (0/79) f
Pulmonary keratinizing cysts (non-neoplastic)		11/74 (0/77) f 1/77 (0/79) m
Cystic keratinizing epitheliomas (benign)	16/100 f (no lesions in controls)	-

Squamous cell carcinomas	4/100 f (no lesions in controls) (including 3 animals with cystic keratinizing squamous cell carcinoma)	1/74 (0/79) f

Note: control incidences in brackets; "-" = none reported

Although it is known that PSLT-induced tumours appear late in the rat (Gebel, 2012), malignant tumours in the Heinrich *et al*. (1995) study were already found after 18 months and at termination of exposure (24 months) (see Table below).

Table: Lung tumors (18 and 24 months of study) in Heinrich et al. (1995)

Study month	**control**	**Incidence at 10 mg/m³ TiO2**
18 (interim sacrifice)	0/18	2/20 adenocarcinoma
		3/20 squamous cell carcinoma
24	0/10	1/9 adenocarcinoma
		2/9 squamous cell carcinoma

Rat lung tumours caused by other PSLT particles

The range of tumour phenotypes observed with TiO2 was similar to that reported for other PSLT substances (Nikula, 2000).

Intra-tracheal instillation of TiO2 (Pott and Roller, 2005)

The dossier submitter presented TiO2 instillation studies as supportive evidence for the carcinogenic effects of inhaled TiO2. Intratracheal instillation is not a realistic route of exposure; this exposure regime bypasses the upper respiratory tract and may produce non-physiological patterns of deposition and clearance. Intratracheal instillation delivers a bolus dose into the lung in a relatively short period of time.

In the study by Pott and Roller (2005), female Wistar rats received up to 30 instillations of suspended particles of different types of TiO2 at weekly intervals. The study was terminated after 30 months. In the controls no primary lung tumours were diagnosed. The dose levels tested with nano-sized (20 to 30 nm) and micro-sized (200 nm) anatase particles resulted in lung tumour incidences from about 50 to 70% for the nano-sized anatase particles, and from about 30 to 60% for the micro-sized anatase particles. The dossier submitter stated that treatment with both types of TiO2 resulted in an increase of benign tumours (adenomas and epitheliomas) and an increase in malignant tumours (adenocarcinomas and squamous cell carcinomas).

Intratracheal instillation did not result in respiratory tract tumours in Syrian golden hamsters (the reference in the CLH report was to IARC monograph 93). The chronic dose schedule was similar to the dosing schedule in the Pott and Roller (2005) rat studies. However, the treated hamster group had a markedly decreased lifespan of 70-80 weeks, as a result of which the detection of late lung tumours was impaired.

Exposure conditions in key TiO2 inhalation studies

Neoplastic lung lesions in the Lee *et al* (1985) study with micro-sized rutile particles of TiO2 were only observed at the high air-borne concentration of 250 mg/m³. No increased tumour incidences were observed at 50 mg/m³. The TiO2 lung burden after 24 months was ~ 665 mg/lung at 250 mg/m³, 124 mg/lung at 50 mg/m³ and 26.5 mg/lung at 10 mg/m³. Corresponding lung clearance rates were not reported, but these are available from other studies. Deposited TiO2 (four-week exposure, pigment-grade rutile type, size of primary particles: 200 to 300 nm) was found to be cleared from the lungs with half-times of 68, 110 and 330 days for the 5, 50 and 250 mg/m³ groups respectively (Warheit *et al*. 1997). Furthermore, pigmentary TiO2 was administered to rats for 13 weeks (0, 10, 50 and 250 mg/m³). Lung clearance half-times reported were 100 days at 10 mg/m³, 324 days at 50 mg/m³ and 838 days at 250 mg/m³ (Bermudez *et al*. 2002).

Neoplastic lung lesions in the Heinrich *et al*. (1995) study (nano-sized TiO2) were reported at the tested dose level of 10 mg/m³. The TiO2 lung burden after 24 months was ~ 40 mg/lung. The lung clearance half-time at this exposure level was 368 days compared to ~ 60 to 90 days in controls.

Comments received during public consultation emphasised the view that the experimental exposure level of 250 mg/m³ in Lee *et al*. (1985) clearly exceeded the MTD and therefore the corresponding tumour data would be inappropriate to be considered as a positive tumour response.

RAC specifically refers to the Guidance on the Application of the CLP Criteria (2015) and the OECD Guidance Document (GD) 116 (2012). The CLP Guidance proposes that testing protocols should maximise the sensitivity of the test without significantly altering the accuracy and interpretability of the biological data observed. Generally the MTD is defined as the highest dose to produce toxic effects without causing death and at which decreases in body weight gain of no more than 10% relative to controls are observed. The CLP Guidance refers to overload conditions in general terms, stating that the "relevance of lung overload in animals to humans is currently not clear and is subject to continued scientific debate" (section 3.9.2.5.3., page 470 of the CLP Guidance). In OECD GD 116 it is noted that inhalation of doses that overwhelm pulmonary clearance may lead to tissue responses that are specific to the tested species. It is proposed that for substances with poor solubility the degree of lung overload and the delay in clearance needs to be estimated. There is the recommendation that the "use of concentrations exceeding an elimination half-time of approximately 1 year due to lung overload at the end of the study is discouraged." It needs to be emphasized however, that there is no specific justification for this concrete definition. More information on the conduct of acute and repeated dose inhalation studies is given in OECD GD 39 (2009); in this guidance no recommendation is given on a maximum lung clearance half-time. It is however expressed that (1) "the study design of repeated inhalation studies … need to reveal and quantify the clearance of test articles" and (2) "exposure concentrations should be selected to cover the entire range of lung burdens, i.e. those which do not delay clearance to those which do delay clearance." (OECD GD 39, page 44).

The Draft ECHA Guidance on Nanomaterials (2016) gives specific advice on testing PSLT particles for repeated dose toxicity. For PSLT particles the rat lung burden is considered to be an important issue when assessing the toxicological outcome of an inhalation study. It was concluded that "lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans unless it can be clearly substantiated otherwise" (page 15).

There is no specific recommendation as to the level of overload that might compromise the relevance of the corresponding toxicological outcome for humans.

RAC acknowledges that rat tumours only developed under conditions of marked reduction of lung clearance. Corresponding lung clearance half-times reached and exceeded 1 year.

RAC notes the overload concept proposed by Morrow (1988 and 1992). Whereas it might not be a generally accepted concept, it might assist in a constructive discussion of the term "overload": Long-term exposure to increasing exposure levels of PSLT particles leads to increasing pulmonary dust burdens whereby the clearance of deposited particles by alveolar macrophages becomes progressively reduced to the point that pulmonary clearance essentially ceases. Parallel to these changes alveolar macrophages change their response pattern within the lung. In the rat lung, chronic inflammation is a key consequence of increased particle lung burdens. Morrow (1988 and 1992) developed an overload concept relating the extent of lung dust burden and reduction of alveolar clearance with the extent of the volumetric loading of alveolar macrophages. According to Morrow (1988 and 1992) increasing inhalation exposure to particles "brings about a significant prolongation of particle clearance" if 6% of the volume of alveolar macrophages is occupied by particles. When volumetric loading of alveolar macrophages reaches about 60%, "pulmonary dust clearance appeared to cease almost completely" (Morrow 1992).

Morrow roughly estimated the levels of lung burdens related to the 6% and 60% particle volume loading of alveolar macrophages (AM): he calculated a total AM pool volume of 25 mm^3 in a rat lung of about 1.5 g (about 2.5 x 10E+7 alveolar macrophages with an AM volume of about 1000 μm^3). A volume of 6% of the AM pool then corresponds to 1.5 mm^3 or 1.5 mg of particles with unit density (AM volume loading of 60% corresponds to 15 mm^3 or 15 mg of particles with unit density).

Table: Substance-specific lung burdens and degree of AM particle volume loading

Density of substance	**Substance-specific lung burden in the rat lung corresponding to a 6% volume loading of alveolar macrophages**	**Substance-specific lung burden in the rat lung corresponding to a 60% volume loading of alveolar macrophages**
1	1.5 mg/rat lung	15 mg / rat lung
1.5 (e.g. coal dust)	2.3 mg / rat lung	23 mg / rat lung
2.7 (talc)	4.1 mg / rat lung	41 mg / rat lung
4.3 (TiO2)	6.5 mg / rat lung	65 mg / rat lung

If one follows this concept for TiO2 with a density of 4.3, a TiO2 lung burden range between 6.5 mg and 65 mg per rat lung would cover a range from significant prolongation to an almost complete cessation of alveolar clearance. As a rule of thumb, for TiO2 with a density of 4.3, the numerical value of the TiO2 rat lung burden is identical to the numerical value of the percentage of AM particle volume loading.

Table: Lung burden and particle volume loading in key inhalation studies

	Heinrich *et al*. 1995	**Lee *et al*. 1985**
Exposure levels	~10 mg/m³	10, 50 and 250 mg/m³
TiO2 lung burden after 24 months	~ 40 mg/lung	26.5 mg/lung (10 mg/m³) 124 mg/lung (50 mg/m³) 665 mg/lung (250 mg/m³)
Particle volume loading in alveolar macrophages	~ 40%	A 60% volume loading is already reached between 10 and 50 mg/m³

Based on this overload concept of Morrow (1988 and 1992) the TiO2 lung burden in Heinrich *et al*. (1995) (40 mg/lung after 24 months) corresponds to an AM particle volume loading of 40% (ie. below the 60% level for almost complete cessation of alveolar clearance). In the Lee *et al*. (1985 study) the corresponding lung burdens at 50 mg/m³ and 250 mg/m³ (with 124 and 665 mg/lung) both exceeded the 60% level for the almost complete cessation of alveolar clearance.

Criteria to agree on a specific guidance level for the maximum reduction of lung clearance in experimental testing are complex in nature. Evidence indicates that highly exposed coal miners experience particle lung burdens that can be reached in the rat lung only under conditions of a marked degree of overloading (Kuempel *et al*. 2009 and 2014): historically lung burdens in coal miners in the range of 10-20 mg/g lung were reported (it was not reported whether coal miners with these lung burdens developed pneumoconiosis). Referring to the Morrow overload concept (as described above) the 6 to 60% range for AM particle volume loading in the rat corresponds to a lung burden of 2.3 to 23 mg coal dust per rat lung (or ~1.5 to 15 mg coal dust per g rat lung). With reference to these considerations, the high lung burden of 20 mg/g lung in coal miners can be reached in rats only under conditions of an almost complete inhibition of lung clearance (15 mg/ g rat lung corresponds to a 60% particle volume loading). Part of an explanation for these relationships may be that lung clearance of particles in humans is considerably slower than in rats (Gregoratto et al. 2010). If particle lung burden can be considered a relevant dose metric, these considerations indicate that experimental testing of PSLT particles in the rat under exposure conditions strictly avoiding a significant degree of overload is not justified. In view of this, RAC acknowledges that high particle loading in rats is a condition that can not be dismissed in the classification of TiO2.

<u>Genotoxicity of TiO2</u>

The dossier submitter did not propose a harmonised classification for the hazard class "germ cell mutagenicity" because in their opinion the existing mutagenicity data showed too many discrepancies which could not be adequately explained. Nevertheless, the dossier submitter decided to use the genotoxicity data as one of the parameters relevant for a mode-of-action discussion on carcinogenicity.

The dossier submitter noted that particle-related inflammatory reactions resulting in oxidative stress can be considered the main pathway explaining positive genotoxic results obtained with TiO2. Although they considered primarily this indirect mode of action as the mechanism of genotoxicity, the dossier submitter did not exclude a direct genotoxic effect. The DS indicated

that TiO2 was found in the cell nucleus in some *in vitro* and *in vivo* studies and thus it might directly interact with DNA. However, the dossier submitter also noted that accumulation of TiO2 in the nucleus had not been systematically investigated and when reported, it was not quantified. Comments received during public consultation questioned the dossier submitter's statement that TiO2 might reach the nucleus and cause primary genotoxicity.

Reporting of the study-specific results in the CLH report was generally limited; there was no detailed study-specific information on parameters related to a possible mechanism of genotoxicity. The way the genotoxicity data were presented in the CLH report did not enable RAC to form an independent opinion on the dossier submitter's conclusion on genotoxicity.

The opinion of the dossier submitter, which considered oxidative stress as the main pathway explaining positive genotoxic results obtained with TiO2, is supported by a recent review on the genotoxicity of titanium dioxide nanoparticles: "Current data indicate that the genotoxicity of TiO2 NPs is mediated mainly through the generation of oxidative stress in cells" (Chen *et al*. 2014).

RAC is expected to appropriately take into account scientific opinions of other European committees/agencies. In 2016 EFSA published their "Reevaluation of titanium dioxide (E 171) as a food additive" The EFSA document contains a thorough and detailed summary and discussion of TiO2 genotoxicity data. Much emphasis was placed on a study-specific discussion of the reliability and relevance of the data, including the possible mechanism of genotoxic action. The Panel concluded that "orally ingested TiO2 particles (micro- and nanosized) are unlikely to represent a genotoxic hazard *in vivo*."

Based on the reviews referred to (CLH report, Chen *et al*. 2014, EFSA 2016) RAC considers it justified to assume that oxidative stress plays a central role in TiO2 genotoxicity.

For the purposes of this TiO2 carcinogenicity assessment, RAC considered possible genotoxicity (and carcinogenicity) in the rat lung as a direct consequence of inflammatory reactions resulting in an increased tissue level of reactive oxygen species. This approach is specifically supported by the findings of Driscoll *et al*. (1997) (see next chapter).

<u>Mode of action in rats</u>

TiO2 particles (without WHO fibre characteristics or surface coating resulting in specific toxicity) are considered to be "poorly soluble low toxicity particles" (PSLT). Inhaled respirable TiO2 particles are phagocytosed by alveolar macrophages. Morrow (1988) proposed that when the macrophage intracellular volume of ingested particles becomes greater than 6% of the cell volume, macrophage mobility slows; when particle content in the macrophages reaches 60% of the macrophage volume , movement of macrophages ceases. High particle volume loading of macrophages is considered responsible for the elicitation of alveolar chronic inflammatory reactions with oxidative stress, formation of reactive oxygen species (ROS) and cytotoxicity. As a consequence, secondary mutagenicity is likely to occur.

Driscoll *et al*. (1997) published a corresponding key study: for rats exposed to poorly soluble particles (quartz, carbon black and micro-sized titanium dioxide) by intratracheal instillation the relationships between particle exposure, inflammation and mutagenesis in rat alveolar type II cells were characterised. The studies demonstrated that *in vivo* exposure to particle doses eliciting a neutrophilic inflammatory response (verified by bronchoalveolar lavage) resulted in increased mutation in rat alveolar type II cells. The mutagenic effect was observed for all three materials and was dose-related.

The relative potency of these materials in causing *in vivo* mutation in lung cells varied (the lowest mutation frequency was seen with TiO2) and corresponded to their relative *in vivo* neutrophilic inflammatory activity. The potential contribution of lung inflammatory cells to *in vivo* mutagenic responses was evaluated by co-culturing *in vivo* particle-elicited BAL cells with a rat alveolar epithelial cell line *in vitro*. Particle-elicited BAL cells were shown to exert a mutagenic effect on these epithelial lung cells *in vitro*. The addition of catalase inhibited the BAL cell-related increase in mutations which the authors considered as an indication that reactive oxygen species are required for the mutagenic activity of particle-elicited rat lung inflammatory cells.

Histological investigations in rats showed the occurrence of alveolar hypertrophy and hyperplasia of type II alveolar cells surrounding clusters of particle-laden macrophages. Hyperplastic reactions seem to be more prominent in alveoli compared to terminal bronchiolar cells. There was evidence of lipoproteinosis in the alveolar lumen. Alveolar metaplasia will eventually result in tumour development in the rat (adenocarcinoma and cystic keratinizing squamous cell carcinoma). With increasing doses, translocation of particle-laden macrophages to the lung interstitium increased and interstitial fibrosis developed as well.

Tumour development is not considered to be triggered by direct contact of TiO2 with epithelial lung cells, but by high particle loading of macrophages with TiO2 particles and subsequent modification of macrophage activity, essentially resulting in marked and sustained inflammatory responses in the lung. RAC considers it plausible to assume a practical threshold for this mode of action.

The information provided to RAC on this mode of action comes partly from studies with TiO2, but also from studies with other PSLT particles (e.g. Lee *et al*. 1985, Heinrich *et al*. 1995, Bermudez *et al*. 2002 and 2004, Warheit *et al*. 1997, Warheit *et al*. 2016).

Interspecies differences and relevance to humans

90-day TiO2 toxicity studies in rats, mice and hamsters (Bermudez et al. 2002 and 2004)

The TiO2 studies by Bermudez *et al*. (2002 and 2004) are directly relevant because they contribute to the understanding of mode of action and enable the assessment of interspecies differences for lung responses in three laboratory rodent species (hamsters, mice and rats).

The main features of the study protocol were as follows: exposure to TiO2 was for 13 weeks (6 h/days, 5 days/week) followed by a recovery period without TiO2 exposure for up to 52 weeks. The main parameters investigated were TiO2 lung burden at different time points, pulmonary inflammation reactions and cytotoxicity via broncho-alveolar lavage, lung cell proliferation (BrdU) and histopathology. TiO2 of both micro- (pigmentary/fine) and nano-(sub-pigmentary/ultra-fine) primary particle sizes were tested.

Pigmentary TiO2 was tested at concentrations up to 250 mg/m³ (0, 10, 50 and 250 mg/m³). Dose-dependent lung retention half-times of TiO2 were 100 days for rats in the low concentration group and 324 days to 838 days in the higher concentration groups. The number of macrophages and the proportion of neutrophils as markers of chronic inflammation along with parameters of cytotoxicity (LDH, total protein) significantly increased in bronchoalveolar lavage (BAL) fluid. The BrdU labelling index in alveolar cells (but not in terminal bronchiolar cells) was significantly increased at the highest concentration. Immediately post-exposure, both mid- and high-dose rats had alveolar hypertrophy and hyperplasia of type II epithelial cells surrounding aggregations of particle-laden macrophages. Infiltration of neutrophils indicated histological evidence of chronic active inflammation. By 52 weeks, post-exposure

mid-dose rats additionally showed alveolar metaplasia; high-dose rats developed more severe alveolar type II hypertrophy, hyperplasia and metaplasia and additionally significant alveolar septal fibrosis and interstitialization of particle-laden macrophages. The alveolar lumens in lesion areas were characterised by lipoproteinosis.

Bermudez *et al*. (2002) compared these responses in rats with pulmonary responses in hamsters and mice: the particle lung retention pattern in hamsters was different to the pattern in mice and rats: the lung retention half-time in hamsters at the high-dose level was lower compared to the other species (110 days compared to 621 days in mice and 838 days in rats). Correspondingly, parameters of chronic inflammation rapidly declined in hamsters compared to mice and rats. Significant lung cell replication (BrdU) was only measured in high-dose rats, not in mice and hamsters. There was minimal type II alveolar hyperplasia in hamsters, but not in mice. The most marked difference between hamsters and mice compared to rats was that alveolar metaplasia and fibro-proliferative changes were exclusively observed in high-dose rats with a retention half-time of 838 days.

Similar investigations were performed with nano-sized TiO2 (Bermudez *et al*. 2004). The primary particle size was approximately 20 nm. The aerosol generated was made up of particle agglomerates with an MMAD of 1.37 µm (similar to the micro-sized TiO2 tested). The maximum concentration tested was 10 mg/m³ (compared to 250 mg/m³ of the micro-sized TiO2). The high dose level of the nanomaterial indicated substantial retardation of alveolar clearance, with lung clearance half-times around 50% of the lung clearance half-times for the high dose micro-sized material (in rats, mice and hamsters; according to the summary of lung clearance rates in Hext *et al*. 2005). According to the authors (Bermudez *et al*. 2004), the toxicity profile of the nanoparticles was consistent with the profile obtained with the micro-sized particles (Bermudez *et al*. 2002).

In comparing these subchronic studies, it is evident that for both nano- and micro-sized TiO2 there is a higher sensitivity of rats compared to mice and hamsters. At high concentrations of nano- and micro-sized TiO2, alveolar metaplasia and fibro-proliferative changes were only observed in rats, not in mice and hamsters. Particle retention patterns and pulmonary responses were different in these rodent species (for mice and hamsters robust carcinogenicity data are not available).

Supporting evidence from PSLT particle data: rats versus monkeys (Nikula et al. 1997)

In comments received during public consultation it was additionally proposed that reference should be made to publications on interspecies differences related to other PSLT particles. The publication by Nikula *et al*. 1997 is one of the corresponding key studies. Male cynomolgus monkeys and F344 rats were exposed to 2 mg/m³ of respirable particles of coal dust (and to diesel exhaust soot as well) for 24 months (7h/day, 5 days/week; for the monkeys an exposure duration of 24 months does not correspond to a lifetime study).

At these identical air-borne concentrations, relatively more particulate material was retained in the lungs of monkeys than in rat lungs. The volume percentage of lung occupied by particulate material was about 0.3% in the rat and about 1% in the monkey. However, the compartmental coal dust retention pattern in the lung was different in rats and monkeys: Rats retained a greater portion of coal dust in lumens of alveolar ducts and alveoli than monkeys. In contrast, monkeys retained a greater portion of the particulate material in the interstitium than rats. The retention percentages for the alveolar versus interstitial compartments was

73% versus 27% in rats and 47% versus 53% in monkeys. The different retention sites are assumed to be linked to differences in sensitivity for tumour development; see chapters below.

The corresponding histopathological findings in rats and monkeys are summarised in the following table (for a complete picture see tables 2 and 3 in Nikula *et al*. 1997). The differences observed in pulmonary responses between rats and monkeys should be interpreted with caution in view of the weak responses observed in the rats. Indeed, less than minimal inflammation was observed in 7/15 animals and less than minimal septal fibrotic reactions was observed in 4/15 animals (see Table below).

Table: Pulmonary responses to identical low concentrations of coal dust in rats and cynomolgus monkeys (Nikula et al. 1997)

	Rat Control	**Rat 2mg/m³**	**Monkey Control**	**Monkey 2 mg/m³**
Type of lung pathology and average grading scores				
Number of animals examined	15	15	14	14
Alveolar macrophage hyperplasia	1(1)	15(1.5)	2(1.0)	14(1.4)
Alveolar epithelial hyperplasia	1(1)	14(1.5)	2(1.5)	3(1.0)
Particle-associated inflammation	0(-)	7(1.0)	1(1.0)	4(1.0)
Septal fibrotic reaction	0(-)	4(1.0)	3(1.3)	0(-)
Alveolar proteinosis	0(-)	2(1.0)	0(-)	0(-)

Footnote: The first number is the number of animals with the findings. The number in parentheses is the average severity score which ranges from 1 (slight) to 5 (marked). Score 2 means minimal.

Overall, RAC recognises that in the monkeys there was still a uniform distribution of particles in alveolar spaces and interstitium of the lung. As compared to the rather weak effect profile in rats (at the dose level tested), a weaker response in monkeys is not considered by RAC sufficient reasoning for claiming alveolar findings to be unique to the rat.

Supporting evidence from PSLT particle data: particle retention pattern in rats and humans (Nikula et al. 2001)

Nikula *et al*. (2001) extended their morphometric lung examinations of particle retention patterns to humans. The authors compared the retention pattern within the compartments of the lung (parenchymal lumen versus interstitium) of Diesel exhaust soot in rats and of coal dust in humans. In this study, lungs from 5 control persons and from 11 low-dose miners and 5 high-dose coal miners were examined for compartmental particle location in the lung.

The major compartment both in the human and rat lung was the alveolar parenchyma with roughly about 85% and the interstitium with roughly about 15% of the total lung volume. About 80% of diesel soot particles were retained in the rat parenchymal lumen (compared to about 20% in the interstitium). In low-dose miners, nearly 70% of coal dust particles were retained in the interstitium (compared to about 30% in the parenchymal lumen). The few data available indicate an even higher percentage in the human lung interstitium for the high-dose miners.

The authors finally calculated a "relative compartmental retention", a lung burden parameter, indicating the density of packaging of particulates in a specific compartment. The relative compartmental retention (figure 7 in Nikula *et al*. 2001) of Diesel soot particles was slightly higher in rat parenchyma than in the rat interstitium (about 1.2 versus 0.8 at the high-dose level). In coal dust miners the particulate material was more densely packed in the interstitium (relative compartmental retention of about 2 in the interstitium versus about 0.5 in the parenchymal lumens; low-dose miners; figure 8 in Nikula *et al*. 2001).

The authors discuss that these different retention patterns for Diesel exhaust soot in rats and coal dust in humans could mean that different lung cells are predominantly in contact with the particles in humans and rats, which suggests that PSLT particle responses may differ between the two species.

- *Dosimetric modelling of human retention patterns (Gregoratto et al. 2010)*

Gregoratto *et al*. (2010) refined the Kuempel *et al*. (2001) lung retention model based on coal miner data including further data from human lung retention studies after the inhalation of radioactive particles. The Gregoratto dosimetry model predicts a clearance from the alveolar space to the mucociliary escalator for 60% of alveolar deposit (T1/2 = 400 days) and a clearance from the alveolar space to the interstitium for 40% of alveolar deposits (with T1/2 of 700 days).

- *Supporting evidence from PSLT particle data: Review on the overload concept by Warheit et al. 2015*

The differences in lung distribution patterns between species is considered to be one reason for the differing pulmonary responses among the several species studied. While inhaled particles are predominantly retained in the alveolar duct compartments of rats, resulting in the rat lung tumours observed, in humans there is a greater tendency of transmigration of alveolar deposits to interstitial sites of the lung. A relatively lower particle load in human alveolar spaces is considered to result in a lower extent of alveolar hyperinflammatory responses. Some authors (e.g. Warheit *et al*. 2015) propose to assume that the interstitialisation of particles appear to serve as a repository in humans which is deemed to be less reactive as to tumour development. According to the authors coal workers exposed to high concentrations may develop interstitial-based progressive fibrosis, but possibly less or no risk of developing pulmonary tumours.

- *Human non-neoplastic lung responses due to PSLT particle exposure by inhalation (Schultz 1996, Green 2007, NIOSH 2011 and ECETOC 2013)*

Schultz (1996) compared the pathology of dust-induced pulmonary lesions in rats and humans. He stated that "lung particle burdens equivalent to those producing overload in rats have occurred in coal workers". Furthermore, he concluded that "lesions commonly seen in overload studies in rats, such as marked accumulation of alveolar macrophages, inflammation, necrosis of pneumocytes, alveolar proteinosis, and cholesterol granulomas, were not present in humans with coal worker's pneumoconiosis."

Significantly, Green *et al* (2007, quoted in NIOSH, 2011) compared the tissue responses in human and rat lungs to a range of poorly soluble particles (coal dust, talc and silica). Criteria for selection of human pathology materials was based on known exposure to dust aerosols, a history of not smoking cigarettes, and a lack of major confounding diseases. Similar criteria were used for selection of rodent pathology material. It is obvious that documentation of exposure to dust aerosols was good in studies with rats and poor in human populations. The

cellular responses in the lungs were graded for severity (scores 1 to 4) using a standardized grading system. Key morphological changes were documented. Based on the corresponding bar charts in the original publication the following scores were estimated for the various morphological changes documented for coal dust and talc. Both similarities and differences in response to the same agent were shown. Specifically, acute intra-alveolar inflammation, alveolar epithelia hyperplasia and alveolar lipoproteinosis were all greater in rats than in humans; relevant fibrosis was observed both in rats and humans. The authors indicate that these differences may account for differences in carcinogenic responses as well. RAC notes that human pathology material still showed low scores for centriacinar alveolar hyperplasia.

Table: tissue responses in human and rat lungs to a range of poorly soluble particles

Scores 1-4		**Coal Low Dose**		**Coal High Dose**		**Talc**	
		Humans	**Rats**	**Humans**	**Rats**	**Humans**	**Rats**
Inflammation, acute,	centriacinar intra-alveolar	< 0.2	0.4	< 0.2	1.1	< 0.2	2.4
Inflammation,	granulomatous nodular	< 0.2	< 0.2	< 0.2	0.3	1.6	1.7
Lipoproteinosis,	alveolar	< 0.2	< 0.2	< 0.2	0.8	< 0.2	3.8
Hyperplasia,	centriacinar, alveolar	0.5	0.6	0.4	2.5	1.1	2.3
Fibrosis,	centriacinar	1.0	< 0.2	1.6	1.4	1.8	2.3

NIOSH (2011) concluded that "collectively, these studies indicate that, while there are uncertainties about the rat as a model for particle-related lung cancer in humans, and specifically for TiO2, there is insufficient evidence for concluding that the rat is not a valid model" (chapter 3.6 in NIOSH 2011). Specifically with respect to non-cancer responses, NIOSH stated that "the rat and human lung responses to poorly soluble particles of low or high toxicity (e.g. coal dust and silica) are qualitatively similar in many of the key steps for which there are data, including pulmonary inflammation, oxidative stress, and alveolar epithelial cell hyperplasia" (again chapter 3.6 in NIOSH 2011).

ECETOC (2013) stated that "that the abundance of available clinical and epidemiological data in occupationally-exposed workers is consistently negative for lung cancer as well as non-neoplastic lung diseases." RAC notes some contradiction in the ECETOC assessment of interspecies non-neoplastic lung responses: stating first that there are no non-neoplastic lung diseases, then describing the basic mechanisms in the etiology of Coal Workers Pneumoconiosis (with direct cytotoxicity, oxidant production by pulmonary phagocytes, mediator release from alveolar macrophages and secretion of growth factors from alveolar macrophages). ECETOC (2013, page 73) concluded: "Chronic particle exposure may cause adverse health effects other than lung cancer in humans, such as systemic inflammation and pneumoconiosis. However, further analysis of these data including toxicokinetic modelling to

evaluate hypotheses about relationships between lifetime exposures, retained lung dust, overloading of lung clearance and disease development are recommended."

ECETOC (2013) stated that there is a low severity of pulmonary inflammation in humans. This statement is supported by Morfeld *et al*. (2015): "The PMNs however, play a unique role in rat experiments, findings that do not appear to occur in high dust exposed workers, such as coal miners." A comprehensive review on coal dust toxicity takes a totally different view on the coal dust related inflammation in humans (Attfield in Patty 2012): "Taken together, these data support the hypothesis that initially disease is prevented by the upregulation of protective antioxidants and the downregulation of inflammatory cytokines. Induction and progression of the disease process are associated with an increase in oxidant generation, loss of antioxidant protection, and progressive pulmonary inflammation and damage."

Overall, RAC notes that a sufficiently detailed and transparent presentation of TiO2- or PSLT-related non-neoplastic lesions in humans is neither provided in the CLH report nor in the references referred to in the comments received during public consultation. The data available to RAC does not provide a sufficiently consistent view. The main concern of RAC is that reporting of dose response relationships for non-neoplastic lesions in humans is essentially lacking. Reporting of human data on non-neoplastic consequences of inhalation exposure to TiO2 and PSLT particles is however considered necessary in order to judge whether the mode-of-action in rats is at least partly operative in humans.

Considerations on human relevance of rat lung tumours

The American Cancer Society considers non-small cell lung cancer the most common type of human lung cancer (percentage of about 85%). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are all subtypes of non-small cell lung cancer (https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html).

Maronpot *et al*. (2004) compared the major morphological categories of pulmonary neoplasia in rodents and humans. They concluded that without the lung cancer consequences of smoking the major subtypes in humans would then be "adenocarcinomas and bronchioalveolar carcinomas, which would correspond very closely to the types of lung tumours generally occurring in rodents".

In Lee *et al*. (1985) and Heinrich *et al*. (1995) and Rittinghausen *et al*. (1997) two different tumour types were observed: (1) cystic keratinizing epitheliomas (benign) or cystic keratinizing squamous cell carcinomas and (2) bronchiolo-alveolar adenoma or adenocarcinoma.

The discussion of interspecies differences preferentially focuses on the cystic keratinizing lung tumours. The WHO list of classification of human lung tumours does not contain cystic keratinizing lung tumours (Travis *et al*. 2015); the absence of this lung tumour type in the WHO list is considered by RAC as an indication that this type of cystic keratinizing lesions so far might not have occurred in humans. Different from the cystic keratinizing lung tumours seen in rats, other types of lung tumours, such as adenoma and adenocarcinoma do occur in humans (Travis *et al*. 2015).

Schultz (1996) stated that "responses such as keratinizing squamous cysts and adenomatous proliferation within areas of bronchiolarization, which are common in rats in lung overload, have not been observed in humans". RAC does not know whether the author considers

"adenomatous proliferations within areas of bronchiolarization" identical to the adenomas and adenocarcinomas observed in the rat lung following exposure to TiO2 and other PSLT particles. In the literature provided to RAC, no convincing evidence is reported which allows considering the adenoma and adenocarcinoma observed in the TiO2 rat inhalation studies as unique to the rat.

RAC acknowledges that TiO2 epidemiology studies provide no consistent evidence of an association of exposure to TiO2 and excess lung cancer risks. But in the opinion of RAC this human TiO2 epidemiology alone does not exclude a carcinogenic potential of TiO2 in humans and does not necessarily question the positive evidence for rat lung tumours, bearing also in mind the methodological limitations of the epidemiology studies available. Epidemiology describes risks: TiO2 lung cancer potency in rats is already comparatively low. If in addition there is low exposure to TiO2, then TiO2 lung cancer potential in humans would not be expected to be shown in the available epidemiology studies.

Available chronic rat inhalation studies with nanosized PSLT particles were used to model and estimate ranges of concentration-risk relationships (www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/pdf/910/nanoscaled-GBP.pdf).

Where dosimetry adjustments of the rat lung cancer data are included, the human work-life adjusted BMD10 values (10% excess risk of lung cancer) for nanosized PSLT particles cover a range of about 5 to 25 mg/m³ respirable PSLT particles (TWA, daily work life-time exposure). In the studies evaluated, cystic keratinizing lesions could not be differentiated as to their specific subtypes and were counted as lung tumours. A slightly lower carcinogenic potency (slightly higher BMD10) should be assumed for microsized PSLT particles. In this estimate of BMD10 values it is conservatively assumed that there is potential for carcinogenicity in humans and that humans possess an identical sensitivity based on identical PSLT particle lung burdens. For the reasons given above, it can be assumed that human BMD10 values are higher than calculated.

The maximum average exposures to the respirable fraction of TiO2 at workplaces covered in the study by Boffetta *et al*. 2004 (mainly microscale TiO2) were reported to be around 0.7 mg/m³(summary of data in Hext *et al*. 2005; corresponding data on the respirable fraction of TiO2 are not available for the other epidemiology studies). If (over-conservatively) a human BMD10 is assumed for workers of 5 to 25 mg/m³ for TiO2 and linearity in the dose range of interest, then a presumed chronic exposure level of 0.7 mg/m³ implies an excess worker lifetime lung cancer risk of 0.3 to 1.4%. Against the background of 5 to 7% lung cancer incidence in the general population, an excess risk of probably less than 0.3 to 1.4% cannot easily be recognized under conditions of current epidemiology studies.

Influence of specifications of TiO2 on lung toxicity

The following chapter reports on selected key studies illustrating the influence of specifications of TiO2 on lung toxicity. Reference is made to these studies to assist RAC in considering whether to relate the TiO2 classification proposal to a narrow or broad definition of chemical identity.

Shape/morphology

The TiO2 materials tested for carcinogenicity by inhalation are known to have a granular shape. RAC is not aware of inhalation studies or studies with other routes of administration where fibrous shapes of TiO2 have been tested. The chronic inhalation toxicity observed is

considered to be related to high concentrations of respirable TiO2 particulates of non-fibrous morphology.

Crystal structure and primary particle size

Micro-sized rutile and nano-sized anatase/rutile primary particles were tested for lung carcinogenicity (Lee *et al*. (1985), Heinrich *et al*. (1995)). Particle size distribution (MMAD) in both chronic inhalation studies was similar and within the range of respirability. Based on these studies the specific influence of the crystal structure or the size of primary particles on experimental lung carcinogenicity in rats cannot be demonstrated. RAC is not aware of toxicity studies with TiO2 with brookite crystal structure.

There is some evidence from other PSLT substances regarding the influence of the size of primary particles. When comparing the findings of different carcinogenicity studies it needs to be recognized that the particle-related tumour rates increase with age and these lung tumours in the rat are known to appear late, mainly after study durations longer than 24 months. Taking the different study durations into account, a comparative analysis indicates that nano-sized particles are somewhat more potent than micro-sized particles. But it was concluded by the author that the difference in carcinogenic potency between PSLT nano- and micromaterials is relatively low in general and can differ by a factor of about 2 to 2.5 if related to the cumulative mass concentration as the dose metric (Gebel, 2012). RAC takes this conclusion cautiously, as the underlying database was limited and because the CLH report and opinion document does not contain a comprehensive analysis of the quantitative differences in the carcinogenic potential of nano- and microsized PSLTs.

Coating of particles (particle surface chemistry)

The key inhalation studies (Lee *et al*. 1985, Heinrich *et al*. 1995) used TiO2 materials without or at most with marginal surface coating (Warheit *et al*. 2016).

The impact of surface treatment on TiO2 particle toxicity was tested in a subacute rat inhalation study (Warheit *et al* 2005):_Several TiO2 materials were tested in a 4-week inhalation study in male Sprague Dawley rats at the very high concentration of 1130 to 1300 mg/m³ (6h/day, a total of 20 exposures). Primary particle sizes ranged from 290 to 440 nm; the particle size distribution in the aerosol is characterised by MMADs between 1.3 and 1.8 µm. Lung tissues were evaluated by histopathology immediately after exposure as well as up to 12 months post-exposure. In summary, particle-laden macrophage accumulation and light alveolar cell hyperplasia were observed in all TiO2-exposed rats; there was a marked reduction of these lesions towards the end of the post-exposure period. Slight collagen deposition was only observed in the TiO2 formulation with the highest percentage of coating materials (7% alumina, 8% amorphous silica, thus 85% TiO2). While the airborne concentrations of the different aerosols were similar (between 1130 and 1300 mg/m³), the surface area per particle mass was significantly higher in the formulation resulting in fibrogenic responses (27.8 m²/g vs 6 to 12.2 m²/g). The authors of the study noted that surface treatments can influence the toxicity of TiO2 particles in the lung, but noted that they viewed these impacts as minor when compared to other types of dusts (without specifying which other dusts were meant).

The CLH report refers to further data on the impact of coating. The dossier submitter concluded that coating can impact the toxicity of TiO2 and that the inflammation response can differ between different forms of TiO2, although a clear pattern cannot be drawn from the existing data.

Reference to IARC TiO2 assessment

The CLH report contains a summary of the IARC assessment. In the opinion of RAC, this IARC summary of results of the carcinogenic studies is consistent with the toxicological TiO2 data presented in the CLH report.

The IARC working group concluded that there was inadequate evidence from epidemiological studies to assess whether TiO2 causes cancer in humans. The IARC working group did not conclude that there was human evidence suggesting lack of carcinogenicity. On the basis of results of increased incidence of lung tumours in rats, the IARC working group concluded that there was sufficient evidence that TiO2 is carcinogenic in experimental animals. The IARC working group considered the available mechanistic evidence for TiO2 not strong enough to conclude that TiO2 is "probably carcinogenic to humans" (group 2A). The IARC working group therefore decided to categorize TiO2 as "possibly carcinogenic to humans" (group 2B).

Although IARC extensively describes the variability of TiO2 (crystal type, size of primary particles, coating), there is no explicit IARC discussion on possible differences in carcinogenic potential or potency.

Human epidemiological data

One case report study, three case-control studies as well as five cohort studies were summarised in the REACH registration dossier or mentioned during public consultation; the majority of them have already been analysed in the IARC monograph (volume 93). Apart from the detailed analysis of the strength of the studies in question given below, RAC concludes that the single case report investigation observed TiO2 in lung tissue of a lung cancer patient, but did not allow any conclusion on a causal relationship. In addition, one case-control study carried out by Siemiatycki (1991) showed some indication (statistically non-significant) of an association between TiO2 exposure in the occupational environment and lung cancer. The other two case-control studies and five cohort studies did not indicate an association between TiO2 exposure and lung cancer. Detailed description and analysis of the epidemiological studies mentioned is given in the summary table (see Annex 3 at the end of the document).

RAC underlines that no information is given on TiO2 particle characteristics, including surface area and size distribution in the occupational environment to which the workers are exposed. Only the cohort study carried out by Boffetta *et al.* (2004) in 11 European companies producing TiO2 specifically considered the respirable TiO2 dust. Three out of five cohort studies assessed the exposure quantitatively by direct long-term stationary and personal sample measurements. In other cases, details of exposure are unclear or they are derived from typical workplace conditions based on self-reported occupational histories. The total exposure time was different starting with 6 month or 1 year employment at TiO2 production industries. Only in some case-control studies was frequency of exposure during a normal work-week clearly considered, however, the cohort studies applied derivation of average intensity, duration and cumulative exposure used for the assessment. Generally, adjustment for different confounders (for example, presence of asbestos, smoking, etc.) was done with few exceptions concerning smoking in cohort studies.

Case reports

Deposits of TiO2 in lung tissue were reported for a 53-year old man engaged in packing of TiO2 for 13 years and suffering from pneumoconiosis accompanied by a papillary adenocarcinoma of the lung (Yamadori *et al*., 1986). However, as the person had also a

smoking history of ~40 years, it is not possible to conclude on the role of TiO2 deposits in causing cancer and the subsequent death of the person.

Case-control studies

The case-control study based on interviews with more than 4000 persons in Montreal (Canada) including patients with different types of cancer as well as population controls indicated a statistically non-significant association between substantial exposure to TiO2 defined as ≥ 10 years in the industry or occupation up to 5 years before onset of a disease and lung cancer (odds ratio or OR 2.0, 95% CI 0.6 – 7.4, based on 5 exposed cases) (Siemiatycki, 1991). However, when considering any exposure to TiO2, only the risk of squamous-cell lung cancer was increased but without statistical significance (OR 1.6, 95% CI 0.9 – 3.0, based on 20 exposed cases). In addition, the risk of urinary bladder cancer was increased when considering any TiO2 exposure (OR 1.7, 95% CI 1.1 – 2.6, based on 28 exposed cases). No information on magnitude or frequency of exposure was given, making it impossible to do dose – response relationship analysis.

Two other related case – control studies carried out in Montreal involved persons with histopathologically confirmed cases of lung cancer (up to 2093 persons) and control groups of randomly selected healthy people (up to 2045 persons) as well as persons with cancer in organs other than the lung (up to 1349 persons) (Boffetta *et al*., 2001 based on Siemiatycki, 1991 and Ramanakumar *et al*., 2008). Low, medium and high exposure as well as frequency of exposure to TiO2 was reconstructed based on self-reported occupational histories and typical workplace conditions being in place. Neither an increased risk of lung cancer caused by TiO2 exposure, nor a correlation between occurrence of lung cancer and the frequency, level or duration of exposure was reported (OR ~1.0).

Cohort studies

Chen and Fayerweather (1988) compared observed numbers of incident cases of lung cancer and associated mortality within a population of 1576 workers exposed to TiO2 in USA with expected numbers based on both company rates and USA national rates. Duration and time-weighted exposure average was used, but it is not clear whether it is based on direct measurements. Mortality from lung cancer was profoundly lower than expected both on the basis of national rates (standardized mortality ratio or SMR 0.52) and company rates (SMR 0.59). Neither was there an increase in the incident cases of lung cancer (SMR 1.04). However, it was indicated that only cancer cases in active employees of the company were included, This applies both to calculation of the cases observed in exposed persons and in calculation of the company reference rates. Nevertheless, RAC considers that no association between TiO2 exposure and lung cancer is indicated.

Another retrospective mortality cohort study included 4241 workers employed at four TiO2 production plants in the USA (Fryzek *et al*. 2003). Workers started their career in 1960 and afterwards, and have been followed-up until the end of 2000. A pool of 1472 participants who had worked exclusively in administration or in jobs not associated with TiO2 exposure formed the control group. Low, medium and high categories of exposure were derived based on historical exposure reconstruction involving long-term stationary as well as full-shift or near full-shift personal samples for total TiO2 exposure estimation. Average intensity, duration and cumulative exposure were adjusted by Cox proportional hazard models. Workers with the highest exposure to TiO2 had significantly smaller number of deaths than that expected for all

causes (SMR 0.7) with no excess for lung cancer (SMR 1.0). RAC therefore considers that no association between TiO2 exposure and lung cancer is indicated.

A large cohort study in 11 European companies covering TiO2 production plants in Finland, France, Germany, Italy, Norway and UK was conducted by Boffetta *et al*. (2004). In this mortality follow-up study, 15017 workers who started their employment from 1927 to 1969 and ended in 1995-2001 were involved. Yearly average cumulative exposure was derived based on exposure reconstruction which in its turn was based on personal sample measurements that were mainly collected during the 1990s. The estimated cumulative exposure to respirable TiO2 dust (recalculated from total dust) was from 0 to ≥ 13.20 mg/m^3 years. When considering any TiO2 exposures mortality from lung cancer was slightly higher than in the general national population (SMR 1.19 - 1.23 depending on the statistical model used) with large variations among countries (from SMR 0.76 in Finland to 1.51 in Germany). Nevertheless, it must be stressed that many of the regions where the factories were located had a higher death rate from lung cancer than the national rate for their country, which implied that the SMR for lung cancer would have been lower if regional reference mortality had been used. Besides, adjustment for smoking was lacking. The analysis with respect to respirable fraction of the TiO2 dust showed no dose – response relationship: cumulative exposure 0 – 0.73 mg/m^3 year – Relative Risk (RR) 1, 0.73–3.43 mg/m^3 year – RR 1.19, 3.44–13.19 mg/m^3 year– RR 1.03, ≥13.20 mg/m^3 year – RR 0.89. In addition, there was no relationship with exposure to TiO2 considering duration of employment and concentration. Irrespective of some possible methodological problems (possible exposure misclassification mentioned, some of the factories were relatively new and therefore follow-up periods were short), RAC considers that no clear association between TiO2 exposure and lung cancer is indicated.

RAC notes however, in the above three cases the issue of level of exposure; this is further discussed at the end of this chapter.

Two cohort studies on individuals (5054 participants for study in 2010 and 3607 participants for study in 2013) employed in three DuPont titanium dioxide production facilities in the US and followed from 1935 through 2006 were conducted by Ellis *et al*. (2010 and 2013). The first study was a general follow-up without an attempt to analyse dose-response, while the latter study used exposure reconstruction taking into account work history and static as well as personal monitoring data for TiO2 and TiCl4 (in total, 3488 industrial hygiene monitoring records from 1971 to 2002 with differing measurement durations). A number of cumulative exposure categories from <5 to >80 mg/m^{3}year were established. No increase in causes of death compared to the US population (all causes of death: SMR 0.81 (95% CI 0.77-0.85); all malignant neoplasms: SMR 0.90 (95% CI 0.82- 0.99); lung cancer: SMR 0.90 (95% CI 0.75- 1.05)) was revealed in the 2010 study.

In the 2013 study, the same conclusion was drawn with respect to overall SMRs. However, compared to the DuPont workers not involved in TiO2 production, SMR for lung cancer was 1.35 (95 % CI 1.07–1.66). Comparing increasing exposure groups to the lowest group, disease risk (lung cancer mortality assessed without lag) did not increase with exposure: cumulative exposure 5–15 mg/m^3 year – Relative Risk (RR) 1.68, 15–35 mg/m^3 year – RR 1.65, 35–80 mg/m^3 year– RR 1.20, >80 mg/m^3 year – RR 1.38. Although the RR was greater than "1" at every exposure level, the CIs were wide and overlapped across all levels (Figure below). RAC considers that no clear association between TiO2 exposure and lung cancer was demonstrated.

Additionally, two publications dealing with systematic review of the literature on both experimental and epidemiological data on TiO2 analysed above were mentioned during public consultation (Hext *et al*., 2005 and Thompson *et al*., 2016). Both came to the overall conclusion that there was no suggestion of any carcinogenic effect associated with workplace exposure to TiO2.

Hext *et al*. stressed that regarding the large cohort study in 11 European companies covering TiO2 production plants (Boffetta *et al*., 2004), the average estimated respirable TiO2 dust concentration fell at most factories over the study period to current typical levels of 0.2–0.4 mg/m^3.

Thompson *et al*. (2016) which is the most recent study, gave a summary of lung cancer risk estimates from epidemiological studies of TiO2 (Figure below) and assessed the internal and external validity of these investigations (the other Figure below)[2].

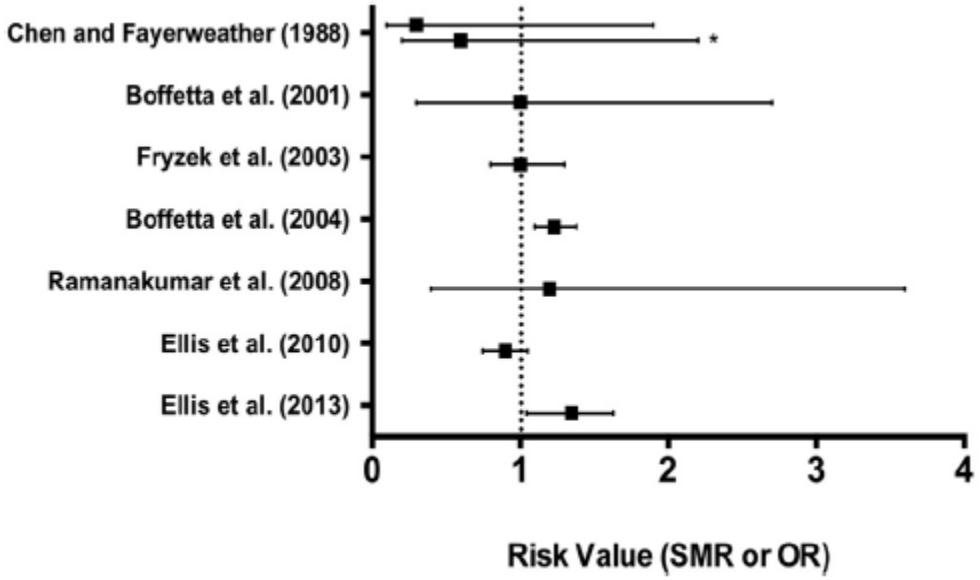


Figure. A summary of lung cancer risk estimates from epidemiologic studies of TiO2 pigment production workers showing standardised mortality ratio (SMR) or odds ratio (OR) for lung cancer. The 90% or 95% confidence interval for each risk estimate is also presented. In most cases, risk estimates were extracted for the highest exposure groups as identified by the study authors. All studies except Chen and Fayerweather (1998) reported SMRs for lung cancer or ORs based on lung cancer cases. Chen and Fayerweather (1998) reported ORs for lung cancer mortality and lung cancer incidence (indicated with an asterisk) (extracted from Thompson *et al*., 2016).

With respect to the Boffetta *et al*. (2004) study reflected in the Figure above, many of the regions where the factories were located had a higher death rate from lung cancer than the national rate for their country. The SMR for lung cancer would have been lower if regional reference mortality had been used. In addition, the Ellis *et al*. (2013) study mentioned here

[2] Study quality and relevance were evaluated by Thompson *et al* (2016) as follows: Internal validity using the National Toxicology Program Office of Health Assessment and Translation (NTP OHAT); Risk of Bias tool, external validity and other quality and relevance elements (e.g., indirectness) using direction from the OHAT handbook and grading of recommendation, assessment, development and evaluation (GRADE) approach (Guyatt *et al*., 2011; NTP OHAT, 2015). Summary characterizations of hazard were generated based on validity assessments, including considerations for strengths and weaknesses, risk of bias, magnitude of effect, dose-response, and consistency. Based on such, a confidence in the body of evidence was assessed and candidate datasets identified.

reflects the SMR compared to workers of the same plants not involved in TiO2 production. The SMR compared to the general populations was below "1".

Study	External Validity (Indirectness)	Internal Validity (Risk of Bias)					
		Study participants (Q3)	*Confounding (Q4)*	*Data completeness (Q7)*	*Exposure characterization (Q8)*	*Outcome assessment (Q9)*	*Reporting (Q10)*
Boffetta et al (2001)	Low	++	++	++	-	++	++
Boffetta et al (2004)	Low	--	-	-	+	++	++
Chen et al (1988)	Low	+	+	+	+	++	+
Ellis et al (2010)	Low	--	-	+	-	++	++
Ellis et al (2013)	Low	++	-	+	+	++	+
Fryzek et al (2003)	Low	++	--	++	++	++	++
Ramanakumar et al (2008)	Low	++	++	++	-	++	++

Figure (extracted from Thompson *et al*., 2016). Internal and external validity assessment results of human TiO2 data. External validity based on the level (very low [dark green] to very high [dark red]) of indirectness. Low indirectness indicates high external validity and vice versa. Internal validity based on risk of bias (definitively low risk of bias [dark green; ++] to definitely high risk of bias [dark red–]). Low risk of bias indicates high internal validity and vice versa.

Thompson *et al*. (2016) concludes that similar to other observational epidemiological studies evaluating risk from chemical exposures, the findings of TiO2 epidemiologic studies are likely to be impacted by exposure misclassification (exposure characterization in the Figure above) and confounding factors. However, when considering all quality elements, Thompson *et al*. (2016) concluded that the data support a moderate level of confidence for the human evidence.

Summarising all the epidemiological data, taking into account the assessment of the internal and external validity of these investigations performed by Thompson *et al*. (2016) and acknowledging that all these studies have their methodological limitations, confounding factors and the level of exposure, type/size of particles as well as dose-metrics reported is debatable, applying a weight of evidence analysis, RAC considers that human data do not consistently suggest an association between occupational exposure to TiO2 and risk for lung cancer. Currently there are no epidemiological data to distinguish any potential carcinogenic effect of specific TiO2 micro and nano particle sizes and/or specific physical forms. However, one cohort study by Boffetta *et al*. (2004) deals specifically with the respirable fraction of TiO2 dust and did not observe a clear dose – response relationship between estimated exposure level and RR for lung cancer. Taking into account the general lack of epidemiological investigations on respirable fraction of TiO2 dust and indications made by Boffetta *et al*. (2004) and repeated by Hext *et al*. (2005) that the investigated TiO2 concentrations in the occupational environment are rather low (for example, in the Boffetta *et al*., 2004 study the median cumulative exposure of workers was 1.98 mg/m^3 years with interquartile range 0.26-6.88 mg/m^3 years) to cause lung cancer, RAC concluded that the epidemiological data was not sufficient to conclude on a carcinogenicity classification as the exposure data was inconclusive and that the epidemiological data could not overrule the outcome of the animal studies.

Comparison of carcinogenicity data with classification criteria

Oral and dermal carcinogenicity

Considerations on classification of TiO2 for the oral and dermal route rely on experimental animal data; for these routes of exposure human evidence is not available.

TiO2 was tested in two oral carcinogenicity studies. TiO2 forms tested were anatase with an unspecified size of primary particles and a mineral silicate covered with TiO2. Experimental animal species tested were rats and mice. The dossier submitter concluded that a carcinogenic concern for the oral route was not identified. This conclusion was not questioned during public consultation. Based on the negative oral carcinogenicity data reported in the dossier and the low oral absorption of TiO2, **RAC concludes that a classification for TiO2 for oral carcinogenicity is not warranted.**

Standard dermal carcinogenicity studies with TiO2 are not available. The dossier submitter reported on the results of five studies with a two-stage skin carcinogenesis testing protocol. The dossier submitter concluded that there is no concern for dermal carcinogenicity of TiO2. Comments during public consultation did not question this conclusion. Based on the negative dermal carcinogenicity data and the low dermal absorption of TiO2, **RAC confirms that available data do not support a classification of TiO2 for dermal carcinogenicity.**

Carcinogenicity by inhalation

Introduction

The dossier submitter proposed a Category 1B classification for TiO2 carcinogenicity by inhalation. As noted in the CLH report, the dossier submitter proposed the following CLP Annex VI entry: "Titanium dioxide in all phases and phase combinations; particles in all sizes/morphologies". Following public consultation the dossier submitter provided the following revised proposal: "Particles of titanium dioxide in all phases, phase combinations and morphologies with at least one dimension below 10 µm." Parallel to this textual definition the CLH report (Part A, chapter 1.1) referred to 3 different substance identifiers: titanium dioxide, anatase and rutile each with specific EC and CAS numbers. This classification proposal was substantially challenged in many comments received during public consultation.

The dossier submitter responded to the comments received during public consultation in the RCOM annex, in which they concluded to retain the proposal for a category 1B classification for TiO2 carcinogenicity by inhalation.

Epidemiological studies

A substance is classified into category 1A if it is known to have a carcinogenic potential in humans. Category 1A is largely based on human evidence. Category 1A requires that human studies establish a causal relationship between human exposure to a substance and the development of cancer.

Taking into account the available information and acknowledging that all these studies have their methodological limitations, RAC is of the opinion that the human data do not consistently suggest an association between occupational exposure to TiO2 and risk for lung cancer. RAC however emphasises that average respirable TiO2 dust concentrations at workplaces (Boffetta *et al*. 2004, Hext *et al*. 2005) are estimated to be at levels below 1 mg/m³ (TWA). Hence, RAC concludes that the animal carcinogenicity studies cannot be overruled by these epidemiological studies (see corresponding chapter above).

The dossier submitter considered the human data insufficient for a category 1A classification. The proposal not to classify TiO2 based on human data was not questioned in comments received during public consultation. It is the opinion of RAC that the human studies were not adequate to establish a causal relationship between exposure to TiO2 and the development of cancer. **RAC concludes that a carcinogenicity category 1A for TiO2 is not warranted.**

Experimental animal studies

RAC considered whether a carcinogenicity classification for TiO2 can be justified based on the experimental animal data available. Carcinogenicity in experimental animals can be evaluated using conventional bioassays, and other *in-vivo* bioassays that focus on one or more of the critical stages of carcinogenicity. For carcinogenicity classification, reference is made to chapter 3.6 of the Guidance on the Application of the CLP Criteria (version 4.1 / June 2015).

The tested TiO2 particles are considered to be "poorly soluble low toxicity" particles. This "grouping" is intended to set these TiO2 particles and other PSLT particles apart from other particle types, such as poorly soluble fibrous particles or poorly soluble particles with specific toxicity.

Based on the available experimental evidence for TiO2, additionally referring to selected carcinogenicity data for poorly soluble low toxicity particles as supporting evidence, RAC takes the view that the tested TiO2 particles experimentally induced lung tumours in rats under conditions of marked particle loading in the lung.

- *Carcinogenic effects in two or more animal species?*

A causal relationship between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two or more species of animals is indicative of a category 1B classification. It is the conclusion of RAC that exposure to respirable TiO2 particles resulted in treatment-related lung tumours in rats. There was no increased lung tumour incidence in a female NMRI mice study (Heinrich *et al*. 1995, but the duration of exposure of 13.5 months may have been to short. Other species have not been tested for carcinogenicity. The specific condition addressed (an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two or more animal species) is not considered to be fulfilled for TiO2. This TiO2 carcinogenicity profile corresponds to the carcinogenicity profile of other PSLT substances.

- *Neoplasms in two or more independent studies in one species?*

In case of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two or more independent studies in one species, Category 1B is indicated. The Lee *et al*. (1985) study revealed an increased incidence of benign neoplasms (bronchio-alveolar adenoma) in male and female rats at the rather high exposure level of 250 mg/m³. This exposure level was linked to cessation of alveolar clearance. RAC takes the view that this marked condition of overload should not have a determining influence on classification of TiO2.

The only TiO2 inhalation study with malignant neoplasms was the Heinrich *et al*. (1995) study. In this study nano-scaled TiO2 was tested at a single exposure level of 10 mg/m³ in female rats. The experimental exposure schedule resulted in a particle volume loading of alveolar macrophages in the region of 40% resulting in a marked, but not total cessation of alveolar clearance. Two types of malignant lung tumours were observed: adenocarcinoma and cystic keratinizing squamous cell carcinoma. Benign cystic keratinizing epitheliomas were also reported in this study. Intratracheal instillation of TiO2 resulted in increased rat lung tumour rates as well; these results are consistent with the results of the chronic rat inhalation studies.

This TiO2 carcinogenicity profile corresponds to the carcinogenicity profile of other PSLT substances (Nikula *et al*. 2000; Gebel, 2012). RAC refers to these PSLT particle carcinogenicity data as supporting evidence. Adding data from other PSLT particles, RAC considers this condition of classification (malignant neoplasms or an appropriate combination of benign and

malignant neoplasms in two or more independent studies in one species) to be fulfilled. For its final recommendation however, RAC used a weight-of-evidence approach integrating other modifying conditions and criteria.

- *Tumours in both sexes of a single species?*

Category 1B might also be indicated if there is an increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practice (GLP). In the context of the formal conditions for "sufficient evidence of carcinogenicity", it is the interpretation of RAC that the wording "increased incidence of tumours" is a short form for an "increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms". The carcinogenicity study with TiO2 nanoparticles was only performed with female rats (Heinrich *et al*. 1995); the only rat study in which both sexes were tested (Lee *et al*. 1985) did report an increased incidence of bronchio-alveolar adenoma in both sexes at the rather high exposure level of 250 mg/m³, but did not report an increased incidence of treatment-related malignant tumours. Data available for other PSLT substances (Nikula *et al*. 2000 and Gebel, 2012) generally indicate a lower sensitivity of the male rat. RAC does not in this case consider this condition for category 1B ("tumours in both sexes of a single species") to be sufficiently fulfilled.

- *Unusual degree of malignant tumours in a single study in one species and sex?*

A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites. The results in the Heinrich *et al*. (1995) rat study with an increased incidence of both adenocarcinoma and cystic keratinizing squamous cell carcinoma in the order of magnitude of 10% are not considered to fulfil this category 1B condition of an "unusual degree of malignant tumours in a single study".

- *Weight-of-evidence approach*

In the context of a preferred weight-of-evidence approach RAC discussed additional considerations for classification (chapter 3.6.2.3.2 of the Guidance on the Application of the CLP criteria). RAC considers it essential to additionally take the following factors into consideration: the overload concept, specifically the related mode of action for genotoxicity and carcinogenicity and species differences, including consideration of human relevance of experimental animal data.

- *Particle clearance and lung dust burden*

In the OECD Guidance Document 116 it is recommended not to use experimental exposure levels for particles "exceeding an elimination half-time of approximately 1 year due to lung overload at the end of the study". This recommendation however lacks a specific justification for the duration of 1 year. The draft ECHA Guidance on Nanomaterials (2016) refers to this issue as well but does not give a specific recommendation as to the level of overload that might compromise the relevance of the corresponding toxicological outcome for humans.

The justification for any specific guidance level for the maximum reduction of lung clearance in experimental testing are considered to be complex: evidence from coal miners indicate that highly exposed coal miners experience particle lung burdens that can be reached in the rat lung only under conditions of a significant degree of overloading (Kuempel *et al*. 2009 and 2014). If particle lung burden can be considered a relevant dose metric, then the experimental

testing of PSLT particles in the rat under exposure conditions which strictly avoid a significant degree of overload may not be sufficient.

RAC is of the opinion that it is generally justified that TiO2 or other PSPs are tested under overload conditions. The maximum degree of overloading of alveolar macrophages necessary remains undecided. RAC does not set aside the available rat carcinogenicity findings because rat lung tumours were only observed under exposure conditions resulting in marked overload. However, RAC acknowledges that overload conditions resulting in a complete cessation of alveolar clearance (beyond a 60% particle volume loading of alveolar macrophages) can be considered "excessive exposure" with questionable relevance for humans. This extreme degree of overload was observed in the Lee *et al*. (1985) study. However, with a particle volume loading of about 40%, there was a marked, but not complete cessation of alveolar clearance in the Heinrich *et al*. (1995) study.

Because of the complete cessation of alveolar clearance, RAC takes the view that the results of the Lee *et al*. (1985) rat study should not have a determining influence on classification of TiO2. In the context of a weight-of-evidence approach RAC recognises that the described experimental conditions for rat lung tumour development indicate that TiO2 can be considered a relatively weak rat lung carcinogen.

- *Mode of action in rats*

In rats, chronic TiO2 exposure levels associated with marked overload resulted in increased incidences of lung tumours. High TiO2 lung burdens cause a functional impairment of rat alveolar macrophages with associated impaired pulmonary clearance and provocation of chronic pulmonary inflammatory responses. The potential contribution of lung inflammatory cells to *in vivo* mutagenic responses was ascertained by co-culturing *in vivo* particle-elicited BAL cells with a rat alveolar epithelial cell line (Driscoll *et al*. 1997). Sustained alveolar inflammation can be considered the causative link to indirect genotoxicity and tumour development in the rat lung.

Based on the overall evidence available, RAC considers it plausible to assume that inflammatory reactions and reactive oxygen species play a central role in TiO2 genotoxicity and carcinogenicity.

In the opinion of RAC, the mode-of-action proposed for the rat is consistent with the assumption a practical threshold. Based on the experimental data available, rat lung tumours develop if exposure levels are associated with marked overloading of macrophages and chronic alveolar inflammation. The Guidance on the Application of the CLP Criteria (chapter 3.6.2.2.4) refers to such considerations and indicates that the assumption of a practical threshold can be viewed as decreasing the level of concern for human carcinogenicity.

- *Are different conclusions necessary for specific TiO2 materials?*

The evidence outlined in the CLH report and in this opinion do not indicate substantial differences in the toxicity profile of the tested TiO2 materials. Rat lung carcinogenicity of the two tested TiO2 materials is characterised as "particle carcinogenicity". Regarding the specific influence of the size of primary particles, nanoscale particles are generally considered to be somewhat more potent than microscale particles, although there are some indications that the difference is not very large. The integrating approach, i.e. to not split up classification for carcinogenicity by inhalation for micro- and nano-sized TiO2, is supported by RAC, the dossier submitter and by many of the comments during public consultation.

RAC is not aware of experimental data on TiO2 materials that may not be considered as PSLT particles (e.g. TiO2 particles fulfilling the WHO fibre criteria or coated TiO2 particles with specific surface toxicity) for which their toxicity profiles must be separately assessed and compared with the CLP criteria.

- *Interspecies differences and relevance to humans*

Substances which have induced benign and malignant tumours in well-performed experimental studies on animals are considered also to be presumed or suspected human carcinogens unless there is strong evidence that the mechanism of tumour formation is not relevant for humans (CLP 3.6.1.1). The Guidance defines a rather strict corresponding condition: "Only if a mode of action of tumour development is conclusively determined not to be operative in humans may the carcinogenic evidence for that tumour be discounted" (Guidance on the Application of the CLP Criteria, page 380).

As shown in subchronic studies only (there are no adequate carcinogenicity studies in animal species other than rats available) there are distinct species differences related to early steps of lung tumour development.

In this context, RAC considered the reported data on species-specificity of lung retention patterns, of site-specific development of non-neoplastic lung lesions and of lung tumour development. Specific reference is made to TiO2 data; however, the CLH report and especially comments during public consultation extensively referred to selected data on other PSLT particles as well.

Based on subchronic inhalation studies (both with nano- and microscale TiO2) rats were more sensitive than other small rodents. For both TiO2 specifications, it has been shown that at high identical respirable concentrations of TiO2 particles alveolar metaplasia and fibrosis was observed in the rat lung, but not in mice and hamsters. Particle retention patterns and pulmonary responses were different in these small rodent species (Bermudez *et al*. 2002 and 2004).

The discussion on species differences of TiO2 toxicity is supported by selected data for other PSLT particles. Species differences were not only observed between rats and other small rodents, but between rats and monkeys as well (Nikula *et al*. 1997).

There is further supporting evidence from PSLT particles as to the relative particle retention pattern in rats and humans based on comparative morphometric lung examinations (diesel exhaust soot in rat lungs versus coal dust in humans) and based on revised human lung retention models (Nikula *et al*. 2001; Kuempel *et al*. 2001; Gregoratto, 2010). Again, data indicate that retention of inhaled particles in the human lung interstitium was much more pronounced than in the rat lung interstitium. Again, these data do not justify a black-white conclusion: the evidence-based Gregoratto human dosimetry model predicts a clearance from the alveolar space to the mucociliary escalator for 60% of alveolar deposits (T1/2 = 400 days) and a clearance from the alveolar space to the interstitium for 40% of alveolar deposits (with T1/2 of 700 days).

Overall, RAC acknowledges that there is a quantitative difference in retention patterns of PSLT particles (no data for TiO2) in lung compartments of rats versus monkeys and humans. The data available document that relative retention in alveolar spaces (compared to lung

interstitium) is higher in rats than in monkeys or humans. The data however do not indicate that retention of particles in human alveolar spaces can be disregarded.

Available data indicate toxicodynamic differences as well. There are various reviews (Schultz, 1996; Green, 2007; NIOSH, 2011; ECETOC, 2013) which report on similarities and dissimilarities of non-neoplastic lung responses in experimental animals and humans.

Overall, these reviews all lack a dose-related description of human non-neoplastic lung responses. Specifically, for important aspects such as chronic inflammation in humans following coal dust exposure, contradictory statements have been published (see summary in preceding chapters).

This gap of analysis is not filled by the CLH report or the comments received during public consultation. Because of this missing link in relating the human non-neoplastic responses to human exposure levels, RAC is not in a position to finally judge the comparability of the adverse outcome pathway for non-neoplastic lesions in rats and humans.

Experimental animal testing of TiO2 resulted in lung carcinogenicity in male rats (tested only in the Lee *et al.* 1985 study) and female rats. TiO2 was not adequately tested for lung carcinogenicity in hamsters and monkeys; nor in mice. Other PSLT particles mainly resulted in lung carcinogenicity in the female rat.

The human relevance of observed types of rat lung tumours is the subject of on-going discussion. TiO2 (and other PSLT particles) essentially resulted in two types of lung tumours in the rat: (1) cystic keratinizing epitheliomas (benign) or cystic keratinizing squamous cell carcinomas and (2) bronchiolo-alveolar adenoma or adenocarcinoma.

The cystic keratinizing lesions tend to occur late in these studies, rarely before 20 months of exposure. These lesions are generally found in female rats under overload conditions; these lesions are uncommon in male rats. RAC acknowledges that the cystic keratinizing lesions can be considered unique to the rat; a corresponding type of lesion is not known in humans. However, bronchio-alveolar adenoma or adenocarcinoma are well-known in humans; based on the scarce data available, RAC does not see the evidence to judge this type of tumour observed in the rat as irrelevant to humans.

RAC acknowledges that TiO2 epidemiology does not consistently provide evidence of an association of workers' exposure to TiO2 and increased incidences of lung cancer. However, average long-term exposure to workers in the study of Boffetta *et al. (*2004) was relatively low (below 0.7 mg/m³; with reference to Hext *et al*. 2005). In association with the given rat lung cancer potency of TiO2 and the species differences already known, RAC is of the opinion that a possible carcinogenic potential in humans cannot easily be recognised by the usual epidemiology studies.

Some authors (e.g. Nikula *et al*. 2001; ECETOC, 2013; Warheit *et al*. 2015) propose to assume different adverse outcome pathways for lung tumour development in rats and humans:

In the rat, inhaled particles are predominantly retained in the alveolar duct compartments. Rat macrophages are considered to be more sensitive to overload conditions than macrophages in other species, resulting in a more pronounced degree of inflammation in alveolar spaces. Inflammation in the alveolar lumen is considered to be the cause for indirect mutagenicity in epithelial lung cells. Lung tumours (at least the keratinizing cysts and

corresponding squamous cell carcinoma) are considered to originate in the alveolar lung compartment.

For humans, the same authors propose a greater tendency for transmigration of alveolar particle deposits to interstitial sites of the lung. A relatively lower particle load in the human alveolar spaces is assumed to result in a lower extent of alveolar inflammatory responses. Warheit *et al*. (2016) propose to assume that interstitialization of particles could be considered to serve as a repository in humans which is deemed to be less reactive as to tumour development. They presume that human cells in closest contact with the particles and macrophages in the interstitium are mesenchymal cells rather than epithelial cells. In this context, the authors refer to coal workers who may develop interstitial progressive fibrosis, but are possibly at less risk of developing pulmonary tumours.

These complex data on interspecies differences need to be thoroughly taken into account in an overall assessment on the possible human potential of TiO2-related lung tumour development (see "overall conclusion" below).

- *Intrinsic properties*

Comments during public consultation generally questioned the adequacy of any classification of TiO2. These comments noted that TiO2 toxicity is particle toxicity, that the adverse effects observed do occur irrespective of the chemical composition of the substance and thus are not to be considered intrinsic properties. The comments emphasised that classification of TiO2 would imply that any insoluble solid matter thus would require a classification for carcinogenicity.

RAC acknowledges that the TiO2 inhalation toxicity observed in rats is particle toxicity and accepts the general understanding that the development of rat lung tumours is mediated by the pathological consequences of a higher loading of macrophages with particles of rather low solubility. The deposited particles, but not solutes of TiO2 molecules, can be assumed to be responsible for the observed toxicity. RAC acknowledges as well that the carcinogenicity profile described for TiO2 is not exclusively characteristic for TiO2 but applies to the whole group of chemicals referred to as "poorly soluble low toxicity particles".

The CLP regulation requires a classification to be based on the intrinsic properties of substances. The CLP Guidance defines the intrinsic property of a substance as the basic properties of a substance as determined in standard tests or by other means designed to identify hazards. RAC considers the toxicity profile observed as a basic property of inhaled and respirable particles of TiO2. With reference to the CLP definition of intrinsic properties, RAC considers that the CLP regulation regards the properties of TiO2 or other substances which are PSLT particles as relevant for classification.

Overall conclusion

Following a weight-of-evidence approach,

- taking note that TiO2 was not shown to be a multisite carcinogen,
- being aware that TiO2 is a lung carcinogen especially in female rats,
- recognising that there are no robust carcinogenicity studies in species other than rats,
- recognising that the majority of rat lung tumours occurred late in life,
- recognising that rat lung tumours only developed under inhalation exposure conditions associated with marked particle loading of macrophages,

- presuming a practical threshold for lung tumour development (mutagenicity in lung cells is considered to depend on chronic inflammation and oxidative stress),
- taking note of experimental, mainly repeated dose toxicity data indicating a lower sensitivity of other small rodents, monkeys and humans compared to rats,
- being aware of TiO2 epidemiology studies which do not consistently suggest an association between occupational exposure to TiO2 and lung cancer mortality

RAC takes the view that the experimental and human evidence does not support titanium dioxide to be classified as Carc. 1A or 1B.

RAC also considered whether TiO2 fulfills the classification criteria for category 2 for carcinogenicity or whether no classification for carcinogenicity is more appropriate. Balancing the reasons for category 2 or no classification, RAC looked closely at the experimental conditions in the rat inhalation studies and at interspecies differences.

The experimental schedule of the Lee *et al*. (1985) study resulted in a complete cessation of alveolar clearance already at the non-carcinogenic exposure level of 50 mg/m³. Alveolar clearance half-times measured in different studies at the exposure level of 250 mg/m³ reached and exceeded 1 year. RAC takes the view, that these exposure conditions represent excessive exposure which invalidates the results of the Lee *et al*. (1985) study on their own for classification purposes. The exposure schedule of the Heinrich *et al*. (1995) study deviated from standard protocols (18 hours exposure/day), but because of the relatively low exposure level tested (10 mg/m³) the degree of particle loading was substantially lower compared to the Lee *et al*. (1985) study (particle volume loading in the Heinrich *et al*. (1995) study did not yet result in a complete cessation of alveolar clearance). The Heinrich *et al*. (1995) study resulted in an excess incidence of lung adenocarcinomas (and benign and malignant cystic keratinizing lesions). Although not performed according to standard testing guidelines, the results of this study are considered reliable and relevant and consistent with rat inhalation carcinogenicity findings of other PSLT substances (Gebel, 2012). Evidence from coal miners indicates that highly exposed workers experience particle lung burdens that can be reached in the rat lung only under conditions of a marked degree of overloading (Kuempel *et al*. 2009 and 2014). These considerations moved RAC to consider TiO2 as a rat lung carcinogen under marked, not yet excessive conditions of particle loading of lung macrophages.

Arriving at a conclusion also involves making a judgement on whether the available information on interspecies differences is already sufficient reasoning for considering TiO2 particles not being operative in humans at all. In this respect RAC specifically points out that:

- the dosimetry-related data and models available document a higher particle sequestration to the human lung interstitium, but do not exclude and in fact still reveal a significant degree of particle retention in human alveolar spaces as well,
- the evidence presented indicates a lower sensitivity of non-human primates and humans to PSLT induced lung inflammation (including alveolar inflammation), but does not sufficiently document a quantitative dose response relationship of alveolar inflammation in humans,
- there is no convincing scientific evidence to question the human relevance of observed rat lung adenocarcinomas,
- the epidemiological studies, although not consistently suggesting associations between occupational exposure to TiO2 and lung cancer mortality, do not allow this to be interpreted as the absence of a human hazard.

According to the CLH guidance, carcinogenic evidence can only be discounted if the mode of action of tumour development is conclusively determined not to be operative in humans. RAC holds the view that a sufficiently detailed and specific adverse outcome pathway for humans is not yet available. In the opinion of RAC the experimental and human evidence currently available supports a lower human sensitivity but does not conclusively exclude a carcinogenic potential or hazard of TiO2 in humans.

Based on the lines of evidence outlined in this opinion document and summarised in this overall conclusion, RAC concludes that TiO2 warrants a Category 2 classification for carcinogenicity. In the context of drafting the Annex VI entry for TiO2 RAC considers it essential to take note of the following:

RAC acknowledges that the mode of action for the rat lung carcinogenicity in rats can not be considered "intrinsic toxicity" in a classical sense: the deposited particles, but not solutes of TiO2 molecules can be assumed to be responsible for the observed toxicity. Nevertheless, this mode of action results in relevant toxicity and carcinogenicity which in principle merits consideration in classification and labelling. The CLP regulation does not exclude a health hazard classification triggered by physico-chemical characteristics of a chemical.

Generally, classification for carcinogenicity does not specify a route of exposure. However, the profile of lung carcinogenicity described for TiO2 is specifically linked to the inhalation route of application. Currently, there is no experimental evidence for TiO2 carcinogenicity for the oral or dermal route of application. TiO2 lung carcinogenicity is associated with inhalation of respirable TiO2 particles. Based on the data available today RAC considers it conclusively proven that no other route of exposure causes the carcinogenicity hazard. Correspondingly, RAC proposes to classify TiO2 as a Category 2 carcinogen, with the hazard statement H351 (inhalation).

Titanium dioxide tested for carcinogenicity by inhalation comprised samples of non-fibrous shape, differing crystal structures and particle sizes (including both nano- and microscale primary particles) and no or minor toxicologically relevant surface treatment. The toxicity profile determined designates the titanium dioxide tested as a "poorly soluble low toxicity" particle. The toxicity profile of fibrous titanium dioxide with WHO fibre characteristics (which has not been tested) is considered substantially different in terms of specific mode of action and cancer potency. Specifications of titanium dioxide with surface coatings resulting in a mode of action which is not any longer defined by the basic "granular particle toxicity" but by additional specific chemical toxicity, are also not covered by the toxicity profile of the tested titanium dioxide substances.

The carcinogenicity profile observed thus is specifically related to exposure to respirable TiO2 particles with different crystal structures and different primary particle sizes, but which do not possess WHO fibre characteristics or additional specific surface toxicity because of coating of the TiO2 particles.

RAC considered various options for the Annex VI entry of TiO2. The dossier submitter proposed a TiO2 Annex VI entry specifying the CAS number 13463-67-7 combined with the supplementary physico-chemical description "Titanium dioxide in all phases and phase combinations; particles in all sizes/morphologies". In the "attachment to the responses to comments" the DS refined the proposed scope as "particles of titanium dioxide in all phases, phase combinations and morphologies with at least one dimension below 10 µm". These

proposed substance identity descriptions include TiO2 with WHO fibre characteristics in the definition of the TiO2 entry.

With such a supplementary physico-chemical description the Annex VI entry would not be adequately based on the hazard assessment of the specific TiO2 materials referred to in this dossier; furthermore the corresponding Annex VI entry runs the risk of incorrect classification for forms of TiO2 with WHO fibre characteristics (and possibly of TiO2 with surface coatings introducing specific chemical toxicity as well).

To ensure that all relevant scientific and regulatory aspects are taken into account RAC proposes the following scope of an entry in Annex VI of CLP: "Titanium dioxide" (without a further physico-chemical description) is proposed to be used as chemical name (international chemical identification). The CAS number to be used is 13463-67-7. In addition to the classification (category 2 carcinogen including the hazard statement H351 (inhalation)) RAC proposes the following "Note": "***If the substance is placed on the market as particles of the substance fulfilling the WHO fibre criteria or as particles with surface coating their hazardous properties must be evaluated in accordance with CLP Title II to assess whether a higher category (Carc. 1B or 1A) and/or additional routes of exposure (oral or dermal) should be applied.***" The classification is based solely on the hazardous properties of the substance. It does not take into account the likelihood of exposure to the substance and therefore does not address the risks of exposure.

RAC acknowledges that the carcinogenicity profile described for TiO2 is not exclusively characteristic for TiO2 but applies to a group of chemicals with similar toxicity profile addressed as "poorly soluble low toxicity particles". RAC is aware that the CLH report and this RAC Opinion concentrates on TiO2 data and do not fully consider the data for other PSLT substances.

5 ENVIRONMENTAL HAZARD ASSESSMENT OTHER INFORMATION

Not assessed.

6 OTHER INFORMATION

7 REFERENCES

Adachi S, Kawamura K, Takemoto K. A trial on the quantitative risk assessment of man-made mineral fibers by the rat intraperitoneal administration assay using the JFM standard fibrous samples. Ind Health. 2001 Apr;39(2):168-74.

Baan RA. Carcinogenic Hazards from Inhaled Carbon Black, Titanium Dioxide, and Talc not Containing Asbestos or Asbestiform Fibers: Recent Evaluations by an IARC Monographs Working Group. Inhal Toxicol. 2007; 19 Suppl 1:213-28.

Baggs R.B, Ferin J, Oberdörster G. Regression of pulmonary lesions produced by inhaled titanium dioxide in rats. Vet Pathol 1997; 34(6): 592-7.

Barillet S., Simon-Deckers A., Herlin-Boime N., Mayne-L'Hermite M., Reynaud C., Cassio D., Gouget B., Carrière M. Toxicological consequences of TiO_2, SiC nanoparticles and multi-walled carbon nanotubes exposure in several mammalian cell types: an in vitro study. J Nanopart Res. 2010; 12(1): 61-73.

Becker H, Herzberg F, Schulte A, Kolossa-Gehring M. The carcinogenic potential of nanomaterials, their release from products and options for regulating them. Int J Hyg Environ Health. 2011 Jun; 214(3):231-8.

Bermudez E, Mangum J.B, Asgharian B, Wrong B. A, Reverdy E.E, Janszen D.B, Hext P.M, Warheit D.B, Everitt J.I. Long-term pulmonary responses of three laboratory rodent species to subchronic inhalation of pigmentary titanium dioxide particles. Toxicological Sciences. 2002; 70(1): 86-97.

Bermudez E, Mangum J.B, Wrong B, Asgharian A B, Hext P.M, Warheit D.B, Everitt J.I. Pulmonary responses of mice, rats and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicological Sciences. 2004; 77(2): 347-57.

Bernard BK, Osheroff MR, Hofmann A, Mennear JH. Toxicology and carcinogenesis studies of dietary titanium dioxide-coated mica in male and female Fischer 344 rats. J Toxicol Environ Health. 1990; 29(4):417-29.

Bischoff F, Bryson G. Tissue reaction to and fate of parenterally administered titanium dioxide. I. The intraperitoneal site in male Marsh-Buffalo mice. Res Commun Chem Pathol Pharmacol. 1982 Nov;38(2):279-90.

Boffetta P, Gaborieau V, Nadon L, Parent MF, Weiderpass E, Siemiatycki J. Exposure to titanium dioxide and risk of lung cancer in a population-based study from Montreal. Scand J Work Environ Health. 2001 Aug; 27(4):227-32.

Boffetta P, Soutar A, Cherrie JW, Granath F, Andersen A, Anttila A, Blettner M, Gaborieau V, Klug SJ, Langard S, Luce D, Merletti F, Miller B, Mirabelli D, Pukkala E, Adami HO, Weiderpass E. Mortality among workers employed in the titanium dioxide production industry in Europe. Cancer Causes Control. 2004 Sep; 15(7):697-706.

Boisen AM, Shipley T, Jackson P, Hougaard KS, Wallin H, Yauk CL, Vogel U. NanoTiO(2) (UV-Titan) does not induce ESTR mutations in the germline of prenatally exposed female mice. Part Fibre Toxicol. 2012 Jun 1;9:19.

Bolis V, Busco C, Ciarletta M, Distasi C, Erriquez J, Fenoglio I, Livraghi S, Morel S. Hydrophilic/hydrophobic features of TiO2 nanoparticles as a function of crystal phase, surface area and coating, in relation to their potential toxicity in peripheral nervous system. J Colloid Interface Sci. 2012 Mar 1;369(1):28-39.

Bonner JC, Silva RM, Taylor AJ, Brown JM, Hilderbrand SC, Castranova V,4 Porter D, Elder A, Oberdörster G,. Harkema JR, Bramble LA, Kavanagh TJ, Botta D, Nel A, Pinkerton KE. Interlaboratory Evaluation of Rodent Pulmonary Responses to Engineered Nanomaterials: The NIEHS Nano GO Consortium. Environmental Health Perspectives. 2013 Jun; 121(6): 676-82

Boorman GA, Brockmann M, Carlton WW, Davis JM, Dungworth DL, Hahn FF, Mohr U, Reichhelm HB, Turusov VS, Wagner BM. Classification of cystic keratinizing squamous lesions of the rat lung: report of a workshop. Toxicol Pathol. 1996 Sep-Oct; 24(5):564-72.

Borm, P.J.A., Schins, R.P.F., Albrecht, C.. Inhaled particles and lung cancer. Part B: Paradigms and risk assessment. Int. J. Cancer. 2004 May 20; 110(1): 3–14.

Botelho MC, Costa C, Silva S, Costa S, Dhawan A, Oliveira PA, Teixeira JP. Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro. Biomed Pharmacother. 2014 Feb; 68(1):59-64.

Browning CL, The T, Mason MD, Wise JP.Titanium Dioxide Nanoparticles are not Cytotoxic or Clastogenic in Human Skin Cells. Environ Anal Toxicol 2014, 4:6.

Brun E, Barreau F, Veronesi G, Fayard B, Sorieul S, Chanéac C, Carapito C, Rabilloud T, Mabondzo A, Herlin-Boime N, Carrière M. Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia.Part Fibre Toxicol. 2014 Mar 25;11:13.

Carlton WW. "Proliferative keratin cyst," a lesion in the lungs of rats following chronic exposure to para-aramid fibrils. Fundam Appl Toxicol. 1994 Aug; 23(2):304-7.

Carmona ER, Escobar B, Vales G, Marcos R. Genotoxic testing of titanium dioxide anatase nanoparticles using the wing-spot test and the comet assay in Drosophila. Mutat Res Genet Toxicol Environ Mutagen. 2015 Jan 15;778:12-21

Catalán J, Järventaus H, Vippola M, Savolainen K, Norppa H. Induction of chromosomal aberrations by carbon nanotubes and titanium dioxide nanoparticles in human lymphocytes *in vitro*. Nanotoxicology 2012 Dec; 6:825-36.

Chen JL, Fayerweather WE. Epidemiologic study of workers exposed to titanium dioxide. J Occup Med. 1988 Dec; 30(12):937-42

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J, Jia G. Genotoxic evaluation of titanium dioxide nanoparticles *in vivo* and *in vitro*. Toxicol Lett. 2014; 226(3):314-9.

Chen T, Yan J, Li Y. Review Genotoxicity of titanium dioxide nanoparticles. J Food Drug Anal. 2014;22(1):95-104.

Chen HW, Su SF, Chien CT, Lin WH, Yu SL, Chou CC, Chen JJ, Yang PC. Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. FASEB J. 2006 Nov; 20(13):2393-5.

Cho WS, Kang BC, Lee JK, Jeong J, Che JH, Seok SH. Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. Part Fibre Toxicol. 2013 Mar 26;10:9.

Corradi S, Gonzalez L, Thomassen LC, Bilaničová D, Birkedal RK, Pojana G, Marcomini A, Jensen KA, Leyns L, Kirsch-Volders M. Influence of serum on *in situ* proliferation and genotoxicity in A549 human lung cells exposed to nanomaterials. Mutat Res. 2012 Jun 14; 745(1-2):21-7.

Creutzenberg O, Pohlmann G, Hansen T, Rittinghausen S, Taugner F, Ziemann C. Nano- and microscaled titanium dioxide: Comparative study on the inflammatory and genotoxic effects after a 3-week inhalation in rats. Toxicology Letters 2009, 189, Supplement Abstracts of the 46th Congress of the European Societies of Toxicology, Pages S182.

Cui Y, Chen X, Zhou Z, Lei Y, Ma M, Cao R, Sun T, Xu J, Huo M, Cao R, Wen C, Che Y. Prenatal exposure to nanoparticulate titanium dioxide enhances depressive-like behaviors in adult rats. Chemosphere. 2014 Feb; 96:99-104.

Demir E, Akça H, Turna F, Aksakal S, Burgucu D, Kaya B, Tokgün O, Vales G, Creus A, Marcos R. Genotoxic and cell-transforming effects of titanium dioxide nanoparticles. Environ Res. 2015 Jan; 136:300-8.

Demir E, Turna F, Vales G, Kaya B, Creus A, Marcos R. In vivo genotoxicity assessment of titanium, zirconium and aluminium nanoparticles, and their microparticulated forms, in Drosophila. Chemosphere. 2013;93(10):2304-10.

Demir E, Burgucu D, Turna F, Aksakal S, Kaya B. Determination of TiO_2, ZrO_2, and Al_2O_3 nanoparticles on genotoxic responses in human peripheral blood lymphocytes and cultured embryonic kidney cells. J Toxicol Environ Health A. 2013; 76(16):990-1002.

Di Virgilio AL, Reigosa M, Arnal PM, Fernández Lorenzo de Mele M. Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. J Hazard Mater. 2010 May 15; 177(1-3):711-8.

Doak SH, Manshian B, Jenkins GJ, Singh N. *In vitro* genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutat Res. 2012; 745(1-2):104-11.

Dobrzyńska MM, Gajowik A, Radzikowska J, Lankoff A, Dušinská M, Kruszewski M. Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats *in vivo*. Toxicology. 2014 Jan 6 ;315:86-91.

Donaldson K, Tran CL. Inflammation caused by particles and fibers. 2002; Inhal Toxicol 14: 5–27

Driscoll KE, Carter JM, Howard BW, Hassenbein DG, Pepelko W, Baggs RB, Oberdörster G (1996) Pulmonary inflammatory, chemokine, and mutagenic responses in rats after subchronic inhalation of carbon black. Toxicol Appl Pharmacol 136: 372–380

Driscoll KE, Deyo LC, Carter JM, Howard BW, Hassenbein DG, Bertram TA. Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. Carcinogenesis 1997 Feb; 18(2):423–30.

ECHA website – registration data (07/08/2015) - http://apps.echa.europa.eu/registered/data/dossiers/DISS-9eaff323-014a-482f-e044-00144f67d031/DISS-9eaff323-014a-482f-e044-00144f67d031_DISS-9eaff323-014a-482f-e044-00144f67d031.html

El-Ghor A, Noshy MM, Galal A, Mohamed HR. Normalization of nano-sized TiO_2-induced clastogenicity, genotoxicity and mutagenicity by chlorophyllin administration in mice brain, liver, and bone marrow cells. Toxicol Sci. 2014 Nov; 142(1):21-32.

Everitt JI, Mangum JB, Bermudez E, Wong BA, Asgharian B, Reverdy EE. Comparison of selected pulmonary responses of rats, mice and Syrian Golden hamsters to inhaled pigmentary titanium dioxide. Inhalation Toxicology. 2000; 12 (supplement 3): 275-82.

Farcal L, Torres Andón F, Di Cristo L, Rotoli BM, Bussolati O, Bergamaschi E, Mech A, Hartmann NB, Rasmussen K, Riego-Sintes J, Ponti J, Kinsner-Ovaskainen A, Rossi F, Oomen A, Bos P, Chen R, Bai R, Chen C, Rocks L, Fulton N, Ross B, Hutchison G, Tran L, Mues S, Ossig R, Schnekenburger J, Campagnolo L, Vecchione L, Pietroiusti A, Fadeel. Comprehensive In Vitro Toxicity Testing of a Panel of Representative Oxide Nanomaterials: First Steps towards an Intelligent Testing Strategy. PLoS One. 2015 May 21;10(5):e0127174.

Ferin, J., Oberdorster, G., Penney, D.P., 1992. Pulmonary retention of ultrafine and fine particles in rats. Am. J. Respir. Cell Mol. Biol. 1992 May; 6(5): 535–42.

Fryzek JP, Chadda B, Marano D, White K, Schweitzer S, McLaughlin JK, Blot WJ. A cohort mortality study among titanium dioxide manufacturing workers in the United States. J Occup Environ Med. 2003 Apr; 45(4):400-9.

Furukawa F, Doi Y, Suguro M, Morita O , Kuwahara H, Masunaga T, Hatakeyama Y, Mori F. Lack of skin carcinogenicity of topically applied titanium dioxide nanoparticles in the mouse. Food and Chemical Toxicology. 2011 Apr; 49(4):744–9.

Gao G, Ze Y, Li B, Zhao X, Zhang T, Sheng L, Hu R, Gui S, Sang X, Sun Q, Cheng J, Cheng Z, Wang L, Tang M, Hong F. Ovarian dysfunction and gene-expressed characteristics of female mice caused by long-term exposure to titanium dioxide nanoparticles. J Hazard Mater. 2012 Dec; 243:19-27.

Gallagher J, Heinrich U, George M, Hendee L, Phillips DH, Lewtas J. Formation of DNA adducts in rat lung following chronic inhalation of diesel emissions, carbon black and titanium dioxide particles. Carcinogenesis. 1994; 15(7):1291-9.

Gerloff K, Fenoglio I, Carella E, Kolling J, Albrecht C, Boots AW, Förster I, Schins RP. Distinctive toxicity of TiO_2 rutile/anatase mixed phase nanoparticles on Caco-2 cells. Chem Res Toxicol. 2012; 25(3):646-55.

German MAK Commission. Titanium dioxide (respirable fraction). The MAK collection for Occupational Health and Safety. 2014 Apr 16. DOI: 10.1002/3527600418.mb1346367e4714

Ghosh M, Chakraborty A, Mukherjee A. Cytotoxic, genotoxic and the hemolytic effect of titanium dioxide (TiO_2) nanoparticles on human erythrocyte and lymphocyte cells *in vitro*. J Appl Toxicol. 2013 Oct; 33(10):1097-110.

Green FH (2000) Pulmonary responses to inhaled poorly soluble particulate in the human. Inhal Toxicol 12: 59–95

Gui S, Sang X, Zheng L, Ze Y, Zhao X, Sheng L, Sun Q, Cheng Z, Cheng J, Hu R, Wang L, Hong F, Tang M. Intragastric exposure to titanium dioxide nanoparticles induced nephrotoxicity in mice, assessed by physiological and gene expression modifications. Part Fibre Toxicol. 2013 Feb 13;10:4.

Guichard Y, Schmit J, Darne C, Gaté L, Goutet M, Rousset D, Rastoix O, Wrobel R, Witschger O, Martin A, Fierro V, Binet S. Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in Syrian hamster embryo cells. Ann Occup Hyg. 2012; 56(5):631-44.

Hackenberg S, Friehs G, Froelich K, Ginzkey C, Koehler C, Scherzed A, Burghartz M, Hagen R, Kleinsasser N. Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells. Toxicol Lett. 2010 May 19;195(1):9-14.

Hackenberg S, Friehs G, Kessler M, Froelich K,Ginzkey C, Koehler C, Scherzed A, Burghartz M, Kleinsasser N. Nanosized Titanium Dioxide Particles do not Induce DNA Damage in Human Peripheral Blood Lymphocytes Environmental and Molecular Mutagenesis. 2011; 52:264-268.

Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard K, Vogel U, Yauk CL, Wallin H. Pulmonary response to Surface-coated nanotitanium Dioxide particles induction of acute phase response Genes, Inflammatory cascades and Changes in MicroRNAs: a toxicogenomic Study. Env and MOl Mutagenesis. 2001; 52:425-439. Hamzeh M, Sunahara GI. *In vitro* cytotoxicity and genotoxicity studies of titanium dioxide (TiO_2) nanoparticles in Chinese hamster lung fibroblast cells. Toxicol In Vitro. 2013 Mar; 27(2):864-73.

Heinrich U, Fuhst R, Rittinghausen S, Creutzenberg O, Bellmann B, Koch W, Levsen K. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inhalation Toxicology. 1995; 7(4):533-56.

Hext PM, Tomenson JA, Thompson P. Titanium dioxide: inhalation toxicology and epidemiology. Ann Occup Hyg. 2005 Aug; 49(6):461-72.

Höhr D, Steinfartz Y; Schins RPF, Knaapen AM, Martra G, Fubini B, Borm PJA. The surface area rather than the surface coating determines the acute inflammatory response after instillation of fine and ultrafine TiO2 in the rat Int. J. Hyg. Environ. Health 205, 239 ± 244 (2002)

IARC (International Agency for Research on Cancer), "Titanium dioxide group 2B," in IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 9, International Agency for Research on Cancer, World Health Organization, Lyon, France, 2006.

IARC (International Agency for Research on Cancer), "Carbon black, titanium dioxide, and talc," in IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 93, International Agency for Research France, 2010.

IARC (International Agency for Research on Cancer), "Diesel and Gasoline Engine Exhausts and some nitroarenes" in IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 105, International Agency for Research France, 2012.ILSI (International Life Sciences Institute) (2000) The relevance of the rat lung response to particle overload for human risk assessment: a workshop consensus report. In: Gardner DE (Ed.) ILSI Risk Science Institute Workshop: the relevance of the rat lung response to particle overload for human risk assessment. March 1998. Inhal Toxicol 12: 1–17

INRS 2013, fiche toxicologique n°291.

Jackson P, Halappanavar S, Hougaard KS, Williams A, Madsen AM, Lamson JS, Andersen O, Yauk C, Wallin H, Vogel U. Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan) in C57BL/6 mice: effects in prenatally exposed offspring on hepatic DNA damage and gene expression. Nanotoxicology. 2013;7(1):85-96.

Jaeger A, Weiss DG, Jonas L, Kriehuber R. Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes. Toxicology. 2012 Jun 14; 296(1-3):27-36.

Jin C, Tang Y, Fan XY, Ye XT, Li XL, Tang K, Zhang YF, Li AG, Yang YJ. *In vivo* evaluation of the interaction between titanium dioxide nanoparticle and rat liver DNA. Toxicol Ind Health. 2013; 29(3):235-44.

Jin C, Tang Y, Yang FG, Li XL, Xu S, Fan XY, Huang YY, Yang YJ. Cellular toxicity of TiO_2 nanoparticles in anatase and rutile crystal phase. Biol Trace Elem Res. 2011; 141(1-3):3-15.

Jomini S, Labille J, Bauda P, Pagnout C. Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO(2) nanoparticles and byproducts from a sunscreen TiO(2)-based nanocomposite. Toxicol Lett. 2012 Nov 23; 215(1):54-61.

Jones K, Morton J , Smith I, Jurkschat K, Harding AH, Evans G. Human *in vivo* and *in vitro* studies on gastrointestinal absorption of titanium dioxide nanoparticles. Toxicology Letters 233. 2015; 95–101.

Jovanović B.Critical review of public health regulations of titanium dioxide, a human food additive. Integr Environ Assess Manag. 2015 Jan;11(1):10-20.

Jugan ML, Barillet S, Simon-Deckers A, Herlin-Boime N, Sauvaigo S, Douki T, Carriere M. Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. Nanotoxicology. 2012 Aug; 6(5):501-13.

Jugan ML, Barillet S, Simon-Deckers A, Sauvaigo S, Douki T, Herlin N, Carrière M. Cytotoxic and genotoxic impact of TiO_2 nanoparticles on A549 cells. J Biomed Nanotechnol. 2011 Feb; 7(1):22-3.

Kain J, Karlsson HL, Möller L. DNA damage induced by micro- and nanoparticles--interaction with FPG influences the detection of DNA oxidation in the comet assay. Mutagenesis. 2012; 27(4):491-500.

Kansara K, Patel P, Shah D, Shukla RK, Singh S, Kumar A, Dhawan A. TiO_2 nanoparticles induce DNA double strand breaks and cell cycle arrest in human alveolar cells. Environ Mol Mutagen. 2015 Mar; 56(2):204-17.

Karlsson HL. The comet assay in nanotoxicology research. Anal Bioanal Chem. 2010; 398(2):651-66.

Karlsson HL, Di Bucchianico S, Collins AR, Dusinska M. Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environ Mol Mutagen. 2015 Mar; 56(2):82-96.

Kermanizadeh A, Vranic S, Boland S, Moreau K, Baeza-Squiban A, Gaiser BK, Andrzejczuk LA, Stone V. An *in vitro* assessment of panel of engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. BMC Nephrol. 2013; 14:96.

Kermanizadeh A, Løhr M, Roursgaard M, Messner S, Gunness P, Kelm JM, Møller P, Stone V, Loft S. Hepatic toxicology following single and multiple exposure of engineered nanomaterials utilising a novel primary human 3D liver microtissue model. Part Fibre Toxicol. 2014; 11(1):56.

Kermanizadeh A, Gaiser BK, Hutchison GR, Stone V. An *in vitro* liver model--assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanomaterials. Part Fibre Toxicol. 2012 Jul 19;9:28.

Kittel B, Ernst H, Dungworth DL, Rittinghausen S, Nolte T, Kamino K, Stuart B, Lake SG, Cardesa A, Morawietz G, et al. Morphological comparison between benign keratinizing cystic squamous cell tumours of the lung and squamous lesions of the skin in rats. Exp Toxicol Pathol. 1993 Oct; 45(5-6):257-67.

Knaapen AM, Seiler F, Schilderman PA, Nehls P, Bruch J, Schins RP, Borm PJ (1999) Neutrophils cause oxidative DNA damage in alveolar epithelial cells. Free Radic Biol Med 27: 234–240

Knaapen AM, Borm PJ, Albrecht C, Schins RP (2004) Inhaled particles and lung cancer. Part A: mechanisms. Int J Cancer 109: 799–809

Knaapen AM, Güngor N, Schins RP, Borm PJ, van Schooten FJ (2006) Neutrophils and respiratory tract DNA damage and mutagenesis: a review. Mutagenesis 21: 225–236

Koizumi A, Tsukada M, Hirano S, Kamiyama S, Masuda H, Suzuki KT. Energy restriction that inhibits cellular proliferation by torpor can decrease susceptibility to spontaneous and asbestos-induced lung tumors in A/J mice. Lab Invest. 1993 Jun; 68(6):728-39.

Landsiedel R, Ma-Hock L, Van Ravenzwaay B, Schulz M, Wiench K, Champ S, Schulte S, Wohlleben W, Oesch F. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology. 2010 Dec; 4:364-81.

Landsiedel R, Ma-Hock L, Hofmann T, Wiemann M, Strauss V, Treumann S, Wohlleben W, Gröters S, Wiench K, van Ravenzwaay B. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Part Fibre Toxicol. 2014 Apr 4;11:16. doi: 10.1186/1743-8977-11-16.

Lee K.P, Trochimowicz H.J, Reinhardt C.F. Transmigration of titanium dioxide (TiO_2) particles in rats after inhalation exposure. Experimental and molecular pathology. 1985 Jun; 42(3): 331-43.

Lee K.P, Norman W. Henry III, Trochimowicz H.J, Reinhardt C.F. Pulmonary response to impaired lung clearance in rats following excessive TiO_2 dust deposition. Environmental Research. 1986 Oct; 41(1): 144-167.

Lee K.P, Trochimowicz H.J, Reinhardt C.F. Pulmonary response of rats exposed to titanium dioxide (TiO_2) by inhalation for two years. Toxicology and applied pharmacology. 1985 Jun 30; 79(2): 179-92.

Lehnert BE (1993) Defense mechanisms against inhaled particles and associated particle-cell interactions. Rev Mineral 28: 427–469.

Leppänen M, Korpi A, Mikkonen S, Yli-Pirilä P, Lehto M, Pylkkänen L, Wolff H, Kosma VM, Alenius H, Joutsensaari J, Pasanen P. Inhaled silica-coated TiO2 nanoparticles induced airway irritation, airflow limitation and inflammation in mice. Nanotoxicology. 2015 Mar;9(2):210-8.

Li N, Ma LL, Wang J, Zheng L, Liu J, Duan YM, et al. Interaction between nano-anatase TiO_2 and liver DNA from mice *in vivo*. Nanoscale Res Lett. 2009 Oct 13; 5(1): 108-15.

Li B, Ze Y, Sun Q, Zhang T, Sang X, Cui Y, Wang X, Gui S, Tan D, Zhu M, Zhao X, Sheng L, Wang L, Hong F, Tang M. Molecular mechanisms of nanosized titanium dioxide-induced pulmonary injury in mice. PLoS One. 2013; 8(2):e55563.

Lindberg HK, Falck GC, Catalán J, Koivisto AJ, Suhonen S, Järventaus H, Rossi EM, Nykäsenoja H, Peltonen Y, Moreno C, Alenius H, Tuomi T, Savolainen KM, Norppa H. Genotoxicity of inhaled nanosized TiO(2) in mice. Mutat Res. 2012; 745(1-2):58-64.

Louro H, Tavares A, Vital N, Costa PM, Alverca E, Zwart E, de Jong WH, Fessard V, Lavinha J, Silva MJ. Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using LacZ plasmid-based transgenic mice. Environ Mol Mutagen. 2014; 55(6):500-9.

MacNicoll A, Kelly M, Aksoy H, Kramer E, Bouwmeester H, Chaudhry Q. A study of the uptake and biodistribution of nano-titanium dioxide using in vitro and in vivo models of oral intake. J Nanopart Res. 2015; 17:66.

Magdolenova Z, Bilaničová D, Pojana G, Fjellsbø LM, Hudecova A, Hasplova K, Marcomini A, Dusinska M. Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. J Environ Monit. 2012; 14(2):455-64.

Mohr U, Ernst H, Roller M, Pott F. Pulmonary tumor types induced in Wistar rats of the so-called "19-dust study". Experimental and toxicologic pathology 58 (2006) 13-20.

MAK Collection Part I, Mak value documentaions 2014, DFG. General threshold limit value for dust (R fraction) (biopersistent granular dusts). 2014 Wiley-VCH Verlag GmbH & Co. KGaA

Maltoni C, Morisi L, Chieco P. Experimental Approach To The Assessment Of The Carcinogenic Risk Of Industrial Inorganic Pigments. Advances in Modern Environmental Toxicology. 1982; 2: 77-92.

Meena R, Kajal K, R P. Cytotoxic and genotoxic effects of titanium dioxide nanoparticles in testicular cells of male wistar rat. Appl Biochem Biotechnol. 2015 Jan; 175(2):825-40.

Meena R, Rani M, Pal R, Rajamani P. Nano-TiO_2-induced apoptosis by oxidative stress-mediated DNA damage and activation of p53 in human embryonic kidney cells. Appl Biochem Biotechnol. 2012 Jun;167(4):791-808.

Msiska Z, Pacurari M, Mishra A, Leonard SS, Castranova V, Vallyathan V. DNA double-strand breaks by asbestos, silica, and titanium dioxide: possible biomarker of carcinogenic potential? Am J Respir Cell Mol Biol. 2010; 43(2):210-9.

Moreno-Horn M & Gebel T. granular biodurable nanomaterials: no convicing evidence for systemic toxicity. Crit Rev Toxicol, 2014; 44(10):849-875.

Muhle H, Mermelstein R, Dasenbrock C, Takenaka S, Mohr U, Kilpper R, MacKenzie J, Morrow P. Lung response to test toner upon 2-year inhalation exposure in rats. Exp Pathol. 1989; 37(1-4):239-42.

Muhle H, Kittel B, Ernst H, Mohr U, Mermelstein R. Neoplastic lung lesions in rat after chronic exposure to crystalline silica. Scand J Work Environ Health. 1995; 21 suppl 2:27-9.

Muhle H, Bellmann B, Creutzenberg O, Dasenbrock C, Ernst H, Kilpper R, MacKenzie J.C, Morrow P, Mohr U, Takenaka S, Mermelstein R. Pulmonary response to toner upon chronic inhalation exposure in rats. Fundamental and applied toxicology. 1991 Aug; 17(2): 280-299.

Nanogenotox deliverables (2013). www.nanogenotox.eu

NCI. Bioassay of titanium dioxide for possible carcinogenicity. Natl Cancer Inst Carcinog Tech Rep Ser. 1979; 97: 1-123.

National Institute for Occupational Safety and Health. Current Intelligence Bulletin 63: Occupational Exposure to Titanium Dioxide, April 2011.

Naya M, Kobayashi N, Ema M, Kasamoto S, Fukumuro M, Takami S, Nakajima M, Hayashi M, Nakanishi J. *In vivo* genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats. Regul Toxicol Pharmacol. 2012 Feb; 62(1):1-6.

Numano T, Xu J, Futakuchi M, Fukamachi K, Alexander DB, Furukawa F, Kanno J, Hirose A, Tsuda H, Suzui M. Comparative study of toxic effects of anatase and rutile type nanosized titanium dioxide particles *in vivo* and *in vitro*. Asian Pac J Cancer Prev. 2014; 15(2):929-35.

Oberdörster G (1988) Lung clearance of inhaled insoluble and soluble particles. J Aerosol Med 1: 289–320

Oberdörster G (1994) Extrapolation of results from animal inhalation studies with particles to humans? In: Mohr U, Dungworth DL, Mauderly JL, Oberdörster G (Eds) Toxic and carcinogenic effects of solid particles in the respiratory tract (ILSI Monographs), ILSI Press, WashingtonDC, USA, 335–353

Oberdörster, G., Ferin, J., Lehnert, B.E. Correlation between particle size, *in vivo* particle persistence, and lung injury. Environ Health Perspect. 1994 Oct; 102 Suppl 5, 173–9.

Oberdörster, G. Lung particle overload: implications for occupational exposures to particles. Regul. Toxicol. Pharmacol. 1995; 21 (1), 123–35.

Oberdörster, G. pulmonary effects of inhaled ultrafine paritcles. (2002) Int. Arch occup Environ Health 74,1-8

Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113: 823–839

Oberdörster G, Oberdörster E, Oberdörster J (2007) Concepts of nanoparticle dose metric and response metric (Abstract). Environ Health Perspect 115: A290

Osman IF, Baumgartner A, Cemeli E, Fletcher JN, Anderson D. Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells. Nanomedicine (Lond). 2010 Oct;5(8):1193-203.

Pan R, Liu Y, Chen W, Dawson G, Wang X, Li Y, Dong B, Zhu Y. The toxicity evaluation of nano-trititanate with bactericidal properties *in vitro*. Nanotoxicology. 2012; 6(3):327-37.

Pauluhn, J. Comparative pulmonary response to inhaled nanostructures: considerations on test design and endpoints. Inhal. Toxicol. 2009; 21 Suppl 1: 40–54.

Petkovic J, Küzma T, Rade K, Novak S, Filipič M. Pre-irradiation of anatase TiO_2 particles with UV enhances their cytotoxic and genotoxic potential in human hepatoma HepG2 cells. J Hazard Mater. 2011a; 196:145-52.

Petkovic J, Zegura B, Stevanovic M, Drnovsek N, Uskokovic D, Novak S, Filipic M. DNA damage and alterations in expression of DNA damage responsive genes induced by TiO_2 nanoparticles in human hepatoma HepG2 cells. Nanotoxicology 2011b; 5:341–353.

Pott F, Roller M. Carcinogenicity study with nineteen granular dusts in rats. Eur J Oncol. 2005; 10(4):249–81.

Pott F, Ziem U, Reiffer FJ, Huth F, Ernst H, Mohr U.Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats. Exp Pathol. 1987; 32(3):129-52.

Prasad RY, Simmons SO, Killius MG, Zucker RM, Kligerman AD, Blackman CF, Fry RC, Demarini DM. Cellular interactions and biological responses to titanium dioxide nanoparticles in HepG2 and BEAS-2B cells: role of cell culture media. Environ Mol Mutagen. 2014 May; 55(4):336-42.

Prasad RY, Wallace K, Daniel KM, Tennant AH, Zucker RM, Strickland J, Dreher K, Kligerman AD, Blackman CF, Demarini DM. Effect of treatment media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle. ACS Nano. 2013 Mar 26; 7(3):1929-42.

Rad JS, Alfatemi MH, Rad MS, Rad MS, Sen DJ, Mohsenzadeh S. *In-vivo* Titanium Dioxide (TiO2) Nanoparticles Effects on Chromosomal Abnormalities and Lactate Dehydrogenase Activity. American Journal of Advanced Drug Delivery. 2013; 1(3):232-7.

Ramanakumar AV, Parent ME, Latreille B, Siemiatycki J. Risk of lung cancer following exposure to carbon black, titanium dioxide and talc: results from two case-control studies in Montreal. Int J Cancer. 2008 Jan 1; 122(1):183-9.

Rehn B, Seiler F, Rehn S, Bruch J, Maier M. Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: untreated and surface treated. Toxicol Appl Pharmacol. 2003 Jun 1; 189(2):84-95.

Rittinghausen S, Mohr U, Dungworth DL. Pulmonary cystic keratinizing squamous cell lesions of rats after inhalation/instillation of different particles. Exp Toxicol Pathol. 1997 Dec;49(6):433-46.

Roller M. Carcinogenicity of inhaled nanoparticles. Inhalation Toxicology. 2009; 21(S1): 144-57.

Rossi EM, Pylkkänen L, Koivisto AJ, Vippola M, Jensen KA, Miettinen M, Sirola K, Nykäsenoja H, Karisola P, Stjernvall T, Vanhala E, Kiilunen M, Pasanen P, Mäkinen M, Hämeri K, Joutsensaari J, Tuomi T, Jokiniemi J, Wolff H, Savolainen K, Matikainen S, Alenius H. Airway exposure to silica-coated TiO2 nanoparticles induces pulmonary neutrophilia in mice. Toxicol Sci. 2010 Feb;113(2):422-33.

Roszak J, Stępnik M, Nocuń M, Ferlińska M, Smok-Pieniążek A, Grobelny J, Tomaszewska E, Wąsowicz W, Cieślak M. A strategy for *in vitro* safety testing of nanotitania-modified textile products. J Hazard Mater. 2013; 256-257:67-75.

Saber AT, Jacobsen NR, Mortensen A, Szarek J, Jackson P, Madsen AM, Jensen KA, Koponen IK, Brunborg G, Gützkow KB, Vogel U, Wallin H. Nanotitanium dioxide toxicity in mouse lung is reduced in sanding dust from paint. Part Fibre Toxicol. 2012 Feb 2; 9:4.

Sadiq R, Bhalli JA, Yan J, Woodruff RS, Pearce MG, Li Y, Mustafa T, Watanabe F, Pack LM, Biris AS, Khan QM, Chen T. Genotoxicity of TiO(2) anatase nanoparticles in B6C3F1 male mice evaluated using Pig-a and flow cytometric micronucleus assays. Mutat Res. 2012; 745(1-2):65-72.

Sagawa Y, Futakuchi M, Xu J, Fukamachi K, Sakai Y, Ikarashi Y, Nishimura T, Suzui M, Tsuda H, Morita A. Lack of promoting effect of titanium dioxide particles on chemically-induced skin carcinogenesis in rats and mice. J Toxicol Sci. 2012; 37(2):317-27.

Sager TM, Kommineni C, Castranova V. Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area. Particle Fibre Toxicol. 2008 Dec 1; 5:17.

Saghiri Z, Saleh-Moghadama M, Nabavi MS. Effect Evaluation of anatase TiO_2 nanoparticles on induction of chromosomal damage in mice bone marrow *in vivo*. Proceedings of the 4th International Conference on Nanostructures (ICNS4) 12-14 March, 2012, Kish Island, I.R. Iran.

SCCS opinion on Titanium Dioxide (nano form). Colipa n° S75. SCCS/1516/13. Revision of 22 April 2014.

Setyawati MI, Khoo PK, Eng BH, Xiong S, Zhao X, Das GK, Tan TT, Loo JS, Leong DT, Ng KW. Cytotoxic and genotoxic characterization of titanium dioxide, gadolinium oxide, and poly(lactic-co-glycolic acid) nanoparticles in human fibroblasts. J Biomed Mater Res A. 2013 Mar; 101(3):633-40.

Shelby MD, Erexson GL, Hook GJ, Tice RR. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. Environ Mol Mutagen. 1993; 21(2):160-79.

Shelby MD, Witt KL. Comparison of results from mouse bone marrow chromosome aberration and micronucleus tests. Environ Mol Mutagen. 1995; 25(4):302-13.

Sheng L, Wang X, Sang X, Ze Y, Zhao X, Liu D, Gui S, Sun Q, Cheng J, Cheng Z, Hu R, Wang L, Hong F. Cardiac oxidative damage in mice following exposure to nanoparticulate titanium dioxide. J Biomed Mater Res Part A 2013; 101(11):3238–46.

Shi H, Magaye R, Castranova V, Zhao J. Titanium dioxide nanoparticles: a review of current toxicological data. Part Fibre Toxicol. 2013 Apr 15;10:15.

Shi Y, Zhang JH, Jiang M, Zhu LH, Tan HQ, Lu B. Synergistic genotoxicity caused by low concentration of titanium dioxide nanoparticles and p,p'-DDT in human hepatocytes. Environ Mol Mutagen. 2010 Apr; 51(3):192-204.

Shukla RK, Kumar A, Gurbani D, Pandey AK, Singh S, Dhawan A. TiO(2) nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. Nanotoxicology. 2013 Feb; 7(1):48-60.

Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S, Dhawan A. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicol In Vitro. 2011; 25(1):231-41

Siemiatycki J. Risk Factors for Cancer in the Workplace, CRC Press, Boca Raton FL. 1991.

Song MF, Li YS, Kasai H, Kawai K. Metal nanoparticle-induced micronuclei and oxidative DNA damage in mice. Clin. Biochem. Nutr. 2012 May; 50 (3): 211–6.

Srivastava RK, Rahman Q, Kashyap MP, Lohani M, Pant AB. Ameliorative effects of dimetylthiourea and N-acetylcysteine on nanoparticles induced cyto-genotoxicity in human lung cancer cells-A549. PLoS One. 2011; 6(9):e25767.

Srivastava RK, Rahman Q, Kashyap MP, Singh AK, Jain G, Jahan S, Lohani M, Lantow M, Pant AB. Nano-titanium dioxide induces genotoxicity and apoptosis in human lung cancer cell line, A549. Hum Exp Toxicol. 2013 Feb; 32(2):153-66.

Stenbäck F, Rowland J, Sellakumar A. Carcinogenicity of benzo(a)pyrene and dusts in the hamster lung (instilled intratracheally with titanium oxide, aluminum oxide, carbon and ferric oxide). Oncology. 1976; 33(1):29–34.

Stone V, Johnson H, Clift MJD. Air pollution, ultrafine and nanoparticle Toxicology: cellular and molecular interaction. IEEE transactions on nanobioscience, 2007 6(4)331:340

Sycheva LP, Zhurkov VS, Iurchenko VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK, Durnev AD. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice *in vivo*. Mutat Res. 2011 Nov 27; 726(1):8-14.

Tassinari R, Cubadda F, Moracci G, Aureli F, D'Amato M, Valeri M, De Berardis B, Raggi A, Mantovani A, Passeri D, Rossi M, Maranghi F. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology. 2014 Sep;8(6):654-62.

Tavares AM, Louro H, Antunes S, Quarré S, Simar S, De Temmerman PJ, Verleysen E, Mast J, Jensen KA, Norppa H, Nesslany F, Silva MJ. Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicol In Vitro. 2014 Feb; 28(1):60-9.

Thyssen J, Kimmerle G. Inhalation studies with polyurethane foam dust in relation to respiratory tract carcinogenesis. J Environ Pathol Toxicol. 1978 Mar-Apr; 1(4):501-8.

Tiano L, Armeni T, Venditti E, Barucca G, Mincarelli L, Damiani E. Modified TiO(2) particles differentially affect human skin fibroblasts exposed to UVA light. Free Radic Biol Med. 2010; 49(3):408-15.

Toyooka T, Amano T, Ibuki Y. Titanium dioxide particles phosphorylate histone H2AX independent of ROS production. Mutat Res. 2012; 742(1-2):84-91.

Tran CL, Cullen RT, Buchanan D, Jones AD, Miller BG, Searl A, Davis JMG, Donaldson K, Investigation and prediction of pulmonary responses to dust. Part II. In: Investigations into the pulmonary effects of low toxicity dusts. Parts I and II. Suffolk, UK: Health and Safety Executive, Contract Research Report 216/1999, 1999

Trochimowicz HJ, Kimmerle G, Dickhaus S. Chronic inhalation exposure of rats to titanium dioxide dust. J. Appl Toxicol. 1988 Dec; 8(6):383-5.

Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH. Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice. Cancer Res. 2009 Nov 15; 69(22):8784-9.

Tsuda H, Xu J, Sakai Y, Futakuchi M, Fukamachi K. Toxicology of engineered nanomaterials - a review of carcinogenic potential. Asian Pac J Cancer Prev. 2009; 10(6):975-80.

Turkez H. The role of ascorbic acid on titanium dioxide-induced genetic damage assessed by the comet assay and cytogenetic tests. Exp Toxicol Pathol. 2011 Jul; 63(5):453-7.

Ursini CL, Cavallo D, Fresegna AM, Ciervo A, Maiello R, Tassone P, Buresti G, Casciardi S, Iavicoli S. Evaluation of cytotoxic, genotoxic and inflammatory response in human alveolar and bronchial epithelial cells exposed to titanium dioxide nanoparticles. J Appl Toxicol. 2014 Nov; 34(11):1209-19.

Valdiglesias V, Costa C, Sharma V, Kiliç G, Pásaro E, Teixeira JP, Dhawan A, Laffon B. Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. Food Chem Toxicol. 2013 Jul; 57:352-61.

Vales G, Rubio L, Marcos R. Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells. Nanotoxicology 2014 Aug 9: 568-78.

Wan R, Mo Y, Feng L, Chien S, Tollerud DJ. DNA damage caused by metal nanoparticles: involvement of oxidative stress and activation of ATM. Chem Res Toxicol. 2012 Jul 16; 25(7): 1402-11.

Wang J, Fan Y. Lung injury induced by TiO_2 nanoparticles depends on their structural features: size, shape, crystal phases, and surface coating. Int J Mol Sci. 2014 Dec 3; 15(12):22258-78.

Wang S, Hunter LA, Arslan Z, Wilkerson MG, Wickliffe JK. Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells. Environ Mol Mutagen. 2011 Oct; 52(8):614-22.

Warheit DB, Reed KL, Webb TR. Pulmonary toxicity studies in rats with triethoxyoctylsilane (OTES)-coated, pigment-grade titanium dioxide particles: bridging studies to predict inhalation hazard. Exp Lung Res. 2003 Dec;29(8):593-606.

Warheit DB, Brock W J, Lee K, Webb T.R, Reed K.L. Comparative Pulmonary Toxicity Inhalation and Instillation Studies with Different TiO_2 Particle Formulations: Impact of Surface Treatments on Particle Toxicity. Toxicological Sciences. 2005; 88(2): 514–524.

Warheit DB, Frame SR. Characterization and reclassification of titanium dioxide-related pulmonary lesions. J Occup Environ Med. 2006 Dec; 48(12):1308-13.

Warheit DB, Webb TR, Reed KL, Frerichs S, Sayes CM. Pulmonary toxicity study in rats with three forms of ultrafine-TiO_2 particles: differential responses related to surface properties. Toxicology. 2007 Jan 25; 230(1):90-104.

Woodruff RS, Li Y, Yan J, Bishop M, Jones MY, Watanabe F, Biris AS, Rice P, Zhou T, Chen T. Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay. J Appl Toxicol. 2012 Nov; 32(11):934-43.

Xia T, Hamilton RF, Bonner JC, Crandall ED, Elder A, Fazlollahi F, Girtsman TA, Kim K, Mitra S, Ntim SA, Orr G, Tagmount M, Taylor AJ, Telesca D, Tolic A, Vulpe CD, Walker AJ, Wang X, Witzmann FA, Wu N, Xie Y, Zink JI, Nel A, Holian A. Interlaboratory Evaluation of in Vitro Cytotoxicity and Inflammatory Responses to Engineered Nanomaterials: The NIEHS Nano GO Consortium. Environ Health Perspect. 2013 Jun;121(6):683-90.

Xu J, Shi H, Ruth M, Yu H, Lazar L, Zou B, Yang C, Wu A, Zhao J. Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. PLoS One. 2013 Aug 8; 8(8):e70618.

Xu J, Futakuchi M, Iigo M, Fukamachi K, Alexander DB, Shimizu H, Sakai Y, Tamano S, Furukawa F, Uchino T, Tokunaga H, Nishimura T, Hirose A, Kanno J, Tsuda H. Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intra-pulmonary spraying. Carcinogenesis. 2010 May; 31(5):927-35.

Xue C, Wu J, Lan F, Liu W, Yang X, Zeng F, Xu H. Nano titanium dioxide induces the generation of ROS and potential damage in HaCaT cells under UVA irradiation. J Nanosci Nanotechnol 2010 Dec; 10(12):8500–7.

Xu J, Sagawa Y, Futakuchi M, Fukamachi K, Alexander DB, Furukawa F, Ikarashi Y, Uchino T, Nishimura T, Morita A, Suzui M, Tsuda H. Lack of promoting effect of titanium dioxide particles on ultraviolet B-initiated skin carcinogenesis in rats. Food Chem Toxicol. 2011 Jun; 49(6):1298-302.

Yamadori I, Ohsumi S, Taguchi K. Titanium dioxide deposition and adenocarcinoma of the lung. Acta Pathol Jpn. 1986 May; 36(5):783–90.

Yokohira M, Hashimoto N, Yamakawa K, Suzuki S, Saoo K, Kuno T, Imaida K. Lung Carcinogenic Bioassay of CuO and TiO(2) Nanoparticles with Intratracheal Instillation Using F344 Male Rats. J Toxicol Pathol. 2009 Mar; 22(1):71-8.

Zheng D, Wang N, Wang X, Tang Y, Zhu L, Huang Z, Tang H, Shi Y, Wu Y, Zhang M, Lu B. Effects of the interaction of TiO_2 nanoparticles with bisphenol A on their physicochemical properties and in vitro toxicity. J Hazard Mater. 2012 Jan 15; 199-200:426-32.

Additional references

Additional references not included in the CLH report or RCOM Annex

Attfield *et al* (2012). Coal. Chapter 86 in Patty's Toxicology. Sixth edition. Volume 5.

EFSA (2016). Re-evaluation of titanium dioxide (E171) as a food additive. EFSA Journal 14(9):4545, adopted 28 June 2016

Gregoratto et al. (2010) Modelling particle retention in the alveolar-interstitial region of the human lungs. J Radiol Prot 30:491-512

Kuempel *et al*. (2009). Rat- and human-based risk estimates of lung cancer from occupational exposure to poorly-soluble particles: a quantitative evaluation. J Phys: Conf Ser 151:1-12

Maronpot *et al*. (2004). Relevance of animal carcinogenesis findings to human cancer predictions and prevention. Toxicologic Pathology, 32(Suppl. 1):40–48

Morrow (1992). Dust overloading on the lungs: update and appraisal. Toxicol Appl Pharmacol 113:1-12

Nikula (2000). Rat lung tumours induced by exposure to selected poorly soluble nonfibrous particles. Inhal Toxicol 12:97-119

Travis *et al*. (2015). The 2015 World Health Organization Classification of Lung Tumors Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. J Thorac Oncol 10:1243–1260

8 ANNEXES

ANNEX I – SUMMARY OF GENOTOXICITY DATA

1. In vitro data

A literature research including published reviews, projects and studies was performed (ended on 30/04/2015). In addition, information from the registration dossier which has been published on ECHA website has been considered (date: 01/08/2015). All forms of TiO_2 have been taken into account. Due to the high number of *in vitro* genotoxicity assays found, an exhaustive reporting of studies was judged neither feasible nor of any added values.

As a first step, only the most recent studies, published between 2010 and 2015, were assessed. After, these studies (2010-2015) were sorted based on the following criteria in order to keep only the more reliable data:

- Characterization of the tested materials (at least size, crystallinity and coating) and description of dispersion of tested materials. Although the publications do not always provide the physico-chemical characteristics of the material tested, the most recent (2010-2015) generally give few characteristics after addition to the cell culture medium as well as their stability during the assay. However, it can be highlighted that the PDI (polydispersity Index) which gives an idea of the stability of a solution is rarely provided in the publications. Papers published before 2010 have generally missing information on the physico-chemical characteristics and were not assessed.

- Assays with recognized protocols and mainly regulatory tests: Micronucleus assay, Gene mutation assay and Chromosomal Aberrations assay. Comet assay was also used as supportive data (even if no harmonized guideline is currently available). The results from the Comet assay were also included as they may bring information on the mechanism of action especially if the genotoxic effect might be due to some oxidative stress induced by the TiO_2. Furthermore, some protocols such as Ames test do not appear to be suitable because bacterial cells will not easily uptake TiO_2 and also because some NM can harbour antimicrobial activity (Doak, 2012). Indeed, Woodruff (2012) showed that TiO_2 NPs (nanoparticle) were not able to enter the bacterial cell. Therefore, the data from Ames tests were not considered for nanoforms of TiO_2, except when a modified protocol which would promote the uptake was proposed as reported by Jomini (2012).

- Studies with data assessing uptake into the cells in particular in case of negative results. Indeed, uptake should be ensured to conclude that the lack of genotoxicity observed is not due to a lack of exposure. If uptake was not assessed, cytotoxicity data are useful as a proof of adequate cell exposure.

- Inclusion of negative and positive controls and use of replicates.

Several studies published between 2010 and 2015 were deleted from this selection because one or more of the above criteria was not fulfilled. After selection, the total number of studies collected was 39, with 14 micronucleus assays, 20 Comet assays, 2 Chromosomal Aberrations studies and 3 others types studies. The studies which were excluded (9 micronucleus assays, 18 Comet assays 1 Chromosomal Aberrations test and 1 Gene Mutation assay and 11 others types of studies) are included in Annex II Table 1 for transparency.

- **Micronucleus assays**

Table I-01. Summaries of *in vitro* micronucleus assays which fulfil our selected criteria (published between 2010-2015; characterization of the tested materials; data on uptake and/or cytotoxicity; presence of negative and positive controls and use of several replicates)

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	Results; Methods	
Jugan et al. 2012	TiO_2-A12 NP 95% anatase, spherical 12 nm; 92 m^2/g; PZC: 6.4	Dispersion in ultrapure sterile water (pH5.5) by sonication for 30 min at 4 C, in pulsed mode (1 s on/1 s off), NPs suspensions were diluted in DMEM without serum.	A549 human lung carcinoma cells (CCL-185)	Cytotoxic MTT assay (1–200 µg/ml of NP suspensions for 4–48 h) No data during MN assay.	Negative CBMN assay. Conditions tested: 50-100-200 µg/ml for24h; negative and positive controls; triplicate.	Unambiguous accumulation of the smallest NPs in the cytoplasm and in the nucleus of cells
	TiO_2-A25 (AEROXIDE P25) 86% anatase/14% rutile, spherical 24 nm; 46 m^2/g; PZS: 7.0					Unambiguous accumulation of the smallest NPs in the cytoplasm
Prasad et al. 2013	P25 AEROXIDE TiO_2 86% anatase/14% rutile; 95.1% purity, 27.5 nm (14.2-64.6 nm); 49 m^2/g	Dispersion in DI water: Hydrodynamic diameter: 273 to 309 nm; ZP: -8.7 to -17.2 mV 3 media tested: (a) KB (keratinocyte growth medium (KGM) + 0.1% BSA), with 0.1% BSA; largest agglomerates (800-2000 nm) (b) DM (PBS + 0.6% BSA + 0.001% surfactant DSPC): agglomerates (400-800 nm) (c) KF (KGM + 10% FBS): agglomerates of 200 nm PDI between 0.2 and 0.8. ZP :-0.53 to -8.47 mV	Human bronchial epithelial cell (BEAS-2B)	Non-cytotoxic (< 10% decrease of cell viability at the highest concentration). Live/dead assay using a propidium iodide/calcein-AM commercial kit. Conditions tested: 24h; 20-100 µg/mL Concentration-dependent decreased CBPI in the MN assay.	Negative with KB and DM media Positive with KF media: from 20 µg/ml – dose-dependent CBMN assay at 10, 20, 50, 100 µg/ml for 24h; negative and positive controls. Two independent experiments conducted for all concentrations in all three treatment media.	The smaller TiO_2 nanoparticle agglomerates, which occur in the KF medium, interact more with the cells than do those formed in the other two media, which are larger agglomerates.

		=> NP dispersions were unstable, aggregating and separating out of the liquid phase.				
Shukla et al 2011	Titanium (IV) oxide nanopowder 99.7%, anatase, 10 nm	Dispersion in two different media (a) milliQ water: mean hydrodynamic diameter: 124.9 nm and ZP: -17.6 mV (b) DMEM supplemented with 10% FBS: mean hydrodynamic diameter: 171.4 nm and ZP: -11.5 mV Probe sonicated treatment at 30W for 10 min for both media	Human epidermal cell line (A431)	Cytotoxic with NRU and MTT assays at 8 and 80 µg/ml after 48 hours. Conditions tested: 0.008- 80 µg/mL for 6, 24, 48 hours. Not cytotoxic with CBPI data in MN assay.	Positive: from 10 µg/ml –dose-dependent CBMN assay: from 0.008 to 80 µg/mL for 6h ; 2000 binucleated cells scored per condition; 3 independent experiments; 2 replicates; negative and positive controls.	Significant concentration-dependent increase in the cellular internalization of TiO_2 NPs after 6 h exposure (flow cytometer method). Subcellular localization of TiO_2 NPs inside cytoplasm and nucleus confirmed using TEM.
Shukla et al, 2013	Titanium (IV) oxide nanopowder 99.7%, anatase 30-70 nm	NP suspended in IMEM (medium without FBS) and probe sonicated for 10 min. Dilution of suspensions in CMEM (medium with 10% FBS) DLS: 192.5 ± 2.00 nm; PDI: 0.18 ± 0.01 nm; ZP: -11.4 ± 0.25 mV	HepG2 cells	Cytotoxic with MTT and NRU assays: significant reduction of enzymatic activity at 40 and 80 µg/mL after 24 and 48 h; but no cytotoxicity after 6h. Conditions tested: 0, 1, 10, 20, 40 and 80 mg/ml Interference checked with assay reagents but not with cells No data on the CBPI in the MN assay.	Positive: from 10 µg/ml – no dose-dependent (max increase at 20 µg/ml) CBMN assay: from 1 to 80 µg/ml for 6h; 2000 binucleated cells scored per condition; 3 independent experiments; negative and positive controls.	Internalization concluded from flow cytometry as well as from TEM (apparently numerous individualized NPs inside the cells)

Valdiglesias et al, 2013	TiO_2-D 80% anatase and 20% rutile, 25 nm ; 35-45 m^2/g TiO_2-S 100% anatase, 25 nm 200-220 m^2/g	TiO_2 NPs suspended in either deionized water or complete cell culture medium (with FBS) and ultrasonicated at 30W for 5 min TiO_2-D: DI water: 160.5 nm; ZP:-27.8 mV Complete medium: 228.3 nm; ZP: -10.7 mV TiO_2-S: DI water: 447.9 nm; ZP:-9.96 mV Complete medium: 504.5 nm; ZP: -10.7 mV	Human SHSY5Y neuronal cells	Non cytotoxic in MTT and NRU assays. Conditions tested: 20-150 µg/ml for 3, 6, 24h. Interference: no interaction between NP and dyes used. No data on CBPI in the MN assay.	Negative (3 h exposure) Positive (6h exposure): all doses - dose-dependent for TiO_2-S ; not clear dose-relation for TiO_2-D (max at 120 µg/ml) MN assay: 80, 120 and 150 µg/ml for 3 or 6 hours; min 3 independent experiments; negative and positive controls.	Uptake increase with time of treatment (flow cytometry) – Uptake was always lower for TiO_2-D NPs than for TiO_2-S NPs

Guichard et al, 2012	TiO_2 A nano Anatase, 14 nm (TEM); BET = 149 m^2/g, chemical impurity < 0.5% TiO_2 A micro Anatase, 160 nm (TEM); BET = 9 m^2/g, chemical impurity < 0.5% TiO_2 R nano Rutile, 62 nm (TEM); BET = 177 m^2/g Coating: 11% SiO_2, 1% Na_2O and 1% SO_4 TiO_2 R micro Rutile, 530 nm (TEM); BET = 3 m^2/g, chemical impurity < 0.5% TiO_2 P25 (Aeroxide) 80% anatase; 20% rutile, 25 nm (TEM); BET = 58 m^2/g, chemical impurity < 0.5%	Sonication for 20 min at 40 Watt using a sonicator bath. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and antibiotics (Penicillin, Steptomycin). At 80% confluence, cells were harvested using 0.25% trypsin and 0.53 mM ethylenediamine-tetraacetic acid (EDTA). All particle suspensions in the SHE culture medium consisted of mainly agglomerated particles: particle diameters determined by DLS (dZ) varied from 300 to 700 nm depending on the particle type. Suspension of anatase TiO_2 nanoparticles led to coarser particle formation than its microsized counterpart. The contrary was observed for rutile TiO_2.	Syrian hamster embryo cells (SHE)	Cytotoxic based on decrease of RCC. Conditions tested:0,5 - 200 $\mu g/cm^2$ for 24 and 72 hours => Nanoparticles were more cytotoxic than their micrometer counterparts. Micronucleus assay: cytotoxic with a decrease in RICC of 50% for all particles.	Negative for all TiO_2 NP tested. The decreases in micronucleus frequency observed at the highest concentration of TiO_2 particles when compared to the control may be explained either by some blockage to division induced by the treatment or by the presence of particles on the slide which disturbed micronucleus scoring. MN assay; 5, 10, 50 $\mu g/cm^2$ for 24 hours; around 1000 cells per slide analyzed; each experiment performed at least 3 times; positive and negative controls.	All particles are able to penetrate cells in the form of individual particles and agglomerates (TEM)
Srivastava et al, 2013	TiO_2 Anatase without coating, < 25 nm SSA = 200-220 m^2/g	Stock solution of TiO_2 NPs was prepared in complete DMEM-F12 medium containing 10% serum and then sonicated intermittently for 5 min with each stretch of 1 min at 100 Hz. Mean hydrodynamic diameter (DLS) = 434.1 nm and ZP = -7.83 mV	Human lung cancer cell line (A549)	Concentration and time-dependent decrease in percentage cell viability following 6–48 h exposure of TiO_2 NPs in MTT and LDH assays. Cytotoxic after 24 h exposure (10, 50 and 100 mg/ml) and more intense during the 48 h exposure period (10, 50 and 100 mg/ml) in MTT assay. The results of LDH assay were similar to MTT assay. Exposure for 6 h was found to be effective only at 100 µg/ml	Positive from10 µg/ml – dose dependent. CBMN: 0, 1, 10, 50 µg/ml for 24 h; minimum of 1000 bi-nucleated cells scored; at least 3 independent experiments; positive and negative controls.	The internalization of TiO_2 NPs in A549 cells through TEM analysis was found to be dose dependent. The particles adhered on the cell surface when incubated for 30 min and subsequently internalized in small vacuoles at cortical cytoplasm in extending incubations and reached to deep cell center near the mitochondria and Golgi apparatus in larger vacuoles over 48 h of exposure

				Conditions tested: 0, 1, 5, 10, 50, 100 µg/ml for 6, 24 and 48 hours. Interferences: no major interaction between colors of dyes and NPs as in cell-free system. No data on CBPI in the MN assay.		
Srivastava et al, 2011	TiO_2 Anatase; tetragonal in crystallographic system, spherical in shape without any coating 5-20 nm (TEM) Specific surface area = 200-220 m^2/g Purity= 99.7%, pure trace metal basis	Stock solutions of nanoparticles were sonicated before being diluted with DMEM/F-12 (Hams) supplemented with fetal bovine serum (FBS), sodium bicarbonate, and antibiotic/antimycotic solution. Mean hydrodynamic diameter (DLS) in complete medium: 417.7 nm; zeta potential : (-) 7.83 mV	Human lung cancer cell line (A549)	Cytotoxic: significant decrease of % cell viability at all doses; MTT (most sensitive), NRU and LDH assays. Conditions tested: 10 or 50 mM for 24h. Pre-treatment with DMTU, OH° radical trapper (30 min), induced significant protection in viability. No data on CBPI in the MN assay.	Positive at all doses- dose dependent. CBMN assay, 10 or 50 µg/ml for 24h; 1000 binucleated cells with well-defined cytoplasm scored; at least 3 independent experiments; positive and negative controls. Both DMTU and NAC (glutathione precursor/H_2O_2 scavenger) were found to be effective in reducing MN significantly.	The particles were adhered on the cell surface between microvilli and pseudopodes, when incubated for 24 h, subsequently internalized in small vacuoles at cortical cytoplasm (TEM)
Vales et al. 2014	NM 102 Anatase, primary particle size: 21.7±0.6 nm	Nanogenotox protocol: pre-wetted in 0.5% absolute ethanol and afterwards dispersed in bovine serum albumin (BSA) in MilliQ water, the nanoparticles in the dispersion medium were sonicated for 16 min to obtain a stock dispersion of 2.56 mg/mL In exposure medium: 575.9 nm; PDI: 0.471 (DLS), ZP: -19.5 mV (LDV)	BEAS-2B cells	Not reported.	Negative CBMN assay, conditions tested: 1, 10, 20µg/ml for 24h, 1 or 3 weeks; 1000 binucleated cells per sub-culture scored; duplicate; negative and positive controls.	Uptake after 24h to 20 µg/ml (TEM)
Demir et al. 2015	Micro TiO_2 99% to 100.5% purity	No information	Human embryonic kidney (HEK293) and mouse embryonic	Not cytotoxic with CBPI at 10-100-1000 µg/ml for 48 h	Negative CBMN; conditions tested: 0-100-1000 µg/ml for 48 h; 2 independent experiments and 2 replicates of each one; negative and positive controls.	Not reported.

			fibroblast (NIH/3T3)		Very high concentrations tested.	
	Nano TiO_2 Anatase, 21 nm; > 99.5% purity	Dispersed at the concentration of 2.56 mg/mL prepared in bovine serum albumin (BSA) in water, subjected to ultrasonication at 20kHz for 16 min in an ice-cooled bath; (in agreement of the proposal from Nanogenotox EU project). No important agglomerations observed following the dispersion protocol used. Nano-TiO_2 (21 nm) : 22.94± 0.3 nm (DLS) and ZP : 8.71mV Nano-TiO_2 (50 nm) : 50.72±0.4 nm (DLS) and ZP: 9.38mV		Cytotoxic at 1000 µg/ml after 48h of exposure with a CBPI decrease around 50% for HEK293 but less important for NIH/3T3	Positive: significant increases in the frequency of BNMN in the two cell lines at 1000 µg/ml. CBMN assay; conditions tested: 0-100-1000 µg/ml for 48 h; 1000 binucleated cells scored; 2 independent experiments and 2 replicates of each one; negative and positive controls. Very high concentrations tested.	
	Nano TiO_2 Anatase, 50 nm, > 98% purity					
Roszak et al 2013	TiO_2 Mixture of rutile and anatase forms TiO_2 aggregates (SEM), irregular shape broad size distribution from 10 nm up to µm (ATM)	Prepared in MilliQ water DLS size = 220 nm (stable at least 72 h); ZP: 40 mV; Specific BET 27.1 m^2/g (non-porous). Then immediately (within 10 min) mixed with fresh culture medium and applied onto the cells. DLS size 300 nm (stable 30 h) in culture medium with FBS (1:9)	Human lymphocytes	No effect on CBPI in the MN assay.	Positive from 60 µg/ml; dose dependent. CBMN assay; conditions tested: 20 -250 µg/ml for 6 or 24 h; duplicate, negative and positive controls.	Not reported.
			Hamster lung V79 fibroblasts	Cytotoxic at 100-250 µg/ml after 72h in WST1 reduction assay Interference with test system excluded. Error or number of cells seeded for cytotoxicity test seems to be very low. Concentration-dependent decreased CBPI.	Negative Identification of micronuclei during microscopic analysis was hampered by the presence of artifacts originating probably from particles interacting with cellular components. CBMN assay, conditions tested: 20 -250 µg/ml for 6 or 24 h; duplicate; negative and positive controls.	

Prasad et al. 2014	Nano P25 86% anatase and 14% rutile	Suspended in DMEM supplemented with 0.1% BSA or DMEM supplemented with 10% FBS, at 1 mg/ml and probe sonicated at 7 W for 2 min on ice. No significant differences on PI and ZP between the two media.	HepG2 and BEAS 2B	No decrease in viability of HepG2 (CellTiter-Blue® Cell Viability Assay) MN assay: Decrease of the CBPI at all concentrations indicating some cytotoxicity.	Positive: Dose dependent increase from 10 µg/ml in both treatment media (statistically significant from 50 µg/ml) CBMN: conditions tested: 10-100 µg/ml for 24h; 2 replicates; 3 independent experiments; negative and positive controls.	Uptake of agglomerates of TiO_2 NP in cytoplasm of BEAS-2B cells exposed 24h to 20-100 µg/ml
Kansara et al 2015	NM-102 99.7% purity, anatase 4-8 nm; 12-15 m^2/g	Suspended in MilliQ water and complete DMEM F-12 medium. In MilliQ water: 106.7 ± 8.0 nm and 213 ±0.9 mV In complete DMEM F-12 medium supplemented with 10% FBS: 23.28 ± 2.0 nm and 10.1 ± 1.0 mV. Particles stable up to 72h.	A549 human pulmonary cells	Cytotoxic at 150 and 200 mg/ml after 48 h but not at 6 and 24 h in the MTT assay. Cytotoxic at 100, 150, and 200 mg/ml after 48 h exposure, but not at 6 and 24 hours in the NRU assay. Conditions tested: 1-200 µg/ml for 6, 24 and 48h. NP interference with assay reagent checked. MN assay: No data on the level of binucleated cells and CBPI data not given.	Positive: Increase from 75 µg/ml – dose dependent CBMN assay. Conditions tested: 25-100 µg/ml for 6 h; 2000 binucleated cells from each concentration scored; three independent experiments; negative and positive controls.	Not reported.
Tavares et al, 2014	**NM-102:** Anatase; Primary particles were polyhedral. SSA: 90 m^2/g, PPS: 20.8-33 nm; aggregates/ agglomerates: 43 nm (25%), 54 nm (median), 72 nm (75%) **NM-103:** Rutile hydrophobic; coating: dimethicone 2%; Primary particles were polyhedral. SSA: 60 m^2/g, PPS: 21.9-37.9 nm, aggregates/ agglomerates: 33 nm	Prewetting powder in 0.5 vol% ethanol (96%) followed by addition of sterile-filtered 0.05 wt% BSA-water and dispersion by 16 min of probe sonication of the sample, cooled in an ice-water bath. According to the protocol, the batch dispersions are metastable and, for most samples, maintained for at least 1 h. Characterization in batch dispersion: **NM-102:** 22.4 nm and ca. 615 nm in the 0.05% BSA batch dispersion; PDI : from 0.135 ± 0.017 to 0.324 ± 0.020	Human peripheral lymphocytes	Not cytotoxic: cell viability and cell cycle progression were not affected by any of the TiO_2 NMs treatments (RI and CBPI).	Positive (NM-102): Increased MN at 125 µg/ml without dose-relationship; Positive (NM-103): Increased MN at 5 and 45 µg/ml without dose-relationship. Positive (NM-104): Increased MN at 5 and 45 µg/ml without dose-relationship. Negative (NM-105). CBMN assay; from 0 to 256 µg/ml for 30 h of exposure; At least 2 replicate culture and 2000	Not reported.

	(25%), 67 nm (median), 129 nm (75%) **NM-104:** Rutile hydrophobic; coating: glycerine; Primary particles were polyhedral SSA: 60 m^2/g; PPS: 19.0-25.8 nm, aggregates/ agglomerates: 33 nm (25%), 60 nm (median), 112 nm (75%) **NM-105:** Rutile/anatase (15-85%); primary particles were polyhedral; SSA: 61 m^2/g; PPS: 20.0-29.6 nm; Aggregates/Agglomerates: 55 nm (25%), 90 nm (median), 144 nm (75%)	**NM-103:** 78.8 nm and ca. 300 nm in the 0.05% BSA batch dispersion; PDI = 0.324 ± 0.020 **NM-104:** 78.8 nm and 122.4 nm; PDI from 0.135 ± 0.017 to 0.324 ± 0.020 **NM-105:** from 78.8 nm to 122.4 nm; PDI = 0.135 ± 0.017 Characterization in exposure media: RPMI 1640 cell media added 15–20% w/v fetal calf serum and phytohemaglutinin A. **NM-102:** Extensive sedimentation after 6 hours: ca 75% **NM-103:** Extensive sedimentation after 6 hours: ca 25%. **NM-104:** Extensive sedimentation after 6 hours: ca 60%. Strong component of total sedimentation.			binuleated cells and 1000 mononucleated cells from 2 independent cultures; positive and negative control valid.	

- **Comet assays**

Table I-02. Summaries of *in vitro* Comet assays which fulfil our selected criteria (published between 2010-2015; characterization of the tested materials; data on uptake and/or cytotoxicity; presence of negative and positive controls and use of several replicates)

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	Results; Methods	
Hamzeh et al, 2013	Nano-TiO_2 MTI5 anatase 5.9 nm; ≥ 99.5% pure P25 83% anatase and 17% rutile 34.1 nm; ≥ 99.5% pure Nanofilament rutile 15 nm; ≥ 99.5% pure Hombitan LW-S Bulk anatase, ≥ 99.5% pure, 169.4 nm Vive Nano Titania Rutile coated 78% (w/w) polymer and 22% nano-TiO_2, ≥ 99.5% pure, 1-10 nm	Suspension in a serum-containing culture media and sonication for 60 s at 30% amplitude and 20 kHz f (ice/water bath). The suspension was immediately added to cultured cells (in DMEM without phenol red and supplemented with FBS, sodium pyruvate, L-glutamine and pen-strep antibiotics) In media: MTI5: 460 nm (DLS); ZP: -12 mV P25: 400 nm (DLS); ZP : -12 mV Nanofilament: 420 nm (DLS); ZP: -12 mV Hombitan LW-S: 365 nm (DLS); ZP : -13 mV Vive Nano Titania: 600 nm (DLS); ZP: -19 mV	Chinese hamster lung fibroblast cells (V79)	Cytotoxic: decrease of cell viability at 10 and 100 mg/l (24h and 48h) for non-coated TiO_2. Nanofilament caused the highest decreased and Hombitan LW-S the lowest. For coated TiO_2: cell viability was decreased at 100 mg/l. MTT assay. Conditions tested: 1, 10, 100 mg/l for 24 or 48h, Negative and positive toxic controls. Increased apoptosis/necrosis rate of cells exposed to 100 mg/L (flow cytometry). Comet assay: cell viability more than 40% at 100 mg/l.	Positive at 100 mg/l (OTM or %Tail DNA) for MIT5, P25 and Nanofilament Negative for H. Bulk and Vive Nano. Alkaline Comet assay. Conditions tested: 10 and 100 mg/l for 24 h; at least 3 independent experiments; 2 replicates; negative and positive controls.	MTI 5 and Hombitan LW-S could penetrate inside the cells and change cellular morphology Vive Nano Titania particles mostly formed large agglomerates and remained outside the cells.
Prasad et al, 2013	P25 AEROXIDE TiO_2 86% anatase/14% rutile 27.5 nm (14.2-64.6 nm); 49 m^2/g; 95.1% purity	Dispersion in DI water: Hydrodynamic diameter: 273 to 309 nm; ZP: -8.7 to -17.2 mV 3 media tested: (a) KB (keratinocyte growth medium (KGM) + 0.1% BSA), with 0.1% BSA; largest agglomerates (800-2000 nm),	Human bronchial epithelial cell (BEAS-2B)	Non-cytotoxic (<10% decrease of cell viability at the highest concentration). Live/dead assay using a propidium iodide/calcein-AM commercial kit. Conditions tested: 24h; 20-100 µg/ml	Positive: Concentration-dependent increase in DNA damage in all three treatment media (% tail DNA) Comet assay. Conditions tested: 10-100 µg/ml for 24h; 3 independent experiments; negative and positive controls.	The smaller TiO_2 nanoparticle agglomerates, which occur in the KF medium, interact more with the cells than do those formed in the other two media, which are

		(b) DM (PBS + 0.6% BSA + 0.001% surfactant DSPC): agglomerates (400-800 nm), (c) KF (KGM + 10% FBS): agglomerates of 200 nm PDI between 0.2 and 0.8. ZP :-0.53 to -8.47 mV => NP dispersions were unstable, aggregating and separating out of the liquid phase.				larger agglomerates.
Saquib et al, 2012	TiO_2-NPs rutile, crystallites with polyhedral morphologies 30.6 nm (heterogeneous dispersion : presence of both primary particles and larger aggregates)	TiO_2 were suspended in Milli-Q water and subjected to sonication for 15 min at 40W. Stock suspension was then instantly diluted in Milli-Q water and RPMI cell culture medium. In DI water: large particle aggregates of 380 nm. In RPMI cell culture medium: small population of an average 13 nm particle size and larger aggregates of 152 nm.	Human amnion epithelial (WISH) cells	Cytotoxic: concentration dependent decline in the cell survival at all doses in MTT assay. Conditions tested: 0.625 to 10 µg/ml for 24 h; min 3 independent experiments Cytotoxic: a concentration dependent decline in the survival of cells (significant from 2.5 µg/ml) in a NRU assay. Conditions tested: 0.625 to 10 µg/ml for 24 h; min 3 independent experiments No interferences between TiO_2 NP and NR dye up to 10 µg/ml	Positive: significant induction of DNA damage at 20 µg/ml (OTM) Neutral Comet assay. Conditions tested: 0.625 to 20 µg/ml for 6hrs; at least 3 independent experiments; negative and positive controls.	Aggregates of NPs, localized either inside the vesicles or free in cytoplasm (more than 85% of the analyzed cell sections exhibited internalized TiO_2-NPs aggregates)
Shukla et al, 2013	Titanium (IV) oxide nanopowder 99.7%, anatase, 30-70 nm	NP suspended in IMEM (medium without FBS) and probe sonicated for 10 min. Dilution of suspensions in CMEM (medium with 10% FBS). DLS: 192.5 ± 2.00 nm; PDI: 0.18 ± 0.01 nm; ZP: -11.4 ± 0.25 mV	HepG2 cells	Cytotoxic with MTT and NRU assays: significant reduction of enzymatic activity at 40 and 80 µg/mL after 24 and 48 h; but no cytotoxicity after 6h (0, 1, 10, 20, 40 and 80 mg/ml) Interference checked with assay reagents but not with cells	Positive Without Fpg: Increased OTM from 10 µg/ml – dose dependent and increased %tail DNA from 20 µg/ml – dose dependent. With Fpg: Increased OTM at all doses – dose dependent and increased %tail DNA from 10 µg/ml.	Internalization concluded from flow cytometry as well as from TEM (apparently numerous individualized NPs inside the cells)

					Standard and Fpg modified Comet assay. Conditions tested: 1, 10, 20, 40 and 80 mg/ml for 6 h; 3 independent experiments; negative and positive controls.	
Guichard et al, 2012	TiO_2 A nano (Sigma 637254) Anatase, 14 nm (TEM); BET = 149 m^2/g, chemical impurity < 0.5% TiO_2 A micro (Sigma 232033) Anatase, 160 nm (TEM); BET = 9 m^2/g, chemical impurity < 0.5% TiO_2 R nano (Sigma 637262), rutile, 62 nm (TEM); BET = 177 m^2/g, 11% SiO_2, 1% Na_2O and 1% SO_4 TiO_2 R micro (Sigma 224227), rutile, 530 nm (TEM); BET = 3 m^2/g, chemical impurity < 0,5% TiO_2 P25 (Aeroxide), 80% anatase; 20% rutile, 25 nm (TEM); BET = 58 m^2/g, chemical impurity < 0,5%	Sonication for 20 min at 40 Watt using a sonicator bath. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and antibiotics (Penicillin, Steptomycin). At 80% confluence, cells were harvested using 0.25% trypsin and 0.53 mM ethylenediamine-tetraacetic acid (EDTA) and were sub-cultured into a culture support appropriate for the type of experiment selected. All particle suspensions in the SHE culture medium consisted of mainly agglomerated particles: particle diameters determined by DLS (dZ) varied from 300 to 700 nm depending on the particle type. Suspension of anatase TiO_2 nanoparticles led to coarser particle formation than its microsized counterpart. The contrary was observed for rutile TiO_2.	Syrian hamster embryo cells (SHE)	Cytotoxic based on decrease of RCC. Conditions tested: 0,5 - 200 $\mu g/cm^2$ for 24 and 72 hours => Nanoparticles were more cytotoxic than their micrometer counterparts.	TiO_2 A nano: Positive at 50 $\mu g/cm^2$ TiO_2 A micro: Positive from 25 $\mu g/cm^2$ Anatase produced the highest level of DNA damage, with no significant difference between nano and microparticles. TiO_2 R nano: Negative TiO_2 R micro: Positive from 25 $\mu g/cm^2$ TiO_2 P25: Positive at all concentrations. Comet assay. Conditions tested: 10, 25, 50 $\mu g/cm^2$ for 24 hours; 3 independent experiments; negative and positive controls. Genotoxicity as %Tail DNA	All particles are able to penetrate cells in the form of individual particles and agglomerates (TEM)

Hackenberg et al, 2011	TiO_2 Anatase < 25 nm (manufacturer specification) - 15-30 nm (TEM)	Sonication and dilution in PBS. In PBS: Only small fraction of particles dispersed. High level of compact aggregations: 285 ± 52 nm (TEM)	Human peripheral blood lymphocytes from 10 healthy male donors	Not cytotoxic: Percentage of stained cells < 20%, viable cells 81-94% in Trypan blue exclusion test. Conditions tested: 20, 50, 100, 200 µg/ml for 24h	Negative (Tail DNA, Tail length and OTM) Alkaline Comet assay. Conditions tested: 20, 50, 100, 200 µg/ml for 24 hours; 2 slides per cells; positive and negative controls.	The rate of cells with NP transferred to the cytoplasm was low: in 100 counted lymphocytes, intracytoplasmatic TiO_2-NPs could be demonstrated in 5 cells. Mainly large-sized particle aggregates up to 500 nm in diameter were seen and NP invasion into the nucleus was observed in one cell.
Woodruff et al, 2012	Anatase 10 nm; dry size distribution: 10x30nm: heavily aggregated not only in dry powder but also in solution (130-170 nm)	Dispersion by vortexing for 5 min followed by 10 min of bath sonication (size in solution treatment: around 130 nm). Then added to RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Size in cell culture: 170 nm.	TK6 human lymphoblastoid cells	Cytotoxic: significant concentration-dependent decrease of cell viability at all doses (at 200 µg/ml: viability about 55%) in Trypan blue exclusion assay. Conditions tested: 50-200 µg/ml for 24h.	Negative (%Tail DNA) Standard alkaline Comet assay and endoIII and hOGG1-modified Comet assays. Conditions tested: 0, 50, 100, 150, 200 µg/ml 24 hours; 3 independent assays; negative and positive controls.	Cells treated with 200 µg/ml TiO_2 in RMPI-1640 culture medium for 24 hours: TiO_2-NPs were found in nearly every TEM image prepared with the TK6 cells treated with the TiO_2-NPs
Vales et al. 2014	NM 102 Anatase; primary particle size: 21.7±0.6 nm	Nanogenotox protocol: pre-wetted in 0.5% absolute ethanol and afterwards dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water, the nanoparticles in the dispersion medium were sonicated for 16 min to obtain a stock dispersion of 2.56 mg/mL.	BEAS-2B cells	Not reported.	Negative (%Tail DNA) Alkaline and Fpg modified Comet assay. Conditions tested: 1, 10, 20µg/ml for 24h, 1 or 3 weeks. Experiment on duplicate cultures but done only once; negative and positive controls; high value of tail DNA in controls.	Uptake after 24h to 20 µg/ml (TEM)

		In exposure medium: 575.9 nm; PDI: 0.471 (DLS), ZP: -19.5 mV (LDV)				
Roszak et al. 2013	TiO_2 Mixture of rutile and anatase forms TiO_2 aggregates (SEM), irregular shape broad size distribution from 10 nm up to µm (ATM)	Prepared in MilliQ water DLS size = 220 nm (stable at least 72 h); ZP: 40 mV; Specific BET 27.1 m^2/g (non-porous). Then immediately (within 10 min) mixed with fresh culture medium and applied onto the cells. DLS size: 300 nm (stable 30 h) in culture medium with FBS (1:9)	Balb/3T3 fibroblasts	Cytotoxic at 100-250 µg/ml after 72h in WST1 reduction assay. Interference with applied detection systems was excluded. Error or number of cells seeded for cytotoxicity test seems to be very low.	Positive: slight but clear dose-effect in tail moment from 100 µg/ml after 3h exposure and at 250 µg/ml after 24h exposure. Not effect on tail intensity. Comet assay. Conditions tested not well developed; 10-250 µg/ml for 3 or 24h; 4 samples; negative and positive controls.	Not reported.
Shukla et al. 2011	Titanium (IV) oxide nanopowder; 99.7%, anatase, 10 nm	Dispersion in two different media (a) milliQ water: mean hydrodynamic diameter: 124.9 nm and ZP: -17.6 mV (b) DMEM supplemented with 10% FBS: mean hydrodynamic diameter: 171.4 nm and ZP: -11.5 mV Probe sonicated treatment at 30W for 10 min for both media	Human epidermal cell line (A431)	Cytotoxic with NRU and MTT assays at 8 and 80 µg/ml after 48 hours. Conditions tested: 0.008-80 µg/ml for 6, 24, 48 hours.	Positive Increased OTM from 8 µg/ml without Fpg and from 0.8 µg/ml with Fpg (dose-dependent) Increased %Tail DNA from 8 µg/ml without Fpg and from 0.8 µg/ml with Fpg (dose-dependent) Standard and Fpg modified Comet assay. Conditions tested: 0.008-80 µg/ml for 6h exposure; 3 independent experiments; 2 replicates; negative and positive controls.	Significant concentration-dependent increase in the cellular internalization of TiO_2 NPs after 6 h exposure (flow cytometer method). Subcellular localization of TiO_2 NPs inside cytoplasm and nucleus was confirmed using TEM
Kansara et al, 2015	NM 102 99.7%, anatase; 1.7±0.6 nm	Suspended in MilliQ water and complete DMEM F-12 medium. In MilliQ water: 106.7 ± 8.0 nm and 213 ±0.9 mV In complete DMEM F-12 medium supplemented with 10% FBS: 23.28 ± 2.0 nm and 10.1 ± 1.0 mV. Particles stable up to 72h.	A549 human pulmonary cells	Cytotoxic at 150 and 200 µg/ml after 48 h but not at 6 and 24 h in the MTT assay. Cytotoxic at 100, 150, and 200 µg/ml after 48 h exposure, but not at 6 and 24 hours in the NRU assay. Conditions tested: 1-200 µg/ml for 6, 24 and 48h.	Positive from 75 µg/ml (OTM and %Tail DNA) – dose-dependent. Comet assay. Conditions tested: 25-100 µg/ml for 6h; 3 independent experiments; 2 replicates; negative and positive controls.	Not reported.

				NP interference with assay reagent checked.		
Valdiglesias et al, 2013	TiO_2-S 100% anatase 25 nm ; 200-225 m^2/g TiO_2-D 80% anatase and 20% rutile; 25 nm ; 35-45 m^2/g	TiO_2 NPs suspended in either deionized water or complete cell culture medium (with FBS) and ultrasonicated at 30W for 5 min TiO_2-D: DI water: 160.5 nm; ZP:-27.8 mV Complete medium: 228.3 nm; ZP: -10.7 mV TiO_2-S: DI water: 447.9 nm; ZP:-9.96 mV Complete medium: 504.5 nm; ZP: -10.7 mV	Human SHSY5Y neuronal cells	Non cytotoxic in MTT and NRU assays Conditions tested: 20-150 µg/ml for 3, 6, 24 h. Interference: no interaction between NP and dyes used.	Positive TiO_2-S: increased %Tail DNA at all concentration in the 3h treatment group – dose-related and at 80 and 120 µg/ml in the 6h treatment group – not dose related. TiO_2-D: increased %Tail DNA at 150 µg/ml in the 3h treatment group – dose-related and at 80 and 120 µg/ml in the 6h treatment group – not dose related. Alkaline Comet assay. Conditions tested: 80, 120 and 150 µg/ml for 3 or 6 h A minimum of 3 independent experiments; 2 replicates; negative and positive controls.	Uptake increase with time of treatment (flow cytometry) – Uptake was always lower for TiO_2-D NPs than for TiO_2-S NPs
Gerloff, 2012	Aeroxide P25 Pyrogenic nanometric Anatase/rutile powder (77%/23%) Purity > 99.6%, ZP: -21.5 (water, pH 7.4); SSA: 52.6 m^2/g; primary particle mean diameter: 25.20 nm; Z-average hydrodynamic diameter: 214.5 nm (water, pH 9) (DLS)	Samples were suspended in serum free cellular media, sonicated for 10 min in a water bath sonicator and used directly. Cell culture media: MEM with Earle's salts and nonessential amino acids, supplemented with L-glutamine, and penicillin−streptomycin. **P25:** CE diameter in serum free cellular media (FPIA-3000): 11.8 µm	Caco-2 cells (human colon adenocarcinoma)	Cytotoxic at 80 µg/cm^2 at 4 (only LDH assay) and at 24h (WST-1 and LDH assays). Conditions tested: 20 or 80 µg/cm^2 for 4 or 24h.	Positive without Fpg The actual level of DNA damage was relatively close to the background, in contrast to the tested positive control and is therefore considered low. Negative with Fpg Standard and Fpg-modified comet assay. Conditions tested: 20 µg/cm^2 for 4 h; 3 experiments; negative and positive controls.	Not reported.

	TUFA/RII Nanometric anatase/rutile powder (90/10%) Purity > 99.7%; ZP: -23.5 (water, pH 7,4); SSA: 52.8 m^2/g; primary particle mean diameter: 21.90 nm; Z-average hydrodynamic diameter: 327.5 nm (water, pH 9) (DLS)	**TUFA/RII**:CE diameter in serum free cellular media (FPIA-3000): 12.0 µm **JRC12:** CE diameter in serum free cellular media (FPIA-3000): 6.5 µm **Hombikat UV100:** CE diameter in serum free cellular media (FPIA-3000): 6.1 µm **TFA**: CE diameter in serum free cellular media (FPIA-3000): 6.2 µm		Cytotoxic at 80 µg/cm^2 at 4 and 24h for WST-1and LDH assays. Conditions tested: 20 or 80 µg/cm^2 for 4 or 24h.	Negative Standard and Fpg-modified comet assay. Conditions tested: 20 µg/cm^2 for 4 h; 3 experiments; negative and positive controls.	
	JRC12 Nanometric anatase powder (100% anatase) Purity > 99.3%; ZP: -36.5 (water, pH 7,4); SSA: 282.3 m^2/g; primary particle mean diameter: 6.7 nm; Z-average hydrodynamic diameter: 455.2 nm (water, pH 9) (DLS)			Not cytotoxic in LDH assay. Cytotoxic in WST-1 assay at 20 µg/cm^2 after 4 hours and at 80 µg/cm^2 after 4 and 24h. Conditions tested: 20 or 80 µg/cm^2 for 4 or 24h.		
	Hombikat UV100 Nanometric anatase powder (100% anatase) Purity > 99.5%; ZP: -38,3 (water, pH 7,4); SSA: 342.4 m^2/g; primary particle mean diameter: 3.94 nm; Z-average hydrodynamic diameter: 291.1 nm (water, pH 9) (DLS)			Not cytotoxic in LDH assay. Cytotoxic in WST-1 assay at 80 µg/cm^2 after24h. Conditions tested: 20 or 80 µg/cm^2 for 4 or 24h.		

	TFA Fine (micrometric) anatase powder (100% anatase) Purity > 99%; ZP: -48,9 (water, pH 7,4); SSA: 10 m^2/g; primary particle mean diameter: 215 nm; Z-average hydrodynamic diameter: 374 nm (water, pH 9) (DLS)			Not cytotoxic in LDH assay. Cytotoxic in WST-1 assay at 20 µg/cm^2 after 4h. Conditions tested: 20 or 80 µg/cm^2 for 4 or 24h.		
Kermanizadeh et al, 2012	NM101: Hombikat UV100 Rutile with minor anatase 9 nm (XRD); 322 m^2/g; no known coating NRCWE 001 TiO_2 Rutile; Irregular euhedral particles; no coating 10 nm; 99 m^2/g NRCWE 002 TiO_2 Rutile; Irregular euhedral particles 10 nm with positive charge; 84 m^2/g NRCWE 003 TiO_2 Rutile; Irregular euhedral particles 10 nm with negative charge;84 m^2/g NRCWE 004 TiO_2 Rutile; 94 nm	Dispersion utilising MilliQ de-ionised water with 2% FCS. The NMs were sonicated for 16 mins without pause following the protocol developed for ENPRA. Following sonication, all samples were kept on ice until dilution in complete medium: MEM with FCS, Lglutamine, Penicillin/Streptomycin, sodium pyruvate, and non-essential amino acids Size in MEM: M101: 185.742 nm NRCWE 001 : 203 nm NRCWE 002 : 287 nm NRCWE 003: 240 nm NRCWE 004 : 339 nm	Human hepatoblastoma C3A cells	NM101: increased % cytotoxicity but not significant. NRCWE 001; 002; 003; 004: cytotoxic at all concentrations Conditions tested: 5-80 µg/cm^2 for 24h. Pre-treatment with Trolox (antioxidant) prevent the cytotoxicity.	NM101: Positive: at 20 µg/cm^2 without Fpg and from 5 µg/cm^2 with Fpg (most evident DNA damage) (%Tail DNA) – dose-dependent. NRCWE 001: Positive at 10 µg/cm^2 only with Fpg (small but significant increase in % tail DNA) – dose-dependent. Negative without Fpg NRCWE 002: Positive: at 5 µg/cm^2 without Fpg and at all doses with Fpg (most evident DNA damage) – dose-dependent. NRCWE 003: Negative NRCWE 004: Positive: at 10 µg/cm^2 only with Fpg (small but significant increase in % tail DNA) (small but significant increase in percentage tail DNA) – dose-dependent. Negative without Fpg	Not reported

					Standard and Fpg modified Comet assay. Conditions tested: 5, 10, 20 µg/cm^2 for 4h for NM101, NRCWE 003 and 004 or 2.5, 5, 10 µg/cm^2 for 4h for NRCWE 001 and 002. All experiments were repeated a minimum of three times; negative and positive controls.	
Kermanizadeh et al. 2013	NM101: Hombikat UV100 Rutile with minor anatase 9 nm (XRD); 322 m^2/g; no known coating NRCWE 001 TiO_2 Rutile; Irregular euhedral particles; no coating 10 nm; 99 m^2/g NRCWE 002 TiO_2 Rutile; Irregular euhedral particles 10 nm with positive charge; 84 m^2/g NRCWE 003 TiO_2 Rutile; Irregular euhedral particles 10 nm with negative charge;84 m^2/g NRCWE 004 TiO_2 Rutile; Appr. 100 nm Five different particle types were identified: 1) irregular spheres, 1–4 nm (av. Diameter); 2) irregular euhedral	NMs were dispersed in MilliQ deionised water with 2% FCS. The nanomaterials were sonicated for 16 mins without pause following the protocol developed for ENPRA. Following the sonication step, all samples were immediately transferred to ice. Size in complete renal cell medium (K-SFM): NM101: 221 nm NRCWE 001 : 349 nm NRCWE 002 : 314 nm NRCWE 003: 384 nm NRCWE 004 : 396 nm Size in RPMI with 10% FCS: M101: 358 nm NRCWE 001 : 337.5 nm NRCWE 002 : 378.8 nm NRCWE 003: 423.6 nm NRCWE 004 : 482.6 nm	Immortalized adult human renal proximal tubule epithelial cells HK-2	All of the TiO_2 were considered to be low toxicity materials as the LC_{50} was not reached. WST-1cell viability assay. Conditions tested: 0.16-80 µg/cm^2 (0.5-256 µg/ml) for 24h. Interferences: No toxicity of the dispersants to HK-2 cells.	NM101: positive at all concentrations witout and with Fpg (dose-dependent) (%Tail DNA) NRCWE 001 : negative (%Tail DNA) NRCWE 002 : positive at all concentrations without Fpg (not dose-related) and from 40 µg/ cm^2 with Fpg (dose-related) (%Tail DNA) NRCWE 003: negative without Fpg and positive at 40 µg/cm^2 with Fpg (not dose-related) (%Tail DNA) NRCWE 004 : positive at 40 µg/cm^2 without Fpg (dose-related) and from 20 µg/ cm^2 with Fpg (not dose-related) (%Tail DNA) Alkaline and Fpg Comet assay. Conditions tested: 20, 40, 80 µg/cm^2 (NRCWE 002, NM101, NRCWE 001) or 10, 20, 40 µg/cm^2 (NRCWE 004, NRCWE 003) for 4h; triplicate; negative and positive controls.	Not reported

	particles, 10–100 nm (longest dimension); 3) fractal-like structures in long chains, 100–200 nm (longest dimension); 4) big irregular polyhedral particles, 1-2 µm (longest dimension); 5) large irregular particles with jagged boundaries, 1–2 µm (longest dimension).					
Hackenberg et al. 2010	TiO_2-NPs Anatase ; < 25 nm	Dispersed in DI water. Then sonicated for 60s at a high energy level of 4.2×10^5 kJ/m^3 using a continuous mode to create an optimal grade of dispersion, BSA was added as a stabilizer at an end concentration of 1.5 mg/ml. Finally, PBS was added to achieve a physiological salt concentration and pH of 7.4. In stock suspension: 15-30 nm, high level of compact aggregations sized 285±52 nm. In particular cases, aggregates could reach diameters up to 2000 nm.	Human nasal mucosa cells from 10 donors	Not cytotoxic for both cytotoxicity tests: Trypan blue test: death cells below 20% and FDA assay: cell viability between 95 and 76%. Conditions tested: 10, 25, 50, 100 µg/ml for 24 h	Negative (tail DNA, tail length and OTM) Alkaline Comet assay. Conditions tested: 10, 25, 50, 100 µg/ml for 24h; negative and positive controls; 10 donors used per concentration; 2 slides per cells.	11% of the nasal mucosa cells presented nanoparticles in the cytoplasm. In cases of cell invasion, large-sized particle aggregates up to 1000 nm in diameter could be described, being surrounded by vesicles. Invasion into the cell nucleus was observed in 4%
Demir et al. 2015	Micro TiO_2 99% to 100.5% purity	No information	Human embryonic kidney (HEK293) Mouse embryonic fibroblast (NIH/3T3)	Only information from CBPI in the MN assay: Micro-TiO_2: no effect on CBPI Nano-TiO_2: decrease of CBPI	Micro-TiO_2: Negative with both cell lines (%DNA tail) Nano TiO_2 (21 and 50 nm): Positive at 1000 µg/ml (%DNA tail) only in the standard Comet assay – dose-dependent. Negative in the Fpg modified assay.	Not reported.
	Nano TiO_2 Anatase, 21 nm; > 99.5% purity	Dispersed at the concentration of 2.56 mg/mL prepared in a 0.05% bovine serum albumin (BSA) in water, subjected to ultrasonication at 20kHz for 16 min in an ice-cooled bath; (in agreement of the proposal from Nanogenotox EU project).				
	Nano TiO_2 Anatase, 50 nm, > 98% purity					

		No important agglomerations observed following the dispersion protocol used. Nano-TiO_2 (21 nm) : 22.94± 0.3 nm (DLS) and ZP : 8.71mV Nano-TiO_2 (50 nm) : 50.72±0.4 nm (DLS) and ZP: 9.38mV			Standard and modified Fpg Comet assay. Conditions tested: 10-100-1000 µg/ml for 1h; negative and positive controls; 2 independent experiments with 2 replicates. Very high concentrations tested.	
Ursini et al. 2014	Purity up to 97%; 1% Mn as dopant. Typical spherical shape characterized by extremely variable dimensions (TEM), 79/21% anatase/rutile, primary diameter: 43.8 + 17 nm (TEM); SSA: 14.9m^2/g (BET)	Solution was prepared in ultrapure sterile water, vortexed for 1 min and sonicated for 5 min. Then diluted in culture medium and sonicated in 2 steps of 5 min with a pause of 30s, before added to the cells. Cell culture medium for A549: RPMI-1640 with FBS. Cell culture medium for BEASB-2B: BEGM BulletKit. DLS: more negative ZP and smaller agglomerate sizes in water in respect to cell culture media. Agglomerate sizes were significantly smaller in RPMI medium with FBS than in BEGM medium (TEM and DLS). TiO_2 remained stable during the entire exposure period although in BEGM, TiO_2 suspension showed a very slight sedimentation after 24h (DLS). In water: ZP: -32.2 mV, 140 nm In RPMI/FBS: ZP: -9.13mV, 151 nm In BEGM: ZP: -11.7mV, 186 nm	A549 human pulmonary cells. Bronchial epithelial BEAS-2B	Cytotoxic with A459 cells only at 40 µg/ml and with BEAS from 10 µg/ml in WST-1 assay. Cytotoxic with A459 cells at 40 µg/ml after 30 min and 2 h and with BEAS-2B cells from 10 µg/ml at 30 min and 2 h in LDH assay. Conditions tested: 1-40 µg/ml for 24h (WST-1 assay) or 30 min, 2 and 24h (LDH assay).	Positive in A549 cells at 40 µg/ml, after 2 h, – with and without Fpg (%Tail DNA). Negative in BEAS-2B Standard and Fpg modified comet assay. Conditions tested: 1, 5, 10, 20, 40 µg/ml for 2 and 24h; 3 independent experiments; negative and positive controls.	Not reported.
	TiO_2-B		HepG2 cells		Non-irradiated TiO_2-A:	Not reported.

Petkovic, 2011a	> 100 nm; anatase, surface area: 8.6 m^2/g (BET) TiO_2-A < 25 nm, anatase, surface area: 129.3 m^2/g (BET)	UV pre-irradiation: 24h irradiation in a UV chamber. For both non-irradiated and UV-irradiated TiO_2 particles, the stock suspensions were prepared in PBS. These were sonicated for 30 min in an ultrasonic bath at a frequency of 60 kHz. These stock suspensions were subsequently diluted in cell-growth medium. These samples were then sonicated for 30 min before addition to the cells in culture. During the experimental work, illumination of the particles was avoided as much as possible; however, the experiments were not conducted in complete darkness. During the exposure of the cells to the TiO_2-A and TiO_2-B, the incubations were kept in complete darkness. In suspension: TiO_2-A: fast sedimentation; ZP in medium: -8.7 mV TiO_2-B: stable for days; ZP in medium: -13.5 mV		Non-irradiated TiO_2-A and TiO_2-B: not cytotoxic. Irradiated TiO_2-A and TiO_2-B: decreased viability at the 2 highest concentrations; already evident after 4h exposure. MTT assay. Conditions tested: 1-250 µg/ml for 4, 24 or 48h; positive control included.	Without Fpg: positive at the highest concentration after 2, 4 and 24h – dose-related. With Fpg: positive at 250 µg/ml at 2h, from 100 µg/ml at 4h and from 10 µg/ml at 24h – dose-related. Non-irradiated TiO_2-B: Without Fpg: negative at 2h but positive from 100 µg/ml at 4 and 24h – dose-related With Fpg: positive at 250 µg/ml at 2 h – dose-related but negative at 4 and 24h. Irradiated TiO_2-A: Without Fpg: positive from 10 µg/ml at 2h, at 250 µg/ml at 4h and 100 µg/ml at 24h – dose-related. With Fpg: positive at all doses at 2, 4 and 24h – dose-related. Irradiated TiO_2-B: Without Fpg: positive from 100 µg/ml at 2, 4 and 24h – dose-related. With Fpg: positive at all doses at 2 and 4h and from 10 µg/ml at 24h – dose-related. Standard and Fpg modified Comet assay. Conditions tested: 1-250 µg/ml for 2, 4 or 24h; 3 independent experiments; negative and positive controls. % tail DNA used.	
Petkovic, 2011b	TiO_2-An < 25 nm, anatase, BET: 129.3 m^2/g TiO_2-Ru	Powdered TiO_2 NPs were suspended in PBS and sonicated for 30 min in an ultrasonic bath at a frequency of 60 kHz, voltage of 220 V and an	HepG2 cells	Not cytotoxic. MTT assay. Conditions tested: 1-250 µg/ml for 4, 24 or 48h.	TiO_2-An Standard assay: positive at the highest dose at 2 and 24h; positive at 1 and 250 µg/ml at 4h – no clear dose-relation.	Not reported.

	< 100 nm, rutile, BET: 116.7 m^2/g	electric current of 0.5 A. This stock solution was then diluted in the complete cell growth medium. These samples were then sonicated for 30 min. Cell culture medium: EMEM containing fetal bovine serum, non-essential amino acid solution, glutamine and penicillin + streptomycin In the medium both types of TiO_2 NPs are highly aggregated and agglomerated with an average size of aggregates and agglomerate size at the micron level (TiO_2-An: 915 ± 453 nm; TiO_2-Ru: 1542 ± 760 nm). However, the portion of submicron-sized particles is much lower in the case of the TiO_2-An than in TiO_2-Ru.			With Fpg: positive from 10 µg/ml at 2 and 24h and from 100 µg/ml at 4h – dose-dependent. With Endo III: positive at 250 µg/ml at 2h, at 100 µg/ml at 4h and at 10 µg/ml at 24h – not dose-dependent. TiO_2-Ru Standard assay: negative at 2 and 24h; positive at 100 µg/ml at 4h – not-dose related. With Fpg: negative at 2 and 4h and positive at 10 and 100 µg/ml at 24h – not dose-dependent. With Endo III: positive at 250 µg/ml at 2h and 24h; but negative at 4h. Standard, Fpg and Endo III modified Comet assays. Conditions tested: 1, 10, 100, 250 µg/ml for 2, 4 or 24h; 3 independent experiments for standard Comet assay and 2 for Fpg and Endo III Comet assays; negative and positive controls.	

- **Chromosomal aberrations**

Table I-03. Summaries of the *in vitro* Chromosomal Aberration assays which fulfil our selected criteria (published between 2010-2015; characterization of the tested materials; data on uptake and/or cytotoxicity; presence of negative and positive controls and use of several replicates)

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	Results; Methods	
Browning et al. 2014	Aeroxide TiO_2 P25 spherical in shape, uncoated, vary between 78-85% anatase, 14-17% rutile and 0-13% amorphous; 25 nm; ZP: -36.4 mV; SSA: 46 m^2/g	Suspension in deionized water and probe sonicated at 10 KHz for 5 min. Dilutions prepared in cold deionized water filtered with a 0.22 µm filter. Dilution in complete media (serum containing) and media without serum. The media contained DMEM/F12 50 : 50 mixture, supplemented with cosmic calf serum, GlutaGRO, penicillin/ streptomycin and sodium pyruvate. In extracellular medium: peak distribution less than 100 nm when cells treated with 10 µg/cm^2 and at 225 nm when cells treated at 100 µg/cm^2. TiO_2 aggregates were comparable in size in the complete medium and the extracellular medium while they were larger in the serum-free media.	Primary human skin fibroblasts (BJ cells) Human skin fibroblast cells immortalized with hTERT (BJhTERT cells)	Not cytotoxic in a clonogenic survival assay. No effect on cell count. Conditions tested: 10-100 µg/cm^2 for 24h.	Negative Clastogenicity assay. Conditions tested: 10-100 µg/cm^2 for 24h; 3 independent experiments; negative and positive controls.	Identified in the cytoplasm, often associated with lysosomes and in the nucleus (monolayer of skin fibroblasts treated with 50 µg/cm^2 TiO_2 for 24h – TEM)
Catalan et al. 2012	TiO_2 from Sigma Aldrich; Anatase; 99.7% purity;< 25 nm; 222 m^2/g	Dispersion in RPMI-1640 medium with 15% foetal bovine serum and ultrasonication at 37 kHz for 20 min in a 37 C water bath.	Human lymphocytes	Not cytotoxic with propidium iodide incorporation method. Cytotoxicity did not reach the 50% level.	Positive: increased frequency of cells with CAs after the 48-h exposure (gap included, excluded and total CA) with a difference to the control at 300 µg/ml for chromatid-type CAs gaps excluded, from 12.5 µg/ml for chromatid type CAs gaps included, and from 100 µg/ml for total aberrations with or without gaps. Effects dose-dependent. Negative with 24 and 72h exposure.	Among the chromosomes, agglomerates of NM which were still left with the cells despite the multiples washes. It was especially difficult to separate TiO_2 from the cells and for a few metaphases, chromosomal

				Conditions tested: 6.25-600 µg/ml for 24, 48 or 72h. Mitotic index not affected in the CA test.	Chromosomal Aberrations assay. Conditions tested: 6.25-300 µg/ml for 24, 48 or 72h; duplicate; negative and positive controls.	aberrations examination could not be performed (microscopic images).

- **Non-standardized studies**

This section includes types of studies which do not follow any current recognized guidelines and are less commonly used than *in vitro* Comet assay. Only ɣ-H2AX assays detecting DNA double strand breaks were sorted based on our criteria.

Table I-04. Summaries of non-standardized *in vitro* studies which fulfil our selected criteria (published between 2010-2015; characterization of the tested materials; data on uptake and/or cytotoxicity; presence of negative and positive controls and use of several replicates)

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	Results; Methods	
Barillet et al. 2010	TiO_2-CEA 95% anatase; spherical, 12 nm (TEM) and 17 nm (BET), 92 m^2/g (SSA), PZC: 6.4	Nanoparticles were dispersed by sonication in ultrapure sterile water (pH 5.5). Then diluted in cell culture medium (DMEM medium supplemented with 50 IU/mL penicillin and 50 µg/mL streptomycin. TiO_2 were slightly agglomerated and/or aggregated when dispersed in water. In cell culture medium, NP agglomerated as 200-400 nm clusters except for TiO_2-Sigma particles which agglomerated to > 3 µm clusters.	NRK-52E rat kidney proximal cells	Not cytotoxic for exposure periods < 24h. After 48h exposure, cell mortality rapidly reached a plateau for concentrations higher than 20 or 40 µg/ml. Statistically lower cytotoxicity with the largest NP. No conclusion on the impact of crystalline phase on toxicity (MTT assay). Similar results in LDH assay. MTT and LDH release assays. Conditions tested: 0.25 to 100 µg/ml for 1-72h.	Negative ɣ-H2AX immunostaining. Conditions tested: 20-200 µg/ml for 24 h; at least 3 assays; negative and positive controls.	All NP were uptaken by cells. NP were localized in the cytoplasm, either in vesicles or isolated. Nanoparticles were rarely observed in cell nuclei (TEM).
	TiO_2–P25 (Aeroxide P25),89% anatase ; spherical, 24 nm (TEM) and 33 nm (BET), 46 m^2/g (SSA), PZC: 7					
	TiO_2-Sigma 100% anatase ; spherical, 142 nm (TEM) and 152 nm (BET), 10 m^2/g (SSA), PZC: 5.2					
	TiO_2-Sigma-R 100% rutile ; elongated, 68*9 nm (TEM), 118 m^2/g (SSA); silicium as impurity					
Jugan et al. 2012	TiO_2-A12 NP 95% anatase, spherical 12 nm; 92 m^2/g; PZC: 6.4	Dispersion in ultrapure sterile water (pH5.5) by sonication for 30 min at 4 C, in pulsed mode (1 s on/1 s off), NPs suspensions were diluted in DMEM without serum.	A549 human pulmonary cells	Cytotoxic MTT assay (1–200 µg/ml of NP suspensions for 4–48 h)	Negative ɣ-H2AX immunostaining. Conditions tested: 50-200 µg/ml for 24h; negative and positive controls.	Unambiguous accumulation of the smallest NPs in the cytoplasm and in the nucleus of cells
	TiO_2-A25 (AEROXIDE P25) 86% anatase/ 14% rutile, spherical 24 nm; 46 m^2/g; PZS: 7.0					Unambiguous accumulation of the smallest NPs in the cytoplasm

Valdiglesias et al, 2013	TiO_2-S 100% anatase, 25 nm ; 200-220 m^2/g TiO_2-D 80% anatase and 20% rutile; 25 nm ; 35-45 m^2/g	TiO_2 NPs suspended in either deionized water or complete cell culture medium (with FBS) and ultrasonicated at 30W for 5 min TiO_2-D: DI water: 160.5 nm; ZP:-27.8 mV Complete medium: 228.3 nm; ZP: -10.7 mV TiO_2-S: DI water: 447.9 nm; ZP:-9.96 mV Complete medium: 504.5 nm; ZP: -10.7 mV	Human SHSY5Y neuronal cells	Non cytotoxic in MTT and NRU assays. Conditions tested: 20-150 µg/ml for 3, 6, 24 h. Interference: no interaction between NP and dyes used	Negative ɣ-H2AX phosphorylation. Conditions tested: 80, 120, 150 µg/ml for 3 or 6h; minimum of 3 independent experiments; negative and positive controls.	Uptake increase with time of treatment (flow cytometry) – Uptake was always lower for TiO_2-D NPs than for TiO_2-S NPs

Conclusion on *in vitro* genotoxicity assays

The list of above studies was based on a literature research including published reviews, projects and studies (2010-2015) and on information from the registration dossier which has been published on ECHA website (date: 01/08/2015). All forms of TiO_2 have been taken into account. Due to the high number of *in vitro* genotoxicity assays found, an exhaustive reporting of studies was judged neither feasible nor of any added values and only the studies fulfilling the following criteria were summarized:

- Characterization of the tested material (at least size, crystallinity and coating);
- Information on dispersion and exposure protocols;
- Inclusion of negative and positive controls to validate the system and avoid under or over responses;
- Use of known or validated protocols;
- Use of replicates or independent experiments for *in vitro* assays;
- Evidence of uptake or cytotoxicity in case of negative results. Indeed, false negative results may be induced if there is no uptake of TiO_2 by the cells. This can be assessed by specific uptake data or by the presence of cytotoxicity. Furthermore, the time point selected for the genotoxicity endpoint measurement should be appropriately chosen.

Although a large number of data on *in vitro* genotoxicity of TiO_2 are available, most of the published results refer to nano-TiO_2 and especially to the anatase form as well as the mixture of anatase and rutile (generally P25). Very few studies assessed the genotoxicity of fine or coated TiO_2 as well as rutile forms (Table I-05).

Table I-05: Number of studies performed depending on the form of the tested TiO_2

Number of studies	MN assay	Comet assay	Chromosomal Aberrations	Non-standardized studies	Total
Nanoforms					
Anatase	12	16	1	2	31
Rutile	0	9	0	1	10
Mixture anatase/rutile	7	8	1	4	20
Coated-rutile	3	5	0	0	8
Total	22	38	2	7	69
Microforms					
Anatase	1	4	0	1	6
Rutile	1	1	0	0	2
Not defined	1	1	0	0	2
Total	3	6	0	1	10

Some studies included different genotoxicity assays and/or were performed with different forms of TiO_2. Each of them is counted in all the corresponding sections.

Most of the positive results were found in MN and Comet assays (Table I-06). Inconsistencies in the results of the studies may be the result of differences in test materials (size, crystallinity, coating…). Based on the table below, nanoforms seem to induce more positive results in *in vitro* genotoxicity studies. However, this impression comes mainly on the fact that very few studies on microforms are available. Furthermore, it can be suggested that anatase forms would be more cytotoxic than rutile or anatase/rutile ones because of photocatalytic properties of anatase (Xue, 2010; Wang, 2014). Based on *in vitro* genotoxicity studies, although some publications showed a higher genotoxicity potential of anatase (Petkovic, 2011b; Guichard, 2012; Tavares, 2014), other reported no difference as function of crystallinity (Jugan, 2012; Valdiglesias, 2013; Guichard, 2012). Despite a systematic review of the different characteristics that may explain the discrepancies observed in the studies, it remains difficult to highlight which parameter(s) can drive them. Inconsistencies in the results can also be explained by the various test conditions used, including dispersal of the material, concentrations and exposure duration, cell/organ examined and parameter assessed. It was also noticed that in several cases the statistical test performed was inappropriate that can lead to inappropriate interpretations and inconsistent results. Moreover, numerous interferences with TiO_2 can occur due to fluorescence and absorbance interaction, but also probable interactions with the proteins, the enzymes… used during the assay; unfortunately, these interferences are not properly tested in most of the publications. All these differences do not permit an easy comparison of the studies.

Table I-06: Number of positive results* depending on the form of the tested TiO_2

Number of experiments	MN assay	Comet assay	Chromosomal Aberrations assay	Others types of assays	Total
Nanoforms					
Anatase	9/15	34/53	1/3	0/2	44/73
Rutile	0/0	14/24	0/0	0/1	14/25
Mixture anatase/rutile	5/13	12/17	0/1	0/3	17/34
Coated-rutile	2/3	5/10	0/0	0/0	7/13
Microforms					
Anatase	0/1	10/16	0/0	0/1	10/18
Rutile	0/1	1/1	0/0	0/0	1/1
Not defined	0/1	0/4	0/0	0/0	0/5
Total	16/34	76/125	1/4	0/7	93/170

*According to the authors

One experiment was defined by one form of TiO_2 and a specific protocol (ex. cells, media, exposure-duration, standard or modified protocol…)

2. *In vivo* data

A literature research including published reviews, projects and studies was performed (ended on 30/04/2015). In addition, information from the registration dossier which has been published on ECHA website has been considered (date: 01/08/2015). All published available studies with any forms of TiO_2 have been summarized below.

- Micronucleus assays

Table I-07. Summaries of *in vivo* micronucleus assays found in the literature

Reference	Nanoparticle characterisation	Protocol	Results	NM uptake	Toxicity
Oral route					
Sycheva, 2011	TiO_2, simethicone Anatase, 33 nm	CBAxB6 male mice (5/group) TiO_2 dispersed in distilled water. 40, 200, 1000 mg/kg, gavage daily for 7 days. Poly-organ karyological assay, including bone marrow micronucleus assay 1000 PCE assessed for MN. 200 erythrocytes assessed for toxicity. Negative control included but no positive control.	Negative (bone marrow) Other results: No micronuclei, nuclear protusions or atypical nuclei in forestomach and colon and no micronuclei in spermatid but TiO_2 was cytotoxic in these tissues (mitotic index).	Not reported	Not reported.
	Micro-TiO_2, anatase, 160 nm		Positive (bone marrow, only at 1000 mg/kg) Other results: No micronuclei, nuclear protusions or atypical nuclei in the forestomach and colon and no micronuclei in spermatid but TiO_2 was cytotoxic in these tissues (mitotic index).		No cytotoxicity (immature PCE/total erythrocytes)
Nanogenotox WP6, 2013	NM-102 Anatase, about 20 nm, no coating	Rats Wistar, male (4-5/group) TiO_2 was prewetted in ethanol followed by dispersion in rat serum albumin in ultrapure water. Sonication on ice for 16 min. 6.5 to 26 mg/kg (NM-102, 103, 105) 7.5 to 31 mg/kg (NM-104) Gavage, 3 consecutive days, samples 3 to 6h after the last administration. Negative control. Methylmethanesulfonate as positive control.	Negative (bone marrow)	Small detectable Ti content only at the highest dose (liver, kidney, spleen, stomach, jejunum and colon). Highest Ti concentration in liver and spleen for NM-105. Ti was also detected in the GI tract.	Gavage was well tolerated in all animals, expect diarrhea in 3 animals on day 3 exposed to 26 mg/kg bw of NM-105. No cytotoxicity as observed by PCE/NCE ratio.
	NM-103 Rutile, about 20 nm, 2% organic coating In suspension: agglomerate: 80-90 nm				
	NM-104 Rutile, about 20 nm, 2% organic coating In suspension: agglomerate: 80-90 nm				
	NM-105 Rutile/anatase, about 25 nm				

	In suspension: agglomerate: 80-90 nm				
Chen et al 2014	Anatase Purity: 99.90% 75 ± 15 nm, specific surface area: 63.95 m^2/g In exposure medium, TiO_2 tend to agglomerate into 473.6 nm and 486.8 nm size when suspended in H_2O and FBS-free DMEM.	Rats SD, male (7/group) TiO_2 dispersed in ultrapure water and ultrasonic vibrated for 15 min. 10, 50, 200 mg/kg by intragastric administration, once a day for 30 consecutive days. Sacrifice immediately after the last administration. 1000 PCE scored for MN and 200 erythrocytes for PCE/NCE ratio. Negative control but no positive control.	Negative (bone marrow)	Not reported.	No abnormal behaviour and symptoms, no significant changes in the body weight. No cytotoxicity as observed by PCE/NCE ratio.
Trouiller et al 2009	P25, purity ≥ 99.5% TiO_2 75% anatase / 25% rutile, 21 nm, specific surface area: 50±15 m^2/g In water: mean size: 160 nm.	C57Bl/6Jp^{un}/p^{un} mice (5/group) TiO_2 dispersed by ultrasonication for 15 min 50, 100, 250, 500 mg/kg for 5 days in drinking water. 2000 erythrocytes scored per animal. Negative control included but no positive control.	Positive at 500 mg/kg bw/day (peripheral blood erythrocytes)	Not reported.	Inflammation: upregulation of pro-inflammatory cytokines.
Registration data 2014-07-22 2014-07-30	TiO_2 pg-1 Rutile= 10.7% Anatase= 89.3% Particle size: D_{50} (laser diffraction, 10mg/mL loading) =3.691 µm D_{50} (TEM ECD) = 23 nm D_{50} (corrected XSDC) =20 nm Density (g/cm^3)= 3.861 BET surface area= 50.4m^2/g	Crl:CD(SD) rat male/female (5/sex; except for 2000 mg/kg bw: 7/sex) TiO_2 in sterile water 500, 1000, 2000 mg/kg bw once by gavage Blood samples collected at 24 and 72h after treatment. Whenever feasible, at least 20,000 reticulocytes analyzed per blood sample Negative and positive control (cyclophosphamide) included.	Negative (peripheral blood erythrocytes)	Single oral gavage administration resulted in no discernible dose-dependent increases of TiO_2 in the blood and liver of treated rats relative to control rats.	No toxicity

	TiO_2 uf-3 Rutile Particle size D_{50} (laser diffraction, 10mg/mL loading): 11.22 µm, D_{50} (TEM ECD):22 nm D_{50} (corrected XSDC):24 nm Density(g/cm³): 3.999 BET surface area: 58.8m²/g			Not reported	No toxicity
Registration data **2014-05-04** **2014-07-30**	TiO_2 pg-2 Rutile Particle size D_{50} (laser diffraction, 10mg/mL loading):1.734 µm, D_{50} (TEM ECD):165 nm D_{50} (corrected XSDC):162 nm BET surface area= 7.1 m²/g TiO_2 pg-3 Rutile Particle size D_{50} (laser diffraction, 10mg/mL loading):0.349 µm, D_{50} (TEM ECD):132 nm D_{50} (corrected XSDC):179 nm Density(g/cm³): 3.976 BET surface area: 17.1 m²/g	Wistar rat male/female (5/sex) TiO_2 in sterile water 500, 1000, 2000 mg/kg bw once by gavage Blood samples collected at 24 and 72h after treatment. At least 20,000 immature erythrocytes per animal scored Negative and positive control (cyclophosphamide) included.	Negative (peripheral blood erythrocytes)	Not reported	No toxicity
Registration data **2014-05-06** **2014-07-30**	TiO_2 uf-2 Anatase Particle size D_{50} (laser diffraction, 10mg/mL loading): 1.349 µm, D_{50} (TEM ECD): 19 nm	Wistar rat male/female (5/sex) TiO_2 in sterile water 500, 1000, 2000 mg/kg bw once by gavage Blood samples collected at 24 and 72h after treatment. At least 20,000 immature erythrocytes per animal scored	Negative (peripheral blood erythrocytes) Statistically significant increase of MN at 200 mg/kg in males at 72h but within the range of	Not reported	No toxicity

	D_{50} (corrected XSDC): 19 nm BET surface area= 82 m^2/g	Negative and positive control (cyclophosphamide) included.	control data mentioned in the literature		
Registration data 2011-02-21	H-29865	ICR mice male/female (10/sex, except high dose with 14/sex) TiO_2 in aqueous methylcellulose prepared with deionized water Single dose 500, 1000, 2000 mg/kg Sacrifice 24 and 48 after treatment At least 20,000 immature erythrocytes per animal scored Negative and positive control (cyclophosphamide) included.	Negative (bone marrow)	Not reported	No toxicity
Inhalation route					
Lindberg et al 2012	74% anatase and 26% brookite Primary particle size: 21 nm, specific surface area = 61 m^2/g Geometric mean mobile diameter of the aerosol was about 80 nm	C57Bl/6 mice (6/group) 0.8, 7.2, 28.5 mg/m^3 4h/day for 5 days, whole body inhalation. Blood sample collected 48h after the last exposure. 2000 PCE and 2000 NCE per mouse scored for MN. Negative control. Ethylene oxide as gaseous positive control.	Negative (peripheral blood erythrocytes)	Content of TiO_2 retained in the lung was less than 10% of the inhaled dose	Inflammatory response as percentage of neutrophils among BALf cells at 28.5 mg/m^3. No cytotoxicity as observed by PCE/NCE ratio.
Intra-tracheal route					
Nanogenotox WP6, 2013	NM101 Anatase, <10 nm, 8% organic coating In suspension: agglomerate: 140-150 nm NM102 Anatase, about 20 nm, no coating In suspension: agglomerate: 140-150 nm NM 103 Rutile, about 20 nm, 2% organic coating In suspension: agglomerate: 80-90 nm NM104 Rutile, about 20 nm, 2% organic coating	Rats SD, male (4-5/group) TiO_2 was prewetted in ethanol followed by dispersion in rat serum albumin in ultrapure water. Sonication on ice for 16 min. 1.15, 2.3, 4.6 mg/kg by IT, 3 consecutive days, samples 3 to 6h after the last administration. A total of 203 slides were received in duplicate. Bone marrow slides of 40/115 animals could not be read due to the abundance of cells in the slide preparation. Both slides from the right and left femurs were scored. Negative control. Methylmethanesulfonate as positive control.	Negative (bone marrow)	Not reported	No cytotoxicity as observed by PCE/NCE ratio.

	In suspension: agglomerate: 80-90 nm NM 105 Rutile/anatase, about 25 nm In suspension: agglomerate: 80-90 nm				
Intravenous route					
Sadiq et al 2012	**TiO_2-NP Anatase, 10 nm, powder form**	**B6C3F1 mice, male (5/group) TiO_2 was suspended in PBS with vigorous mixing ans sonication. 0.5, 5.0, 50 mg/kg, 3 consecutive days by IV Blood collected on day 4 was used to measure MN. Frequency of MN-RET was determined on approx. $2x10^4$ CD71-positive RETs for each animal. Negative control and ENU as positive control.**	**Negative (blood)**	**Analysis of Ti level in bone marrow after administration of 50 mg/kg: Ti levels increased at 4, 24 and 48h (x 12.1-14.2) after the last treatment.**	**Reduction in %RET on day 4 suggested a treatment related cytotoxicity. A rebound was recorded on week 1 and level was normal later.**
Xu et al 2013	TiO_2-NP Purity: 99.99% Anatase, 40 nm Impurities: Pb < 2 ppm, Cd < 1 ppm, As < 1 ppm, Hg < 1 ppm, Ni < 1 ppm. Size distribution of TiO_2 aggregates in saline.	ICR mice, male and female (4/sex/group) TiO_2 was suspended in saline by sonication for 30 seconds. The particle suspensions were kept on ice for 15 seconds and sonicated on ice for 3 min. 140, 300, 645, 1387 mg/kg by IV, once. Sacrifice 14 days after treatment. Due to mortality, the dose of 1387 mg/kg was only used for histopathology of tissues and not assessed for MN. 1000 PCE/animal analysed for MN. Negative control and cyclosphamide as positive control.	Negative (bone marrow)	Not reported.	Decreased food and water intake, decreased physical activity, mortality at 1387 mg/kg. Biochemical changes with damage in brain, lung, spleen, liver and kidney.
Dobrzynska et al 2014	NM 105 (P25) Anatase/rutile, 21 nm	Rats Wistar, male (7) TiO_2 dispersed in H_2O with DMSO and sonicated for 5 min 5 mg/kg by IV, sacrifice after 24h, 1 and 4 weeks 200 PCE and 100 reticulocytes per rat analyzed for MN. Negative control included but no positive control.	Negative (bone marrow reticulocytes) Positive only after 24 hours (bone barrow PCE) – not after 1 or 4 weeks.	Not reported.	No cytotoxicity to bone marrow's red and white blood cells
Louro et al 2014	NM102 Anatase non coated, 22 nm	LacZ transgenic C57Bl/6 mice (5-6/group) TiO_2 was pre-wetted in ethanol followed by addition of sterile-filtered serum albumin and probe sonication for 16 min. 10 and 15 mg/kg on 2 consecutive days by IV, sacrifice 42 h after the last injection.	Negative (peripheral blood)	Uptake in the liver: colorless, irregularly sized and shaped particles (about 1-1.5 µm) of refractory material diffusively present in the tissue,	No changes in body weight, behaviour or general health. No gross macroscopic changes at necropsy. Leukocytic aggregation and infiltration suggest a

		2000 reticulocytes per mouse scored for MN. Percentage of reticulocytes as measure of toxicity. Negative control. N-ethyl-N-nitrosurea as positive control.		either inside of between hepatocytes as well as inside macrophages (including Kupffer cells) at both doses. Particles also found inside some of the nuclei of hepatocytes without clear dose-related effect.	low-moderate inflammatory response. No decrease in the percentage of reticulocytes suggesting that NM-102 was not cytotoxic.
Nanogenotox WP6, 2013	NM 103 Rutile, about 20 nm, 2% organic coating NM104 Rutile, about 20 nm, 2% organic coating	Rats male and female TiO_2 was prewetted in ethanol followed by dispersion in rat serum albumin in ultrapure water. Sonication on ice for 16 min. 8.7 – 9.7 mg/kg for males and 12.4 – 13.7 mg/kg for females by IV, single or 5 days injection, sacrifice 24h after the last injection Negative control. Methylmethanesulfonate as positive control.	Negative (bone marrow)	Not reported	Not reported
Intraperitoneal route					
Saghiri et al 2012	TiO_2-NP Anatase, 20 nm	Balb/c mice, male (4/group) TiO_2 was diluted in sterile double-distilled water and ultrasonicated. 100, 500, 1000 mg/kg single IP administration, sacrifice 24h after. 2000 PCE scored per animal for MN. Negative control included but no positive control.	Positive (bone marrow at 1000 mg/kg– dose dependent) To be noted: no MN detected in the control	Not reported.	Increase of mitotic index in bone marrow.
Rad et al 2013	TiO_2-NP Purity: 98.8%, spherical Anatase, 10 nm	Balb/C mice, male (4/group) TiO_2 dissolved in distilled water by ultrasonic for 5 min. 10, 50, 100, 500, 800 mg/kg by IP, once. Sacrifice 24h after injection. 200 PCE per animal scored for MN. Negative control included but no positive control.	Positive (bone marrow at 500 and 800 mg/kg)	Not reported.	Decreased LDH at 10, 50 and 100 mg/kg.
Song et al 2011	TiO_2-NP 19.7-101.0 nm, surface area: 15-77 m^2/g	ICR mice, female (4-6/group) TiO_2 was suspended in saline with Tween 80. 1 and 3 mg/mouse by IP once. Peripheral blood collected from the tail 48 h after injection. 3000 reticulocytes per animal scored for MN. Negative control included but no positive control.	Positive (peripheral blood at 3 mg/mouse; result at 1 mg not presented)	Not reported.	Not reported.

El Ghor et al 2014	TiO_2 NP rutile and anatase < 100 nm In H_2O: particle size was between 45 to 51 nm (XRD, TEM or DLS) In CHL: particle size was between 41 to 44 nm (XRD, TEM or DLS).	Swiss Webster mice, male (5/group) TiO_2 suspended in deionized distilled water or chlorophyllin. 500, 1000, 2000 mg/kg by IP, for 5 consecutive days 2000 PCE/animal were scored for MN. Negative control. Cyclophosphamide as positive control.	Positive at all tested doses (bone marrow) – dose-response relationship Co-administration with chlorophyllin (free radical scavenger) decreased MN frequency and increased PCE/NCE.	Accumulation in bone marrow > liver > brain (inductively coupled plasma-mass spectrometry) at all doses.	No cytotoxicity as observed by PCE/NCE ratio.
Shelby, 1993 & 1995	TiO_2	Male B6C3F1 mice (5/group) 250, 500, 1000 mg/kg by IP administration for 3 consecutive days, sacrifice 24h after the third treatment. 2000 PCE/animal were scored for MN. Negative control included. Dimethylbenzanthracene and mitomycin used as positive controls. Study repeated with 500, 1000, 1500 mg/kg.	Positive (bone marrow at 1000 mg/kg in both studies)	Not reported.	No decrease in % PCE.

All the above studies were summarized regardless of their reliability. However, in order to make a reliable assessment of these results, different key parameters need to be taken into account. First, the tested material needs to be characterized (at least size, crystalline phase and coating). Secondly, the inclusion of a negative and a positive control is required to validate the system and thus the results. Finally, the negative results should be taken into account only when it has been proven that the nanoparticles have reached the organ investigated. This could be confirmed with data on uptake or if (cyto)toxicity was detected. However, most of the studies did not show any cytotoxic effect in bone marrow or have not investigated uptake in this tissue. In summary, only one study (Sadiq, 2012 – study reported in bold in the table) fulfills the above criteria (characterization data, negative and positive controls and evidence of uptake or cytotoxicity in case of negative results).

- Comet assays

Table I-08. Summaries of *in vivo* Comet assays found in the literature

Reference	Nanoparticle characterization	Protocol	Results	NM uptake	Toxicity
		Oral route			
Carmona, 2015	**TiO_2-NP** **Anatase, < 25 nm, surface area : 45-50 m^2/g, 99.7% purity** **High level of agglomeration (average: 85.88 nm) in dry form and larger agglomeration in water suspension (average: 405.3 nm)**	***Drosophila melanogaster* flr^3 strains** **TiO_2-NP diluted in ultrapure water and dispersed by sonication for 30 min.** **TiO_2 bulk was diluted in distilled water by mixing for 10 min.** **Third-instar larvae placed in vials with medium and TiO_2 at 0.08, 0.40, 0.80, 1.60 mg/ml and fed during 24 ± 2h. Then hemocytes were collected for Comet assay.** **Genotoxicity measured as % DNA tail.** **Negative control. Ethyl methane sulphonate used as positive control.**	**Positive (hemocytes from 0.40 mg/ml – dose-dependent)**	**Not reported.**	**Larval viability was increased up to 1.60 mg/ml (> 90%) in a preliminary tryptan blue assay.** **Significant dose response damage for midgut and imaginal discs for 0.80 and 1.60 mg/ml TiO_2 NP for 24 and 48h.**
	TiO_2-bulk 45 µm, 99% purity		Negative (hemocytes)		
Nanogenotox WP6, 2013	NM-102 Anatase, about 20 nm, no coating	Rats Wistar, male (4-5/group) TiO_2 was prewetted in ethanol followed by dispersion in rat serum albumin in ultrapure water and sonication on ice for 16 min. Standard and Fpg modified Comet. 6.5, 13, 26 mg/kg (NM-102 and 105) 6, 12, 24 mg/kg (NM-103) 7.5, 15, 31 mg/kg (NM-104) Gavage, 3 consecutive days, samples 3 to 6h after the last administration. Genotoxicity measured as median %DNA in the tail. Negative control and Methylmethanesulfonate as positive control.	Without Fpg: Positive (spleen at all doses – not dose-related) Negative (colon, liver, kidney, lymphocytes, bone marrow, jejunum) With Fpg: Positive in colon at 26 mg/kg and negative in jejunum	Small detectable Ti content only at the highest dose (liver, kidney, spleen, stomach, jejunum and colon). Highest Ti concentration in liver and spleen for NM 105. Ti was also detected in the GI tract. Biokinetics studies were performed independently and there was no quantification done in the bone marrow.	Gavage was well tolerated in all animals, expect diarrhea in 3 animals on day 3 exposed to 26 mg/kg bw of NM-105.
	NM-103 UV Titan M262 Rutile, about 20 nm, 2% organic coating	Without Fpg: Positive (spleen at 13 mg/kg – not dose-related, jejunum at 24 mg/kg – dose-related) Negative (liver, kidney, lymphocytes, bone marrow, colon) With Fpg: Negative in colon and jejunum			
	NM-104 UV Titan M212	Without Fpg:			

	Rutile, about 20 nm, 2% organic coating		Positive (spleen at all doses – dose-related and bone marrow at 31 mg/kg) Negative (liver, kidney, lymphocytes, colon, jejunum) With Fpg: Negative in colon and jejunum		
	NM-105 Rutile/anatase, about 25 nm, no coating		Without Fpg: Positive (spleen and colon at 26 mg/kg bw) Negative (liver, kidney, lymphocytes, bone marrow and jejunum) With Fpg: Negative in colon and jejunum		
Sycheva et al 2011	TiO_2, simethicone Anatase, 33 nm	CBAxB6 male mice (5/group) TiO_2 dispersed in distilled water. 40, 200, 1000 mg/kg, gavage daily for 7 days. Genotoxicity measured as % tail DNA at 40 and 200 mg/kg. Negative control included but no positive control.	Positive (bone marrow at 40 and 200 mg/kg and liver at 200 mg/kg) Negative (brain)	Not reported	Not reported
	Micro-TiO_2 Anatase, 160 nm		Positive (bone marrow at 40 and 200 mg/kg) Negative (brain and liver)		
Trouiller et al 2009	P25 Purity ≥ 99.5% TiO_2 75% anatase / 25% rutile, 21 nm, specific surface area: 50±15 m^2/g In water: mean size: 160 nm.	C57Bl/6Jp^{un}/p^{un} mice (5) TiO_2 dispersed by ultrasonication for 15 min in distilled water. 500 mg/kg for 5 days in drinking water Genotoxicity measured as tail moment. Negative control included but no positive control.	Positive (blood)	Not reported	Inflammation: upregulation of pro-inflammatory cytokines.
Inhalation route					
Landsiedel et al 2010	T-LiteTM SF Rutile TiO_2 (purity of TiO_2 ≥ 99.9%) coated with aluminium hydroxide and dimethicone/methicone copolymer (TiO_2 content: 79-89%) 10*50 nm, mean agglomerates about 200	Wistar Crl:WI Han male rats (3 animals) Comet assay included in a 5-day head-nose inhalation study performed at 0.5, 2 and 10 mg/m^3, 6h/day on 5 consecutive days. The Comet assay was performed in the lungs of "recovery animals" at 10 mg/m^3 (at post-exposure 3 weeks). Genotoxicity measured as relative tail intensity, tail moment and mean tail length. Negative control included but no positive control.	Negative (lung)	Not reported	No effects on clinical signs, mean body weights. Slight to moderate increases of neutrophils and monocytes, of total protein and of activities of LDH, GGT, ALP and NAG in the BALf at 2 and 10 mg/m^3. Effects partially reversible within the post-exposure

	nm, specific surface area: 100 m^2/g				observation period of 3 weeks. Number of hedgehog images (highly damaged cells) was not influenced by TiO_2. Mean cells viability was 88.7% vs 95% in control.
Lindberg et al 2012	74% anatase and 26% brookite Primary particle size: 21 nm, specific surface area = 61 m^2/g Geometric mean mobile diameter of the aerosol was about 80 nm	C57Bl/6 mice (6/group) 0.8, 7.2, 28.5 mg/m^3 4h/day for 5 days, whole body inhalation. Alveolar type II and Clara cells were collected immediately after the last exposure. Genotoxicity measured as %DNA in the comet tail. Negative control. Ethylene oxide as gaseous positive control.	Negative (lung)	Content of TiO_2 retained in the lung was less than 10% of the inhaled dose	Inflammatory response as percentage of neutrophils among BAL cells at 28.5 mg/m^3
Creutzenberg et al 2009 (only abstract – limited level of details)	P25 80% anatase and 20% rutile 20 nm Substantial number of particles < 100 nm in phosphate buffer	Rats (no further information) TiO_2 dispersed in phosphate buffer with mechanical and ultrasonic treatment. 2, 10 mg/m^3, 6h/day for 21 days, inhalation. Post-observation after 3, 28 and 90 days. hOGG-1-modified comet assay.	Positive (alveolar macrophages on day 28)	Estimated final lung burdens amounted to 205 and 1240 µg/lung for P25 and 1150 and 5760 µg/lung for microscale TiO_2.	Decreases in white blood cells in the high dose at days 28 and 90 for both dusts and on day 3 for P25
	Microscale TiO_2 (Bayertitan T) Rutile type, 1.1 µm	Rats (no further information) TiO_2 dispersed with pressurized air. 9 and 45 mg/m^3, 6h/day for 21 days, inhalation. Post-observation after 3, 28 and 90 days. hOGG-1-modified comet assay.	Negative (alveolar macrophages on day 28)		
Jackson et al 2013	UV Titan L181 Rutile surface coated, 17 nm, surface area: 70 m^2/g Chemical composition: Na_2O (0.6%), SiO_2 (12.01%), Al_2O_3 (4.58%), ZrO_2 (1.17%), TiO_2 (70.81%). UV-Titan is coated with polyalcohol adding to the remaining wt %. Geometric mean size during inhalation exposure: 97 nm	Time-mated C57BL/6Bom-Tac female mice (22-23/group) 42 mg/m^3 (total inhaled dose: 840 µg/animal), 1h/day, inhalation, whole body exposure during gestation days 8-18. Genotoxicity was measured as %DNA in the tail and tail length. Negative control included but no positive control.	Negative (BAL and liver in the non-pregnant females and dams; liver in the newborn at PND 2 or weaned offspring at PND 22).	Estimated deposition: 73 µg/animal in pulmonary region; 315 µg/animal in extra-pulmonary region; 365 µg/animal in gastro-intestinal tract.	Persistent inflammation in mothers and affected gene expression in the liver of offspring, with increased response in female offspring. The observed changes in gene expression in the newborn offspring 2 days after birth suggest that anti-inflammatory processes were activated in the female offspring related to retinoic acid signaling.

Intra-tracheal route					
Naya et al 2012	**Anatase (ST-01), 5 nm** **In the DSP (disodium phosphate) solution: no change in secondary particle size (19 nm), no surface coating, purity = 99.99%**	**Rats SD, male (5/group)** **TiO_2 dispersed in DSP and agitated for 2h.** **Single IT administration of 1 or 5 mg/kg, sacrifice 3 or 24h later or repeated IT administration of 0.2 or 1 mg/kg bw once a week for 5 weeks.** **DNA damage measured as % DNA in the tail.** **Negative control. Ethylmethanesulfonate as positive control.**	**Negative (lung)**	**Deposition of test compound at 1 mg/kg in the repeated instillation experiment.**	**Deposition of test compound and infiltration of alveolar macrophages at 5 mg/kg in the single instillation experiment.** **Deposition of test compound, infiltration of alveolar macrophages and neutrophils and thickening of alveolar wall at 1 mg/kg in the repeated instillation experiment.**
Nanogenotox WP6, 2013	NM-101 Anatase, < 10 nm, 8% organic coating NM-102 Anatase, about 20 nm, no coating NM-103 Rutile, about 20 nm, 2% coating NM-104 Rutile, 20 nm about 20 nm, 2% coating NM-105 Rutile/anatase, about 25 nm, no coating	Rats SD, male (4-5/group) TiO_2 was prewetted in ethanol followed by dispersion in rat serum albumin in ultrapure water and sonication on ice for 16 min. 1.15, 2.3, 4.6 mg/kg by IT, 3 consecutive days, samples 3 to 6h after the last administration. Genotoxicity measured as median %DNA in the tail. Negative control. Methylmethanesulfonate as positive control. It should be noted that the positive control was not valid for the lungs.	Negative (BAL, lung, spleen, liver, kidney) Very slight increase of DNA damage (BAL) for NM-105	Not reported	Viability of the BAL cells was between -9% and + 11%. All materials showed a dose dependent increase in neutrophils (greater with NM-103, 104 and 105) suggesting a pulmonary inflammation.
Saber et al 2012	UV Titan L181 Rutile (coated with Si, Al, Zr and polyalcohol). Crystalline size: 20.6 nm, specific surface area: 107.7 m^2/g. In suspension used for instillation: aggregates of ca. 100 nm or larger.	C57BL/6 mice, female TiO_2 was suspended by sonication for 16 min in NaCl MilliQ water with acellular BAL from mice. Single exposure to 18, 54, 162 µg per animal by IT. Sacrifice 1, 3 and 28 days after exposure. Genotoxicity measured as normalized tail length. Results were normalized to the positive assay control with H_2O_2 exposed A549 cells. Negative control included, but no positive control.	Negative (BAL fluid) Positive (liver at day 1 only at 162 µg – dose-response relationship)	Not reported	Inflammation in BAL: higher total number of BAL cells on day 1 at 54 µg and at all time-points at 162 µg. Higher neutrophils counts on days 1 and 3 at 54 µg and at all time-points at 162 µg (moderate after 28 days). Slight histopathological changes in the liver (small foci of inflammatory cells, hyperplasia of connective tissue perivascular, necrosis in cenrilobular area, binucleate hepatocytes,

					increased number of Kupffer cells) at 162 µg.
Intravenous route					
Louro et al, 2014	**NM102** **Anatase non coated, 22 nm**	**LacZ transgenic C57Bl/6 mice (5-6/group)** **TiO_2 was pre-wetted in ethanol followed by addition of sterile-filtered serum albumin and probe sonication for 16 min.** **10 and 15 mg/kg by IV on 2 consecutive days, sacrifice 28 days after the last injection.** **Genotoxicity measured as %DNA in tail and OTM (olive tail moment).** **Negative control. N-ethyl-N-nitrosurea (ENU) as positive control.**	**Negative (liver and spleen)** **To be noted: ENU did not induce an increase of the mean percentage of tail DNA in the spleen.**	**Uptake in the liver: colorless, irregularly sized and shaped particles (about 1-1.5 µm) of refractory material diffusively present in the tissue, either inside of between hepatocytes as well as inside macrophages (including Kupffer cells) at both doses. Particles also found inside some of the nuclei of hepatocytes without clear dose-related effect.**	**No changes in body weight, behaviour or general health. No gross macroscopic changes at necropsy.** **Leukocytic aggregation and infiltration suggest a low-moderate inflammatory response.**
Dobrzynska et al 2014	NM 105 (P25) Anatase/rutile 21 nm	Rats Wistar, male (7) TiO_2 dispersed in H_2O with DMSO and sonicated for 5 min 5 mg/kg by IV; sacrifice after 24h, 1 week and 4 weeks. Genotoxicity measured as tail moment and %DNA in Comet tail. Negative control included but no positive control.	Negative (bone marrow leukocytes)	Not reported	No cytotoxicity to bone marrow's red and white blood cells
Meena et al 2015	TiO_2-NP containing of elemental titanium 66% and oxygen 34% atoms. 10-20 nm	Wistar male rats (6/group) TiO_2 dissolved in distilled water and ultrasonicated for 10 min. 5, 25, 50 mg/kg TiO_2 in PBS by IV weekly for 30 days. Sacrifice after treatment. Genotoxicity measured as tail length, tail movement and tail migration. Negative control included but no positive control.	Positive at 25 and 50 mg/kg (sperm cells) Clear dose-response relationship.	Dose-related accumulation of TiO_2 in the testes (energy dispersive X-ray fluorescence spectroscopy). TiO_2 was localized in the cytoplasm, mostly in membranous compartments, including lysosomes and mitochondria, thecal organelles, besides particles	Decreased activity of antioxydative enzymes (SOD and GPx); increased activity of CAT at 25 and 50 mg/kg. Increased lipid peroxidation activity (MDA) at 25 and 50 mg/kg. Increase of mean value of creatinine kinase activity (sperm energy transport) at 50 mg/kg. Activation of caspase-3 (apoptosis) at 50 mg/kg.

				existing out of cells, surrounding cells.	Induction of apoptosis was confirmed by DNA fragmentation assay. Decrease in total sperm count and increase in apoptotic cell population at 50 mg/kg. Decreased serum testosterone level at 25 and 50 mg/kg. Moderate pathological change at 50 mg/kg: abnormal testicular morphology with some inflammation in testicular cells.
Intra-peritoneal route					
El Ghor et al 2014	TiO_2 NP Rutile and anatase < 100 nm In H_2O: particle size was between 45 to 51 nm (XRD, TEM or DLS) In CHL: particle size was between 41 to 44 nm (XRD, TEM or DLS).	Swiss Webster mice, male (5/group) TiO_2 suspended in deionized distilled water or CHL (chlorophyllin) as free radical scavenger. 500, 1000, 2000 mg/kg by IP, for 5 consecutive days Genotoxicity measured as tail length, %DNA in tail and tail moment. Negative control. Cyclophosphamide as positive control.	Positive (bone marrow > liver > brain) at all tested doses – dose-response relationship. Co-administration with CHL decreased DNA damage.	Accumulation in bone marrow > liver > brain (inductively coupled plasma-mass spectrometry) at all doses.	Increased MDA level, decreased GSH level, SOD, CAT and GPx in the liver at 500 and 2000 mg/kg. CHL protected against oxidative stress induced by TiO_2.

All the above studies were summarized regardless of their reliability. However, in order to make a reliable assessment of these results, different key parameters need to be taken into account. First, the tested material needs to be characterized (at least size, crystalline phase and coating). Secondly, the inclusion of negative and positive controls is required to validate the system and thus the results. Finally, the negative results should be taken into account only when it has been proven that the nanoparticles have reached the organ investigated. This could be confirmed with data on uptake or if cytotoxicity was detected. In summary, only 3 publications (Carmona, 2015; Naya, 2012; Louro, 2014, in bold in the table) fulfill the above criteria (characterization data, negative and positive controls and evidence of uptake or cytotoxicity in case of negative results) with 2 publications reporting only negative results (Naya, 2012 and Louro, 2014) and the other showing positive (dose-related) and negative results depending on the tested material (Carmona, 2015).

- Mutation assays

Table I-09. Summaries of *in vivo* Mutation assays found in the literature

Reference	Nanoparticle characterization	Protocol	Results	NM uptake	Toxicity
Oral route					
Carmona, 2015	TiO_2-NP Anatase, < 25 nm, surface area : 45-50 m^2/g, 99.7% purity High level of agglomeration (average: 85.88 nm) in dry form and larger agglomeration in water suspension (average: 405.3 nm) TiO_2-bulk 45 µm, 99% purity	*Drosophila melanogaster*, *multiple wing hairs* and *flare-3* strains TiO_2-NP diluted in ultrapure water and dispersed by sonication for 30 min TiO_2 bulk was diluted in distilled water by mixing for 10 min. Third-instar larvae placed in vials with medium and TiO_2 at 0.08, 0.40, 0.80, 1.60 mg/ml and fed during 48h. Negative control. Ethyl methane sulphonate used as positive control.	Negative (small single, large single, and twin spot)	Not reported.	Larval viability was increased up to 1.60 mg/ml (> 90%) in a preliminary tryptan blue assay. Significant dose response damage for midgut and imaginal discs for 0.80 and 1.60 mg/ml TiO_2 NP for 24 and 48h.
Demir et al 2013	TiO_2-NP Anatase, 2.3 nm (manufacturer and TEM in suspension) Zeta potential: 70.2 mV Micro-TiO_2	*Drosophila melanogaster*, *multiple wing hairs* and *flare-3* strains (3-day-old larvae) Wing somatic mutation and recombination assay 0.1, 1, 5, 10 nM in food until pupation in Drosophila instant medium rehydrated. Negative control. EMS as positive control.	Negative (small single, large single, twin, total *mwh* and total spot)	Not reported.	No important difference in percentage of emerging adults in a preliminary study for dose selection.
Inhalation route					
Boisen et al 2012	UV Titan L181 Rutile TiO_2 (70.8%) coated with 1.17% zirconium, 12.01% silicon, 0.60% sodium oxide and 4.58% aluminium. 20.6 nm, surface area: 107.7m^2/g.	Pregnant C57Bl/6JBomTac mice 42.4 mg/m^3, 1h/per day, GD 8-18, inhalation whole body exposure. Female offspring were raised to maturity and mated with unexposed males. F2 descendants were collected and ESTR (expanded simple tandem repeat) germline mutation rates estimated. Mutation analysis and scoring were successful for 388 offspring. Negative control, but no positive control.	Negative (*Ms6-hm* and *Hm-2* mutation rates)	Not reported.	TiO_2 did not affect viability of the F2 offspring.
Intra-tracheal route					
Driscoll et al 1997	TiO_2 fine Anatase 0.18 µm, surface area: 8.8 m^2/g	Rats Fischer F344, female (6-9/group) TiO_2 suspensions were sonicated briefly prior to each instillation. 5 and 50 mg/kg, 2 consecutive days. *Hprt* mutation assay, 15 months after the last administration.	Positive (alveolar epithelial cells at 50 mg/kg – dose dependent)	Histopathology of the lung: accumulation of particle-laden macrophage in the alveoli and interstitium.	Inflammation: Decreased macrophages and increased neutrophils at 50 mg/kg bw/day. Increased lymphocytes in BAL at all doses.

		Negative control included but no positive control.			Minimal centriacinar alveolitis.
Intravenous route					
Sadiq et al 2012	**TiO_2-NP** **Anatase, 10 nm, powder form**	**B6C3F1 mice, male (5/group)** **TiO_2 was suspended in PBS with vigorous mixing and sonication.** **0.5, 5.0, 50 mg/kg, 3 consecutive days by IV** **Blood collected on weeks 1, 2, 4 and 6 was used for RET/RBC *Pig-a* assay** **Negative control and ENU as positive control.**	**Negative (RET and total RBCs)**	**Analysis of Ti level in bone marrow after administration of 50 mg/kg: Ti levels increased at 4, 24 and 48h (x 12.1-14.2) after the last treatment.**	**Reduction in %RET on day 4 suggested a treatment related cytotoxicity. A rebound was recorded on week 1 and level was normal later.**
Louro et al 2014	**NM102** **Anatase non coated, 22 nm**	**LacZ transgenic C57Bl/6 mice (5-6/group)** **TiO_2 was pre-wetted in ethanol followed by addition of sterile-filtered serum albumin and probe sonication for 16 min.** **10 and 15 mg/kg on 2 consecutive days by IV, sacrifice 28 days after the last injection.** **Mutant frequency for liver and spleen was calculated.** **Negative control. N-ethyl-N-nitrosurea as positive control.**	**Negative (liver and spleen)**	**Uptake in the liver: colorless, irregularly sized and shaped particles (about 1-1.5 µm) of refractory material diffusively present in the tissue, either inside of between hepatocytes as well as inside macrophages (including Kupffer cells) at both doses. Particles also found inside some of the nuclei of hepatocytes without clear dose-related effect.**	**No changes in body weight, behaviour or general health. No gross macroscopic changes at necropsy.** **Leukocytic aggregation and infiltration suggest a low-moderate inflammatory response.**
Intraperitoneal route					
El Ghor et al 2014	TiO_2 NP Rutile and anatase < 100 nm In H_2O: particle size was between 45 to 51 nm (XRD, TEM or DLS) In CHL: particle size was between 41 to 44 nm (XRD, TEM or DLS).	Swiss Webster mice, male (5/group) TiO_2 suspended in deionized distilled water or chlorophyllin (CHL). 500, 1000, 2000 mg/kg by IP, for 5 consecutive days Cyclophosphamide as positive control. PCR-based SSCP used to screen for the presence of p53 mutation in liver and brain cells. Amplification of p53 exons (5-8) by PCR and electrophoresis. Negative control included but no positive control.	Positive at all tested doses (liver and brain) – dose-response relationship. Exons 5, 7 and 8 of p53 gene highly mutated by TiO_2 in liver cells. Exons 5 and 8 of p53 gene mutated by TiO2 in brain cells. Decreased frequencies of mutation with co-administration with CHL.	Accumulation in bone marrow > liver > brain (inductively coupled plasma-mass spectrometry) at all doses.	Increased MDA level, decreased GSH level, SOD, CAT and GPx in the liver at 500 and 2000 mg/kg. CHL protected against oxidative stress induced by TiO_2.

All the above studies were summarized regardless of their reliability. However, in order to make a reliable assessment of these results, different key parameters need to be taken into account. First, the tested material needs to be characterized (at least size, crystalline phase and coating). Secondly, the inclusion of negative and positive controls is required to validate the system and thus the results. Finally, the negative results should be taken into account only when it has been proven that the nanoparticles have reached the organ investigated. This could be confirmed with data on uptake or if cytotoxicity was detected. In summary, only 2 publications are judged as reliable (Sadiq, 2012 and Louro, 2014 in bold in the table) based on our criteria.

- **Non-standardized studies**

This section includes studies with specific protocols such as measurement of 8 oxo-dG, identification of DNA adducts and H2Ax phosphorylation assays.

Table I-10. Summaries of non-standardized *in vivo* studies found in the litterature

Reference	Nanoparticle characterization	Protocol	Results	NM uptake	Toxicity
DNA oxidative lesions					
Oral route					
Sheng et al 2013	TiO_2-NP Anatase In HPMC K4 solvent: 5-6 nm, surface area: 174.8 m^2/g.	CD-1 female mice (20/group) TiO_2 was dispersed in HPMC and treated by ultrasonication for 30 min and mechanically vibrated for 5 min. 2.5, 5, and 10 mg/kg, daily, 90 days by intragastric administration. Measurement of 8 oxodG formation in the heart. Negative control included but no positive control.	Positive (heart at all doses – dose-related)	Accumulation in heart – dose-related.	Sparse cardiac muscle fibers (from 2.5 mg/kg/d), inflammatory response (from 5 mg/kg/day), cell necrosis (at 10 mg/kg/day) and cardiac biochemical dysfunction (all doses). Promotion of oxygen species production, increase of malondialdehyde and carbonyl at all doses. Attenuation of activity of antioxidative enzymes and level of antioxidant at all doses.
Gui et al 2013	TiO_2-NP Anatase, 5-6 nm, surface area: 174.8 m^2/g	Mice CD1, male (30/group) TiO_2 was dispersed in HPMC and treated by ultrasonication for 30 min and mechanically vibrated for 5 min. 2.5, 5, and 10 mg/kg, daily for 90 days by intragastric administration. Measurement of 8 oxodG formation in the kidney. Negative control included but no positive control.	Positive (kidney at all doses – dose-related)	Black agglomerates of TiO_2 in kidney at 10 mg/kg/d. Ti content detected at all doses in kidney.	Reduction of renal glomerulus number, apoptosis, infiltration of inflammatory cells, tissue necrosis, disorganization of renal tubules, decreased body weight, increased kidney indices, unbalance of element distribution, production of ROS and peroxidation of lipid, protein and DNA in kidney. Alteration of 1,246 genes assessed at 10 mg/kg/d, including genes associated with immune/inflammatory responses, apoptosis, biological processes, oxidative stress, ion transport, metabolic processes, cell cycle, signal transduction, cell component, transcription, translation and cell differentiation.

Gao, 2012	TiO_2 Anatase, 5-6 nm in HPMC and surface area: 174.8 m2/g	CD-1 (ICR) female mice (30/group) TiO_2 dispersed on the surface of hydroxypropylmethylcellulose K4M solution and treated ultrasonically for 15-20 min and then mechanically vibrated for 2-3 min. 10 mg/kg for 90 days, intragastric route. DNA adduct 8-OHdG measured in ovaries using ELISA kit. Negative control included but no positive control.	Positive (ovary)	Accumulation in the ovaries. TiO_2-NP conglomerates in the cytoplasm and nuclei of ovarian cells.	Decreases in the mating rate, pregnancy rate, number giving birth, survival rate and body weight of young mice. Increases in E2 and FSH and reduction of P1, LH and Testosterone. Abnormal pathologic changes in ovaries (atrophy, disturbance of follicle development, irregular arrangement of cells, shapeless follicular antrum). Mitochondrial swelling, cristae beakage, nucleus chromatin condensation and margination, irregularity of the nucleau membrane suggesting ovarian apoptosis. ROS production (O_2^- and H_2O_2)
Trouiller et al 2009	P25, purity ≥ 99.5% TiO_2 75% anatase / 25% rutile, 21 nm, , specific surface area: 50±15 m^2/g In water: mean size: 160 nm.	C57Bl/6Jp^{un}/p^{un} mice (5) TiO_2 dispersed by ultrasonication for 15 min 500 mg/kg for 5 days in drinking water 8 oxodG measured in liver by HPLC. Negative control included but no positive control.	Positive (liver)	Not reported.	Inflammation: upregulation of pro-inflammatory cytokines.
Intra-tracheal route					
Rehn et al 2003	P25 Anatase/rutile, hydrophilic, 20 nm T805 Anatase/rutile, hydrophobic (silane coating), 20 nm	Rats Wistar, female (30/group) TiO_2 suspended in physiological saline and lecithin. Suspension and intensive sonication did not lead to primary particles of 20 nm in size. 0.15, 0.3, 0.6, 1.2 mg/lung by IT Sacrifice after 3, 21 or 90 days after instillation. Quantification of 8 oxodG in lung tissue on day 90. Negative control. Quartz type DQ12 used as positive control.	Negative (alveolar epithelial cells)	Not reported.	Non-persistent inflammation: Increase in the number of cells and macrophages in BALf, dose-dependent more clearly with P25 and reversible within 90 days. Increase of neutrophils in the BALf was not fully reversible at 1.2 mg/lung. Protein also increased in the BAL and was not fully reversible for T805 at 1.2 mg/lung. Increased TNF-α only at day 21 for P25 from 0.6 mg/lung. Fibronectin was decreased at 0.15 mg/lung of P25 and T805 and increased from 0.6 mg/lung for T805 only on day 3. Elevated amount of phosphatidylcholine in BALF, more pronounced with P25, only on day 3.
Numano et al 2014	TiO_2-NP Anatase without coating,	Rats SD, female (6/group)	Negative (lung)	Alveolar macrophages with phagocytosed	Few small lung inflammatory lesions. Alveolar macrophage infiltration.

	25 nm TiO_2-NP Rutile without coating, 20 nm	TiO_2 was suspended in saline, then autoclaved. Suspensions were sonicated for 20 min. 0.5 ml of suspension at 500 µg/ml, once every the other day over a 2 week period (total of 8 treatments) by trans-tracheal intra-pulmonary spraying. Total amount: 2 mg/rat. Sacrifice 6h after the last spray. Measurement of 8 oxodG formation in the lung. Negative control included but no positive control.	Positive (lung)	TiO_2 particles. Both TiO_2 deposited in various size in cytoplasm of alveolar macrophages (TEM). No found in other types of cells in the lung.	Increase of MIP1α mRNA expression and MIP1α protein in the lung (lower with anatase)
Xu, 2010	TiO_2-NP Rutile type without coating; 20 nm	Female SD rats (20) TiO_2 was suspended in saline, autoclaved and then sonicated for 20 min just before use. 500 µg/ml (1.25 mg/rat) by IPS 5 times for 9 days. Measurement of 8 oxodG formation in the lung and inguinal mammary gland by ELISA kit. Negative control included but no positive control.	Positive (lung) Negative (inguinal mammary gland)	TiO_2 particles were observed in the cytoplasm of cells. TiO_2 aggregates of various sizes were found in macrophages, and aggregates larger than a single macrophage were surrounded by multiple macrophages	Increased SOD activity in the lung but not in the mammary gland. Upregulation of MIP1α expression and Il-6 in the lung.
Intra-nasal route					
Li et al 2013	TiO_2-NP Anatase, 6 nm In HPMC solvent: 5-6 nm, surface area: 174.8 m^2/g	CD1 female mice (30/group) TiO_2 was dispersed in HPMC treated ultrasonically for 30 min and mechanically vibrated for 5 min. 2.5, 5, 10 mg/kg by nasal instillation every day for 90 days. Measurement of 8 oxodG formation in the lung. Negative control included but no positive control.	Positive (lung at all doses – dose-dependent)	Ti detected in lung at all doses. Black agglomerates in the lung at 10 mg/kg.	Decreased body weight. Increased relative lung weight from 5 mg/kg. Increase of inflammatory cells and biochemical changes in the BALf at all doses. Infiltration of inflammatory cells, thickening of the pulmonary intestitium and oedema. Pneumonocytic ultrastructure with characteristic of apoptosis. Generation of ROS, lipid and protein peroxidation at all doses. At 10 mg/kg, gene expression was analysed: modification of expression of 847 genes with 521 involved in immune response, inflammatory responses, apoptosis, oxidative stress, metabolic processes, stress responses, signal transduction, cell proliferation, cytoskeleton, cell differentiation and cell cycle.
Intraperitoneal route					

Song et al 2011	TiO_2-NP 19.7-101.0 nm, surface area: 15-77 m^2/g	ICR mice, female (3/group) TiO_2 was suspended in saline with Tween 80. 1 and 3 mg/mouse by IP once. 8-oxodG measurement in the urine at 24, 48 and 72h (HPLC-ECD method) and liver. Negative control included but no positive control.	Positive (24h urine collection at 3 mg/animal; result with 1 mg not presented). Negative (liver)	Not reported.	Not reported.
Subcutaneous route					
Cui et al 2014	TiO_2-NP Anatase, 5 nm	Rats SD females, pregnant (8/group) 500 µg on gestational day 6, 9, 12, 15, and 18 by SC. 8-oxodG measurement in the brain of male pups (8/group) of 2 days age. Negative control included but no positive control.	Positive (hippocampus of pups)	Not reported.	Oxidative stress (CAT, MDA, T-AOC) in the rat hippocampus.
DNA adducts					
Gallagher et al 1994	P25 Anatase/rutile, 15-30 nm, surface area: 40 m^2/g	Rats Wistar females, Inhalation 10.4 mg/m^3 (7.5 mg/m^3 increased to 15 mg/m^3 after 4 months and then lowered to 10 mg/m^3 following another 4 months) 18h/d, 5d/w for 2 years, whole body exposure (dry aerosol dispersion technique). ^{32}P-postlabeling assay for determination of adduct level in peripheral lung tissue. TiO_2 used as negative control.	Negative (lung) Decreased adduct 1 (adduct migrated outside the region), possibly due to cell proliferation or de novo cell synthesis. No modification of adduct 2 (nuclease sensitive adduct).	Lung particle load between 23 to 39 mg/lung.	Not reported.
H2Ax phosphorylation assay (DNA double-strand breaks)					
Trouiller et al 2009	P25 Purity ≥ 99.5% TiO_2 75% anatase / 25% rutile, 21 nm, specific surface area: 50±15 m^2/g In water: mean size: 160 nm.	C57Bl/6Jp^{un}/p^{un} mice (5/group) TiO_2 dispersed by ultrasonication for 15 min 50, 100, 250, 500 mg/kg for 5 days, in drinking water γ-H2AX assay in bone marrow cells. Negative control included but no positive control.	Positive at all tested doses (bone marrow) Clear dose-response relationship	Not reported.	Not reported.
Chen et al 2014	Anatase Purity: 99.90% 75 ± 15 nm, specific surface area: 63.95 m^2/g In exposure medium, TiO_2 tend to agglomerate into 473.6 nm and 486.8 nm size when suspended in H_2O and FBS-free DMEM.	Rats SD, male (7/group) TiO_2 dispersed in ultrapure water and ultrasonic vibrated for 15 min. 10, 50, 200 mg/kg by intragastric administration, once a day for 30 consecutive days. Sacrifice immediately after the last administration. Immunofluorescence staining for phosphorylation of histone H2AX assay on bone marrow cells. Negative control included but no positive control.	Positive (bone marrow at 50 and 200 mg/kg)	Not reported.	No abnormal behaviour and symptoms, no significant changes in the body weight. No cytotoxicity as observed by PCE/NCE ratio.

All the above studies were summarized regardless of their reliability. However, in order to make a reliable assessment of these results, different key parameters need to be taken into account. First, the tested material needs to be characterized (at least size, crystalline phase and coating). Secondly, the inclusion of negative and positive controls is required to validate the system and thus the results. Finally, the negative results should be taken into account only when it has been shown that the nanoparticles have reached the organ investigated. This could be confirmed with data on uptake or if cytotoxicity was detected. In summary, none of these publications fulfills these criteria.

- **Summary of *in vivo* genotoxicity studies**

The list of *in vivo* studies summarized above was based on a literature research including published reviews, projects and studies (ended on 30/04/2015). In addition, information from the registration dossier which has been published on ECHA website has been considered (date: 01/08/2015). All forms of TiO_2 have been taken into account.

As a first step, no reliability assessment was made on these studies and all were reported. Among them, most of the studies were performed with nano-TiO_2 and referred to the anatase form. Fourty-three experiments over 138 reported positive results. Most of the positive results were found in Comet assays, 8-oxodG tests and H2Ax phosphorylation assays (Table I-11).

Table I-11 Summary of positive responses in function of crystalline phase of TiO_2 according to the authors

Assays	**Micronucleus assay**	**Comet assay**	**Mutation assay**	**DNA oxidative lesions**	**DNA adducts**	**H2Ax phosphorylation assay**	**Total**
Nanoforms							
Anatase	2/9	5/22	0/7	5/6	0/0	1/1	13/45
Rutile	0/2	0/0	0/0	1/3	0/0	0/0	1/5
Anatase/rutile	3/7	8/20	2/2	1/2	0/1	1/1	15/33
Anatase coated	0/1	1/5	0/0	0/0	0/0	0/0	1/6
Rutile coated	0/6	5/22	0/1	0/0	0/0	0/0	5/29
Anatase/rutile coated	0/0	0/0	0/0	0/1	0/0	0/0	0/1
Brookite/anatase	0/1	0/1	0/0	0/0	0/0	0/0	0/2
Unspecified	1/1	1/1	0/0	1/2	0/0	0/0	3/4
Microforms							
Anatase	1/1	2/3	1/1	0/0	0/0	0/0	4/5
Rutile	0/2	0/1	0/0	0/0	0/0	0/0	0/3
Unspecified	0/0	0/1	0/2	0/0	0/0	0/0	0/3
Undefined							
Unspecified	1/2	0/0	0/0	0/0	0/0	0/0	1/2
Total	8/32	22/76	3/13	8/14	0/1	2/2	43/138

Some studies include several experiments with different NM and some NM can show negative and positive results within a study, depending on the organ examined. Each result was counted in all the relevant sections. An experiment is defined by a tested material and a specific protocol (ex. organ examined, duration…).

However, in order to make a reliable assessment of these results, different key parameters need to be taken into account as in the *in vitro* genotoxicity section:

- Characterization of the tested material (at least size, crystallinity and coating);
- Information on dispersion and exposure protocols;
- Inclusion of negative and positive controls to validate the system and avoid under or over responses. It should be noted that in some case, it is difficult to find a positive control for all the endpoints examined;
- Use of known or validated protocols;
- Evidence of uptake or (cyto)toxicity in case of negative results. Indeed, false negative results may be induced if there is no uptake of TiO_2 by the cells. This can be assessed by specific uptake data or by the presence of cytotoxicity. Furthermore, the time point selected for the genotoxicity endpoint measurement should be appropriately chosen.

Considering these criteria, one MN assay, 3 Comet assays and 2 mutations assays were considered reliable (Table I-12). None of the 8-OHdG assays and none of the ɣ-H2AX assays reach the above criteria. Among these studies, positive results were only found in the Comet assays with most of them showing a dose-response

Table I-12. Summary of positive responses in function of crystalline phase of TiO_2 according to the authors in the selected reliable *in vivo* studies

Assays	**Micronucleus assay**	**Comet assay**	**Mutation assay**	**DNA oxidative lesions**	**DNA adducts**	**H2Ax phosphorylation assay**	**Total**
Nanoforms							
Anatase	0/1	1/3	0/2	-	-	-	1/6
Rutile	-	-	-	-	-	-	-
Anatase/rutile	-	-	-	-	-	-	-
Anatase coated	-	-	-	-	-	-	-
Rutile coated	-	-	-	-	-	-	-
Anatase/rutile coated	-	-	-	-	-	-	-
Brookite/anatase	-	-	-	-	-	-	-
Unspecified	-	-	-	-	-	-	-
Microforms							
Anatase	-	-	-	-	-	-	-
Rutile	-	-	-	-	-	-	-
Unspecified	-	-	-	-	-	-	-

Undefined							
Unspecified	-	-	-	-	-	-	-
Total	0/1	9/36	-	-	-	-	9/37

Some studies include several experiments with different NM and some NM can show negative and positive results within a study, depending on the organ examined. Each result was counted in all the relevant sections. An experiment is defined by a tested material and a specific protocol (ex. organ examined, duration…).

-: No study fulfilling our selected criteria

Inconsistencies in the results of the studies may be the result of differences in test materials (size, crystallinity, coating…). Based on the above tables, nanoforms seem to induce more positive results in *in vivo* genotoxicity studies. However, this impression comes mainly on the fact that very few studies on microforms are available (and none fulfilling our criteria). Furthermore, some data suggested that anatase forms are more cytotoxic than rutile or anatase/rutile ones because of photocatalytic properties of anatase (Xue, 2010; Wang, 2014). Very few studies compare the *in vivo* genotoxicity of different crystalline forms of TiO_2: no difference as function of crystallinity was noted in Nanogenotox (2013) and registration data (2014-07-22/2014-07-30) while Numaro (2014) reported a higher effect of rutile form. Despite a systematic review of the different characteristics that may explain the discrepancies observed in the studies, it remains difficult to highlight which parameter(s) can drive them. Inconsistencies in the results can also be explained by the various test conditions used, including dispersal of the material, concentrations and exposure duration, route of exposure, animal model, cell/organ examined and parameter assessed. All these differences do not permit an easy comparison of the studies.

Mechanism of action

Several *in vivo* genotoxicity studies indicate that TiO_2 may cause genotoxic effects *via* secondary mechanisms. Indeed, when assessed, positive results were often associated with oxidative stress and inflammation. Inflammation was characterized by up-regulation of pro-inflammatory cytokines (Trouiller, 2009) and increased cells such as neutrophils in the BALf (Lindberg, 2012; Landsiedel, 2010; Naya, 2012; Saber, 2012; Nanogenotox, 2013; Driscoll, 1997; Rehn, 2003; Numaro, 2014; Li, 2013). Evidence for induction of oxidative stress was observed by decrease of intracellular antioxidant defenses (such as SOD, GSH-Px), increase of lipid peroxidation (Meena, 2015; El Ghor, 2014; Cui, 2014), production of ROS (Sheng, 2013; Gui, 2013; Li, 2013; Gao, 2012) or alteration of genes expression involved in stress responses from transcriptomic analyses (Li, 2013; Gui, 2013; Sheng, 2014; Gao, 2012). Oxidative damage can also be supported by measurement of 8-oxo-dG which were increased in different organs in 9/10 studies by different routes of exposure (Sheng, 2013; Gao, 2012; Gui, 2013; Trouiller, 2009; Numaro, 2014; Xu, 2010; Li, 2013; Song, 2012; Cui, 2014). Modified Comet assays using for example Fpg can also bring information on the induction of oxidative lesions but a clear profile of responses cannot be observed, with various results depending on the test material and the organ examined (Nanogenotox, 2013). **All these results indicate that an oxidative stress pathway is probably involved in the genotoxic effect of TiO_2, even if a consistent response was not observed among the studies.**

However, in some studies, inflammation was not associated with genotoxic effects (Lindberg, 2012; Louro, 2014; Landsiedel, 2010; Jackson, 2013; Naya, 2012; Saber, 2012; Nanogenotox WP6; Rehn, 2003; Noumaro, 2014) suggesting that inflammation and oxidative stress alone may not be sufficient to drive to genotoxic effect. Indeed, a direct genotoxic effect by direct DNA interaction cannot be excluded since there is some evidence that TiO_2 can locate in nuclei. Accumulation in the nucleus was reported in 2 *in vivo* studies (Gao, 2012 and Louro, 2014) but was associated with a genotoxic effect in only one of these publications (Gao, 2012). Additional publications (from a not exhaustive bibliographic research) not summarized in the tables above since they were of low quality also reported some accumulation in the nucleus and possible interaction with DNA after *in vivo* administration of TiO_2 (Li, 2009; Jin, 2013). Accumulation in the nucleus was also observed in some *in vitro* studies reported in Tables I-01 to I-04 (Jugan, 2012; Shukla, 2013; Hackenberg, 2011; Barillet, 2010). However, it should be noted that accumulation in the nucleus was not systematically investigated in the studies and was not quantified when reported. Since the nuclear pore complex is less than 8 nm in diameter, Karlsson (2010 & 2015) hypothesized that direct interactions with DNA could occur during mitosis and interfere with the microtubules, causing clastogenic effects. **These data suggest that TiO_2 can enter into nucleus and directly interact with DNA.**

In summary, oxidative stress seems to be the main pathway explaining positive genotoxic results obtained with TiO_2. A direct genotoxic effect cannot be totally excluded since accumulation in the nucleus was reported in some *in vitro* and *in vivo* studies.

ANNEX II. *IN VITRO* STUDIES ASSESSED BUT NOT SELECTED ACCORDING TO OUR CRITERIA.

- **Micronucleus assays**

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	**Results; Methods**	
Turquez et al, 2011	TiO_2 < 100nm No data on crystallinity.	TiO_2 was prepared with sterile dimethylsulfoxide (DMSO). Culture media:5 ml of culture medium (Chromosome Medium B) with 5 mg/ml of phytohemag-glutinin	Peripheral blood lymphocyte culture	Not cytotoxic at 3 and 5 µM: no alteration of PI values. Cytotoxic at 10 µM: statistically important decrease in the rate of PI.	Positive: significant from 5 µM– dose-dependent. CBMN assay. Conditions tested: 3, 5 and 10 µM for 72h; 2000 binucleated lymphocytes examined per concentration (2 cultures/concentration); negative control but no positive control. Positive effect of adding acid ascorbic (AA) in decreasing the incidence of MN.	Not reported.
Jaeger et al 2012	P25 70% anatase/30% rutile 20 nm TiO_2-MP Anatase, 200 nm	TiO_2 particles were sterilized by heating to 120°C for 2 h, suspended in sterilized phosphate-buffered saline (1× PBS) to a 12.5 mM stock solution (1 mg/ml) and kept at 4 ◦C until used. Before application to the cells, treatment of NP with an ultrasonicator for 3 min at cycle count 20% and 70% In DMEM : SSA = 52.7 ± 3.6 m^2/g (N_2-BET); Zeta potential : −11.6 ± 1.2 mV (P25)	Ha CaT keratinocytes	Cytotoxic: Slight decrease of the number of binucleated cells showing some toxicity; no use of the CBPI parameter recommended; % of BN in control low (40 to 50%) compare to what is expected with cell lines (up to 70-80%) – MN assay.	Positive P25: increase at 10 µg/ml (24h exposure) and at all doses (48h exposure) – not dose dependent TiO_2-MP : increase at 1 and 5 µg/ml (24h exposure) and from 0.5 to 10 µg/ml (48h exposure) MN assay. Conditions tested: 0.5-50 µg/ml for 24 or 48h between 1 and 50 µg/ml; at least 3 replicates of 3 * 500 binucleated cells per data. No positive control.	As soon as 1 h after exposure to TiO_2-NPs (10 g/ml) many TiO_2 particles were observed as electron-dense, highly contrasted bodies in small endosomes and bigger vesicles inside HaCaT cells
Zheng et al 2012	P25 Anatase/rutile 80/20, 25-50 nm	Sterilized in an autoclave and freshly suspended in distilled water. Dispersion in DMEM media with ultra-sonication of 10 min.	Human embryo hepatic L-02 cells	ATP level was not affected nor the cell viability. Conditions tested; 1, 5, 10 mg/l for 6, 12, 24 h exposure	Negative MN assay. Conditions tested: 1, 5, 10 mg/l in the dark for 24h; 3 independent	NPs internalized (SEM observation; 10 mg/l for 24h)

		In DMEM: Size: from 322 to 482 nm; zeta potential: from -25.3 to -8.015 mV		No data on the % of binucleated cells, on the toxicity during the assay (ex: CBPI)	experiments, 3000 binucleated cells scored. No positive control.	
Corradi et al. 2012	NM-101 Anatase with occasional trace of rutile, uncoated 7-9±1 nm NRCWE-002 Rutile, positively coated TiO_2 10±1 nm NRCWE-003 Rutile, negatively coated TiO_2 10±1 nm	Dispersion in MilliQ-filtered water with 2% bovine serum at the concentration of 2.56 mg/ml and 16 min sonication (EU ENPRA protocol). Then dilution in culture media: DMEM with and without 10% FBS. TiO_2 NMs formed denser agglomerates than in the presence of 2% serum in the dispersion medium. Multiple rinses of the culture slides were performed, without obtaining adequate slides for analysis. In MilliQ-filtered water: NM-101: 580.8 nm (PDI: 0.326) NRCWE-002: 175.6 nm (PDI: 0.251) NRCWE-003: 163.3 nm (PDI: 0.242) In DMEM (bimodal distribution): NM-101: 211 and 964 nm NRCWE-002: 139 and 3052 nm NRCWE-003: 190 and 2517 nm In DMEM + 10% FBS NM-101: 129 and 591 nm NRCWE-002: 233 nm NRCWE-003: 109 and 1184 nm	A549 human pulmonary cells	Not cytotoxic at concentrations up to 250 µg/ml (CBPI data). No difference between the CBPI and the 3 TiO_2 NM. No difference in the presence or absence of serum. Conditions tested: 5-75 µg/ml for 40h.	Results are not available as the MN were obscured by NM agglomerates over the cells and thus could not be scored (40h up to 250 µg/ml with one experiment performed)	Big agglomerates of TiO_2 NMs were detected in samples cultured in the presence or absence of 10% serum and colocalized with cells. Increasing the number of rinses of the slides did not decrease the number of agglomerates. Whether those clusters were attached at the cell surface or were internalized by the cells requires further investigation
Jugan et al. 2011	Rutile, spherical 20 nm	No information	A549 human pulmonary cells	Low cytotoxicity: max 25% cell death after 48h exposure; MTT, LDH, Trypan blue,	Negative MN assay. Conditions tested: exposure for 4-48h. No further details.	NPs internalized, located mostly in the cytoplasm and got

	Anatase, spherical 12 nm			clonogenic assays (results not presented). Lowest interference with Trypan blue and clonogenic assay Conditions tested not detailed.		rapidly entrapped into vesicles and vacuoles
Shi et al. 2010	P25 Anatase:rutile crystalline ratio (8:2) Primary particle diameter approx. 25 nm; surface area (BET) of 50 m^2/g.	Nano-TiO_2 was sterilized by heating to 120°C for 2 h, and freshly suspended in distilled water immediately before use. Culture medium: DMEM containing FBS, penicillin G and streptomycin.	Human hepatocytes L-02	Not cytotoxic with the ATP kit. Conditions tested: 0.01-1 µg/ml for 12, 24 or 36h. No CBPI data.	Negative CBMN assay. Conditions tested: 0.01 -1 µg/L for 24 h; triplicate and repeated three times; 3000 binucleated cells scored for each group; negative control but no positive control.	Not reported.
Landsiedel et al, 2010	T-LiteTM SF Coated-rutile. Acicular-shaped. The coating consists of aluminium hydroxide and dimethicone/methicone copolymer. TiO_2 content = 79-89 % Purity TiO_2 core ≥ 99% PPS length: 50 nm; width: 10 nm Mean agglomerate: about 200 nm. SSA = 100 m^2/g	FCS was used as sole vehicle. The dispersion contained still a significant amount of ultrafine particles (diameter <100 nm) including some non-aggregated primary particles. T-LiteTM SF agglomerates strongly. T-LiteTM SF in the vehicle FCS (50 mg/ml) showed a diameter of 239 nm, in FCS/MEM (0.6 mg/ml) a diameter of 562 nm as determined by analytical ultracentrifugation.	V79 cells (lung fibroblasts from Chinese Hamster)	Range finding cytotoxicity test: no reduced cell numbers of below 50% of control up to 5 mg/ml. Strongly reduced quality of the cells from 625 µg/ml onward after 4h treatment and from 156.3 µg/ml onward after 24h treatment. MN assay: not cytotoxic: no reduced proliferation index after 4 and 24h of exposure time, up to the highest concentration scorable for MN induction.	Negative MN assay. Conditions tested: 0, 75, 150, 300 µg/ml for 4 hours or 0, 18.8, 37.5, 75 µg/ml for 24 hours in the dark; quadruplicate culture and 2000 cells analyzed for each group, negative and positive controls.	Not reported.
Di Virgilio et al. 2010	Average particle size: 20 ± 7 nm; SSA: 142 m^2/g (BET). No information on crystallinity.	Suspension prepared in PBS, vortexed for 10 minutes and stored at 4°C in the dark. Cell culture: Ham F10 culture medium supplemented with 10% inactivated fetal calf serum, 50 IU/mL penicillin and 50 g/mL streptomycin sulfate (complete culture medium).	Chinese hamster ovary (CHO-K1)	Cytotoxic: significant decrease from 25 µg/ml with neutral red (population growth) and at all concentrations with MTT assay. NR and MTT assays. Conditions tested: 0, 5, 10, 25, 50, 100 µg/ml for 24 hours.	Positive at from 0.5 µg/ml (only statistically significant at 0.5 and 1 µg/ml) The highest concentration could not be measured due to cytotoxic effects. At this concentration, nuclei (and eventually micronuclei) were covered by NPs. CBMN assay. Conditions tested: 0, 0.5, 1, 5, 10 µg/ml under complete darkness for 24 hours; 3 independent experiments in	TiO_2 (50 µg/ml) for 24 hours: cells formed perinuclear vesicles containing phagocyted material. Agglomerates on both of the surface and inside the cells. Only present in the cytoplasm and no NP was detected in the nuclei. The nucleus shape is modified in

					triplicate; 1000 binucleated cells scored per slide; negative control but no positive control.	the presence of some large vesicles which seem to press it. In some cells with many large vesicles, the membrane was disintegrated.
Osman, 2010	Anatase TiO_2 NP Purity: 99.7% No data on primary size.	Nanoparticles were suspended in 10 ml EMEM– EBSS medium at concentrations of 10, 20, 50 and 100 µg/ml. Suspensions were probe-sonicated at 30 W for 5 min on and off, and then allowed to equilibrate for different times: 0, 2, 4, 24 and 48 h. In culture media: aggregation with increasing dose, but remained constant over a 48h period, except at higher doses (from 50 µg/ml) of TiO_2. Size from 384 nm (up to 20 µg/ml) to 722 nm (100 µg/ml).	Human epithelial Hep-2 cell line	Cytotoxic MTT and NRU assays. Conditions tested: 20-100 µg/ml for 2, 4, 24 and 48h. In comet assay, cell viability was between 70-85% except at 100 µg/ml which showed 65% viability. High membrane integrity. Trypan Blue assay. Conditions tested: 10, 20, 50, 100 µg/ml for 4h.	Positive at 50 µg/ml – dose related. The dose of 100 µg/ml was toxic and precipitated. CBMN assay. Conditions tested: 10, 20, 50 µg/ml for 2h; 1000 binucleated cells scored; 2 independent experiments, negative and positive controls.	Not reported.

- **Comet assays**

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity Results; Methods; Interferences	Genotoxicity Results; Methods	Uptake
Jugan et al, 2012	TiO_2-A12 NP 95% anatase, spherical 12 nm; 92 m^2/g; PZS: 6.4	Dispersion in ultrapure sterile water(pH5.5) by sonication for 30 min at 4 C,in pulsed mode(1 s on/1 s off), at the concentration of 10 mg.ml-1 Suspensions were diluted in cell culture medium (DMEM without serum)	A549 human lung carcinoma cells (CCL-185)	Cytotoxic in MTT assay (less than 25% of cell death after 48h) Conditions tested: 1–200 µg/ml for 4–48 h.	TiO_2-A12 NP: Positive for all duration exposures. TiO_2-R20: Positive for 4h and 24h treatment. TiO_2-A25: Positive for 4h and 24h treatment. TiO_2-R68: Positive only for 4h treatment TiO_2-A140: Positive only for 4h treatment. Alkaline Comet assay: 100 µg/ml 4h -24h -48h; triplicate, negative control but no positive control.	An unambiguous accumulation of the smallest NPs in the cytoplasms and in the nucleus of cells
	TiO_2-R20 NPs 90% rutile, spherical 21 nm; 73 m^2/g					
	TiO_2-A25 (AEROXIDE P25) 86% anatase/14% rutile, spherical 24 nm; 46 m^2/g; PZS: 7.0					
	TiO_2-R68 100% rutile, elongated L: 68 nm d: 9 nm; 118 m^2/g			Cytotoxic (less than 1% of cell death at the highest dose after 48h) in MTT assay (1–200 µg/ml for 4–48 h)		Cytoplasmic accumulation was also observed but not in cell nuclei
	TiO_2-A140 100% anatase, spherical 142 nm; 10 m^2/g; PZS: 5.2			Cytotoxic (less than 10% of cell death at the highest dose after 48h) in MTT assay (1–200 µg/ml for 4–48 h)		
Kermanizadeh et al. 2014	NRCWE 002 NM produced from the NRCWE 001 Rutile, 10 nm (XRD size), 80-400 (TEM size), BET: 84 m^2/g, coated with triethylpropylaminosaline	NM was dispersed in cell culture grade water with 2% FCS. Then it was sonicated for 16 min without pause (instruction of ENPRA project). Then immediately transferred to ice before being diluted in medium just prior to the experiments. Size in human liver maintenance medium: 278±151 nm.	3D human liver microtissues	Not cytotoxic. Concentration-dependent decrease in cell membrane integrity over time (not significant) in Adenylate kinase assay Live/dead staining supported AK data. Conditions tested: 16, 31.25, 62.5, 125 and 250 µg/ml single exposure for 24h or repeated exposure at 72, 144, 216 and 288h. No interference.	Positive Without Fpg single exposure: positive at 16, 125 and 250 µg/ml – not dose-dependent. With Fpg single exposure: positive only at 31.25 µg/ml – not dose-dependent. Without Fpg repeated exposure: positive at 16, 62.5 and 125 µg/ml – not dose-dependent. With Fpg repeated exposure: positive at 31.25 and 125 µg/ml – not dose-dependent.	Not reported.

					Standard and Fpg modified comet assay. Conditions tested: 16, 31.25, 62.5, 125 and 250 µg/ml – single exposure for 24h or repeated exposures at 72, 144, 216 and 288h; All experiments were repeated a minimum of three times; negative but no positive control	
Ghosh et al, 2013	TiO_2 nanoparticles Anatase and rutile; spherical in shape Particle size (~100 nm), surface area (14.0 m^2/g), and density (4.26 g/ml at 25°C) (supplier information) About 50 nm (TEM, SEM), 106 nm (AFM)	Suspension in filter-sterilized PBS and dispersed by ultrasonic vibrations (100 W, 30KHZ) for 30 min In dispersion: symmetric, spherical in shape, well distributed without much aggregation and size range: 90-110 nm; at least 45% of the particles with hydrodynamic diameter of ~200 nm	Human lymphocytes	Not cytotoxic with Trypan blue dye exclusion, resazurin and NR uptake assays. Conditions tested: 0, 25, 50 and 100 µg/ml for 3 h. Cytotoxic with MTT and WST-1 assays: at all concentrations. Conditions tested: 0, 25, 50 and 100 µg/ml for 3 h at 37 °C	Positive only at 25 µg/mL – not dose-dependent. Comet assay; 25-50-100 µg/ml for 3h ; Each experiment was repeated twice; 3 replicates; negative control but no positive control	No information
Botelho et al, 2014	TiO_2 nanopowder 637254 (titanium (IV) oxide anatase Anatase ; < 25 nm	TiO_2 was suspended in two different dispersion media: Milli-Q water and RPMI supplemented with 10% FBS or 2% BSA in phosphate-buffered saline (PBS) and probe sonicated at 30 W for 5 min (1.5 min pulse on and 1 min pulse off for two times and a final pulse of 2 min). In Milli Q water: 420.7 nm; zeta potential: -9.96mV. No information in RPMI media culture	AGS (gastric epithelial cancer) cells	Not cytotoxic: increase of cell proliferation and overall survival in cell in CellTiter 96 AQ nonradioactive cell proliferation assay. Conditions tested: 20-150 µg/ml for 3, 6 or 24h. Confirmation of this result in Trypan blue exclusion assay. Conditions tested: 150 µg/ml for 3h.	Positive (% Tail DNA) Alkaline Comet assay. Conditions tested: 150 µg/ml for 3h; 2 replicates; negative controls but no positive control.	Not reported.
Wang et al, 2011	Anatase (100%) Purity = 99.7%, < 25nm	Nano-TiO_2 was suspended in DMSO and vortexed for 1 min. Culture media: Ham's F12 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin TiO_2 appeared to aggregate in cell culture medium (generally > 100 nm).	Chinese hamster ovary cells (CHO-K1)	Not cytotoxic for concentration below 50 µg/ml with acute or chronic exposure Cytotoxic at 100 and 200 µg/ml with acute exposure XTT assay. Conditions tested: 0, 10, 20, 40 µg/ml and higher for 1, 2 or 60	Negative (tail length) Alkaline comet assay. Conditions tested: 0, 10, 20, 40 µg/ml for 60 days; duplicate culture; negative control but no positive control.	TiO_2 aggregates were internalized by CHO cells that were chronically exposed (TEM). Internalized TiO_2 appeared to be restricted to the cytoplasm and did not appear to localize to

				days (results only shown for concentrations up to 40 µg/ml for 24 and 48h).		any specific organelles and none appeared within cell nuclei.
Wan et al. 2012	Nano-TiO_2. 90 % anatase and 10% rutile ; 28 nm, BET = 45 m^2/g	TiO_2 dispersed in physiological saline and ultrasonicated for 30 min. Then added to culture medium (Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) In cell culture medium: size (DLS): 280 nm	A549 human lung epithelial cells	Not cytotoxic with *in vitro* cytotoxicity kit (SBR) and AlamarBlue™ assay (colorimetric/fluorimetric method).at 2.5-40 µg/ml for 24h	Negative (OTM) Alkaline comet assay. Conditions tested: 5 and 15 µg/ml for 12h; 3 independent experiments; 3 slides per concentration; no positive control.	The uptake of Nano-TiO_2 in A549 cells was 2.75×10^{-12} and 4.34×10^{-12} (g/cell) with exposure to 5 and 15 µg/ml respectively for 12h - clear dose dependent (ICP-MS)
Meena et al. 2012	Anatase < 25 nm	Nano-TiO_2 was dissolved in distilled water and ultrasonicated for 30 min. TiO_2 was then suspended in cell culture medium containing Dulbecco's modified Eagle's medium, supplemented with 10 % heat-inactivated fetal calf serum and IX Penstrep antibiotic solution. In suspension: 10-20 nm (TEM); 43-103 nm (DLS) In cell culture medium: 17–40 nm (TEM); 43-336 nm (DLS)	Human embryonic kidney cell line HEK-293	Cytotoxic: cell viability decreased as function of both concentration (at 100 and 200 µg/ml) and time (24, 48, 72h) with LDH and MTT assays. Conditions tested: 50-200 µg/ml for 24, 48 and 72h.	Positive Increased tail length and tail migration at all concentrations – dose-dependent. Increased tail migration from 100 µg/ml – dose-dependent. Comet assay: Conditions tested not well developed. 50-200 µg/ml for 48h. No positive control.	Not reported.
Zheng et al. 2012	Anatase/rutile forms of 80/20; 25-50 nm	Sterilized in an autoclave and freshly suspended in distilled water. Dispersion in DMEM media with ultra-sonication of 10 min. In DMEM: Size: from 322 to 482 nm; zeta potential: from -25.3 to -8.015 mV	Human embryo hepatic L-02 cells	ATP level was not affected nor the cell viability. Conditions tested; 1, 5, 10 mg/l for 6, 12, 24 h exposure	Negative (Olive Tail Moment) Neutral Comet assay. Conditions tested: 0.1-10 mg/l for 24h; between; no information on the number of replicates; 3 independent assays. No positive control	NPs internalized (SEM observation; 10 mg/l during 24h)
Turquez et al, 2011	< 100nm No information on cristallinity.	TiO_2 was prepared with sterile dimethylsulfoxide (DMSO).	Peripheral blood lymphocyte culture	Not cytotoxic at 3 and 5 µM: no alteration of PI values.	Positive from 5 µg/ml – dose-dependent (tail length)	No information

		Culture media:5 ml of culture medium (Chromosome Medium B) with5 mg/ml of phytohemag-glutinin		Cytotoxic at 10 µM: statistically important decrease in the rate of PI.	Alkali Comet treatment: 3, 5, 10 µg/ml for 72h; negative control but no positive treatment AA had an inhibitory effect on DNA damage in human lymphocytes induced by TiO_2.	
Ghosh, 2010	Bulk TiO_2 No characterization data TiO_2 100 nm; surface area: 14.0 m^2/g; density: 4,26 g/ml No information on cristallinity. Particle symetric, spherical in shape, well distributed without much aggregation and in the size range of 90–110 nm as specified by the supplier (AFM images).	TiO_2 was suspended in filter sterilized double distilled water and were sonicated to prepare stock solutions. Then diluted, followed by sonication and vigorous vortexing. TiO_2 was added to culture media: RPMI-1640	Human peripheral blood lymphocytes	Not cytotoxic (TiO_2-NP and bulk) with Trypan blue assay. Conditions tested: particles at 0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM for 3 h. Cytotoxic (TiO_2-NP) with MTT and WST-1 assays from 0.25 mM. Conditions tested: 0, 0.25, 0.50, 0.75, 1, 1.25 mM for 3 h.	Bulk TiO_2: Positive at concentrations from 1.25 mM (% tail DNA) – not dose dependent. TiO_2 NP: Positive: only at 0.25 mM for TiO_2 NP (% tail DNA), followed by gradual decrease – not dose-dependent. Alkaline Comet assay. Conditions tested: 0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM for 3 h; triplicate per concentration; each experiment repeated twice; negative control but no positive control.	No information
Demir et al, 2013	TiO_2 ionic and nano forms NP characterization: Spherical; 2.3 nm; purity: 99-100.5%; density (1.05 g/ml) No information on cristallinity	Distilled in water and ultrasonication at 20 kHz for 16 min in an ice-cooled bath After dispersion: 2.3 ± 0.5 nm,70.2 mV, no marked agglomerations	Human peripheral blood lymphocytes and cultured human embryonic kidney cells (HEK293)	Not cytotoxic for NP or ionic form (FDA/EB viability assay) in both cells tested. Conditions tested: 1, 10 and 100 µg/mL; EMS used as a positive control	Positive for NP at 100 µg/mL with or without fpg and endo II enzymes (%Tail DNA for standard and modified Comet assay; Tail moment for standard assay) Negative for ionic form without enzyme treatment (%Tail DNA and Tail moment) Standard alkaline Comet assays: 1, 10 and 100 µg/mL for 3h for NP and ionic forms; Modified Comet assay (endo III and Fpg): 100 µg/ml for NP form 2 independent experiments and 2 replicates; positive and negative controls.	No information
Hackenberg et al. 2010	TiO_2-NPs Anatase ; < 25 nm	Dispersed in DI water. Then sonicated for 60s at a high energy level of 4.2×10^5 kJ/m^3	Human nasal mucosa cells	Not cytotoxic for both cytotoxicity tests: Trypan blue test: death cells below	Negative (tail DNA, tail length and OTM)	11% of the nasal mucosa cells presented nanoparticles in the

		using a continuous mode to create an optimal grade of dispersion, BSA was added as a stabilizer at an end concentration of 1.5 mg/ml. Finally, 10× concentrated phosphate buffered saline (PBS) was added to achieve a physiological salt concentration and pH of 7.4. In stock suspension: 15-30 nm, high level of compact aggregations sized 285±52 nm. In particular cases, aggregates could reach diameters up to 2000 nm.	from 10 donors	20% and FDA assay: cell viability between 95 and 76%. Conditions tested: 10, 25, 50, 100 µg/ml for 24 h	Alkaline Comet assay. Conditions tested: 10, 25, 50, 100 µg/ml for 24h in the text but 10, 25, 50, 100 µg/l in the tables; negative and positive controls; 10 donors used per concentration; 2 slides per cells.	cytoplasm. In cases of cell invasion, large-sized particle aggregates up to 1000 nm in diameter could be described, being surrounded by vesicles. Invasion into the cell nucleus was observed in 4%
Jugan et al. 2011	Rutile, spherical 20 nm Anatase, spherical 12 nm	No information	A549 human pulmonary cells	Low cytotoxicity: max 25% cell death after 48h exposure; MTT, LDH, Trypan blue, clonogenic assays (results not presented). Lowest interference with Trypan blue and clonogenic assay	Positive (tail DNA) Comet assay. Conditions tested: 100 µg/ml for 4, 24 or 48h; negative control but no positive controls; no statistics; no information on replicates or independent assays. High level of damage in control increasing with the time of exposure.	NPs internalized, located mostly in the cytoplasm and got rapidly entrapped into vesicles and vacuoles
Shi et al. 2010	P25 Anatase:rutile crystalline ratio (8:2) Primary particle diameter approx. 25 nm; surface area (BET) of 50 m^2/g.	Nano-TiO_2 was sterilized by heating to 120°C for 2 h, and freshly suspended in distilled water immediately before use. Culture medium: DMEM containing FBS, penicillin G and streptomycin.	Human hepatocytes L-02	Not cytotoxic with the ATP kit. Conditions tested: 0.01-1 µg/ml for 12, 24 or 36h.	Negative (OTM) Alkaline and Neutral Comet assays. Conditions tested: 0.01 -1 µg/L for 24 h; triplicate and repeated three times; negative control but no positive control.	No information
Pan, 2012	P25 80/20% anatase/rutile No further characterization	Cells cultured in RPMI 1640 medium with 10% FBS, penicillin, and streptomycin at 37°C, 5% CO_2-humidified environment.	Hep-2 cells	Cytotoxic: IC_{50} = 178.98 µg/ml MTT assay. Conditions tested: 7.8-500 µg/ml for 24h.	Positive at both doses (tail length) and only at the highest dose (%Tail DNA) – dose dependent Alkaline Comet assay. Conditions tested: 7.8 and 62.5 µg/ml for 2	No information

				Results consistent with trypan blue assay.	hours; duplicate; negative control but no positive control.	
Tiano, 2010	TiO_2 99.7%, anatase, no treatment, 10-20 nm Optisol Rutile, 20-100 nm, manganese coated MTD-25 Rutile, 20-40 nm, treated with Aluminum hydroxide UV-Titan Rutile, 20-40 nm, coated with Alumina, sodium M263 Rutle, 20 nm, coated hexametaphosphate, Polyvinylpyrrolidone, Aluminium phosphate Eusolex T2000 Rutile, 20 nm, coated with alimunia, dimethicone TAQ40 Anatase/rutile (80/20), 20 nm, coated with Glycerin, isolaureth-4 Phosphate, vinyl buteth-25/sodium maleate copolymer	Stock solutions of TiO_2 were prepared by suspending the particles in water at a concentration of 1.5 mg/ml and sterilized by heating at 120 °C for 20 min, followed by refrigeration at 4 °C until use. Culture medium: MEM (minimum essential medium) supplemented with foetal bovine serum (FBS), glutamine, and antibiotics (penicillin and streptomycin)	Human dermal fibroblasts	Decrease in viability with MTD-25, T2000 and TAQ40 without UV and with non-coated TiO_2 with 30min UV MTT assay. Conditions tested: 5 µg/cm^2 with no UV or UV treatment for 30 or 60 min	Non-coated TiO_2: positive with and without UV Optisol: positive only without UV MTD-25: positive with and without UV UV-Titan : positive with and without UV M263 : negative Eusolex T2000 : positive only with UV TAQ40 : positive only with UV Comet assay. Conditions tested: 5 µg/cm^2 +/- 12 min UV; duplicate; tail length, tail migration, %tail DNA and tail moment. Negative control, non-coated TiO_2 used as positive control	No information
Magdolenova et al. 2012	NM 105, Aeroxide P25 anatase/rutile ; 21 nm (supplier information). 15-60 nm (TEM), purity > 99%, uncoated, anatase/rutile (TEM/SAED), impurities : Co, Fe (ICP-OES)	2 dispersion protocols : (a) FBS in PBS was added to TiO_2 and sonicated for 15 min at 100 Watt in an ice/water bath. Suspension was added to cell culture medium. (b) TiO_2 suspended in culture medium with HEPES buffer without FBS. Then sonicated for 3 min at 60 W on ice and water mixture. Before use, solution was vortexed for 10s before being immediately sonicated for 1 min at 60 W and	TK6 cells (human lymphoblast cells) Cos-1 monkey kidney fibroblasts EUE human embryonic epithelial cells	TK6 cells: not cytotoxic in Trypan blue exclusion and proliferation assays. Cos-1 cells: Inhibition of plating efficiency and proliferation activity at 15 and 75 µg/cm^2 for 24h. EUE cells: No inhibition of proliferation activity with the first dispersion protocol but decreased proliferation at 6 and 30 µg/cm^2 with the second dispersion.	TK6 cells: Positive only at 75 µg/cm^2 after 2h of exposure, with the second dispersion protocol and with Fpg (tail intensity). Negative with the first dispersion protocol and in the second dispersion without Fpg (both durations) and with Fpg after 24h. Cos-1 cells: Positive (strand-breaks) only at 75 µg/cm^2 after 2 and 24h exposure without Fpg with the second	Not reported.

		added to the cell culture medium. Culture medium for TK6 cells: RPMI 1640 with heat-inactivated FBS, penicillin and streptomycin. Culture medium for other cells: DMEM medium containing FBS, penicillin and streptomycin. First protocol gives more stable (up to 2 days) bimodal dispersion with 2 peaks more or less in the nanosized range (about 100-300 nm) while the second protocol results in large agglomerates (about 700-800 nm) and less stable dispersion. Culture media did not influence the final dispersion.		Conditions tested: 0.12-75 µg/cm^2 for 2 and 24h.	protocol. With Fpg, only a slight but significant increase at 3 µg/cm^2. Negative with the first dispersion protocol. EUE cells (only standard Comet assay performed): Positive (strand-breaks) only at 75 µg/cm^2 after 24h exposure with the second dispersion protocol (tail intensity). Negative with the first dispersion protocol. Standard alkaline and Fpg modified Comet assay; conditions tested: 0.12, 0.6, 3, 15 and 75 µg/cm^2 for 2 or 24 h; no information on replicates or independent assays; negative and positive controls (data not presented for positive control)	
Osman, 2010	Anatase TiO_2 NP Purity: 99.7% No information on size.	Nanoparticles were suspended in 10 ml EMEM– EBSS medium at concentrations of 10, 20, 50 and 100 µg/ml. Suspensions were probe-sonicated at 30 W for 5 min on and off, and then allowed to equilibrate for different times: 0, 2, 4, 24 and 48 h. In culture media: aggregation with increasing dose, but remained constant over a 48h period, except at higher doses (from 50 µg/ml) of TiO_2. Size from 384 nm (up to 20 µg/ml) to 722 nm (100 µg/ml).	Human epithelial Hep-2 cell line	Cytotoxic MTT and NRU assays. Conditions tested: 20-100 µg/ml for 2, 4, 24 and 48h. In comet assay, cell viability was between 70-85% except at 100 µg/ml which showed 65% viability. High membrane integrity. Trypan Blue assay. Conditions tested: 10, 20, 50, 100 µg/ml for 4h.	Positive at all doses – dose related (OTM and %tail DNA) The dose of 100 µg/ml was toxic and precipitated. Alkaline Comet assay. Conditions tested: 10, 20, 50 µg/ml for 4h; 3 independent experiments, negative and positive controls.	Not reported.

- Chromosomal Aberrations assays

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity	Genotoxicity	Uptake
				Results; Methods; Interferences	Results; Methods	
Registration data 2011-01-31	TiO_2 No further information	Vehicle: water. The test substance formed a homogenous white suspension in the vehicle at the highest stock concentration prepared, 50 mg/mL. Cell culture: Complete medium (supplemented with FBS, L-glutamine, penicillin and streptomycin).	CHO cells	Preliminary assay: Substantial toxicity (> 50% reduction in cell growth relative to the vehicle control) at 250 µg/ml for the 4-hour test condition and 100 µg/ml for the 20 hour test condition. Main test: toxicity (> 50% reduction in cell growth relative to the vehicle control) at 100 µg/ml in the 4 hour non-activated and S9 activated test conditions; and 75 µg/ml in the 20 hour non-activated test condition.	Negative Chromosome aberration assay. Conditions tested: 25, 50, 75, 100, 150 µg/ml for 4 and 20h without metabolic activation and 4h with metabolic activation; duplicate; 200 metaphase per concentration scored; negative and positive controls.	Not reported.

- Gene Mutation assays

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity Results; Methods; Interferences	Genotoxicity Results; Methods	Uptake
Wang et al. 2011	Anatase (100%) Purity = 99.7%, < 25nm	Nano-TiO_2 was suspended in DMSO and vortexed for 1 min. Culture media: Ham's F12 medium supplemented with FBS, L-glutamine, and penicillin/streptomycin. TiO_2 appeared to aggregate in cell culture medium (generally > 100 nm).	Chinese hamster ovary cells (CHO-K1)	Not cytotoxic for concentration below 50 µg/ml with acute or chronic exposure. Cytotoxic at 100 and 200 µg/ml with acute exposure. XTT assay. Conditions tested: 0, 10, 20, 40 µg/ml and higher for 1, 2 or 60 days (results only shown for concentrations up to 40 µg/ml for 24 and 48h). HPRT assay: cell viability as measured by colony forming ability was not affected.	Negative HPRT gene mutation assay. Conditions tested: 10, 20, 40 µg/ml for 60 day; repeated 3 separate times using 3 plates for plating efficiency and mini 5 plates for 6TG resistance ; negative control but no positive control.	Nano-TiO_2 aggregates were internalized by CHO cells that were chronically exposed. Internalized TiO_2 appeared to be restricted to the cytoplasm and did not appear to localize to any specific organelles and none appeared within cell nuclei.

- Non-stanardized studies

Reference	Nanoparticles Characterization	Characterization in dispersion medium/culture media	Cells/organism	Cytotoxicity Results; Methods; Interferences	Genotoxicity Results; Methods	Uptake
Di Virgilio et al. 2010	TiO_2 Average particle size: 20 ± 7 nm; SSA: 142 m^2/g (BET)	Suspension prepared in PBS, vortexed for 10 minutes and stored at 4°C in the dark. Cell culture: Ham F10 culture medium supplemented with 10% inactivated fetal calf serum, 50 IU/mL penicillin and 50 g/mL streptomycin sulfate (complete culture medium).	Chinese hamster ovary (CHO-K1)	Cytotoxic: significant decrease from 25 µg/ml with neutral red assay and at all concentrations with MTT assay. NR and MTT assays. Conditions tested: 0, 5, 10, 25, 50, 100 µg/ml for 24h. SCE assay: proliferative rate index reduction (not statistically significant) at concentrations higher than 10 µg/ml. Absence of metaphase at 10 and 25 µg/ml.	Positive at 1 and 5 µg/ml. Highest concentrations could not be measured due to cytotoxic effects. Sister chromatid exchange assay. Conditions tested: 0, 1, 5, 10, 25 µg/ml under complete darkness for 24 hours; 3 independent experiments in triplicate; 100 metaphase scored per treatment; negative control but no positive control.	TiO_2 (50 µg/ml) for 24 hours: cells formed perinuclear vesicles containing phagocyted material. Agglomerates on both of the surface and inside the cells. Only present in the cytoplasm and no NP was detected in the nuclei. The nucleus shape is modified in the presence of some large vesicles which seem to press it. In some cells with many large vesicles, the membrane was disintegrated.
Turquez et al, 2011	TiO_2 < 100nm No further information.	TiO_2 was prepared with sterile dimethylsulfoxide (DMSO). Culture media:5 ml of culture medium (Chromosome Medium B) with5 mg/ml of phytohemag-glutinin.	Peripheral blood lymphocyte culture	Not cytotoxic at 3 and 5 µM: no alteration of PI values. Cytotoxic at 10 µM: statistically important decrease in the rate of PI.	Positive at all concentrations – dose-dependent. Sister chromatid exchange assay. Conditions tested: 3, 5, 10 µg/ml; 25 well-spread second division metaphases scores for each dose; negative control but no positive control. AA (ascorbic acid) during the treatment of the cells with TiO_2 reduced the number of SCEs significantly	Not reported.
Jomini et al. 2012	P25 Anatase/rutile (80/20) ; 25 nm ; SSA: 50±15 m^2/g (supplier data) Characterization : 84% anatase ; 16% rutile, 23 ± 4.9 nm	NP-TiO_2 dispersed in sterile ultrapure water. The resultant suspension was then probe-sonicated for 30 min at 4°C. P25: Average hydrodynamic diameter of the nanoparticle stock suspension obtained after dispersion in milli-Q	*Salmonella typimurium* strains (TA97a, TA98, TA100 and TA102)	Not reported	Negative in conventional fluctuation test P25: Positive: mainly in TA 98 and 102 TiO_2-NA: Positive in TA102	NP strongly adsorb on the surface of the bacterial wall in saline solution, whereas this is not the case in Ames test.

	TiO_2-NA 15% (w/v) stable suspension in acidified water; 100% anatase; 5-30 nm; SSA: 200-220 m^2/g; purity > 99.5% (supplier data) Characterization: 86% anatase, 14% brookite; 5.7 ± 1.9 nm	water and probe sonication ranged between 60 and 80 nm (DLS). TiO_2-NA: average hydrodynamic diameter of the nanoparticles between 5 and 10 nm. No statistical difference between TiO_2-P25 aggregate sizes in Ames test and saline solution (700-800 nm). For TiO_2-NA: average size of aggregates approximately 3800 nm in Ames test and only 67 nm in saline solution.			Conventional fluctuation test and modified Ames tests (with pre-exposure of .0.1, 10 or 20h). Conditions tested: S. *typhimurium* TA97a, TA98, TA100, TA102; 0.875, 8.75, 87.5 mg/l; 2 to 3 independent assays; negative and positive controls; level of revertants for the negative controls are low.	
Setyawati et al. 2012	P25 Range of 73–85% anatase, 14–17% rutile and 2–13% amorphous TEM: 22 ± 6.4 nm	Nanoparticles were dispersed in PBS. The suspensions were then sterilized by 15 min of UV exposure and bath sonicated for 10 min. Thereafter, the stock suspensions were diluted 1:9 in complete media and further sonicated for 10 min to make up the final working concentration of NP. In water: hydrodynamic sizes: 180.9±3.15 nm; ZP: +20.9±10.3 mV. In complete media: 255.8±2.65 nm. It was suggested that NP readily adsorbed proteins upon introduction to serum supplemented cell culture medium.	Human neonatal foreskin fibroblast cells (BJ)	Cytotoxic: dose-dependent from 250 µg/ml. Cell proliferation assay. Conditions tested: 10-1000 µg/ml for 24h.	Positive - dose-dependent. Phosphorylation of ɣ-H2Ax assay. Conditions tested: 10 and 500 µg/ml for 24h; no positive control; one experiment; 150 cells scored per condition.	Not reported.
Demir et al. 2015	Micro TiO_2 99% to 100.5% purity Nano TiO_2 Anatase, 21 nm; > 99.5% purity	No information Dispersed at the concentration of 2.56 mg/mL prepared in a 0.05% bovine	human embryonic kidney (HEK293)	Not reported. Some information from CBMN assay : micro-TiO_2 was non cytotoxic but nano-	Micro- TiO_2: negative Nano-TiO_2 (21 and 50 nm): Positive at 1000 µg/ml in both cell lines (increase in both the	Not reported.

	Nano TiO_2 Anatase, 50 nm, > 98% purity	serum albumin (BSA) in water, subjected to ultrasonication at 20kHz for 16 min in an ice-cooled bath; (in agreement of the proposal from Nanogenotox UE project). No important agglomerations observed following the dispersion protocol used. Nano-TiO_2 (21 nm) : 22.94± 0.3 nm (DLS) and ZP : 8.71mV Nano-TiO_2 (50 nm) : 50.72±0.4 nm (DLS) and ZP: 9.38mV No agglomeration in media (soft-agar colony assay)	Mouse embryonic fibroblast (NIH/3T3) cells	TiO_2 were cytotoxic at 1000 µg/ml.	number and the diameter) – dose-dependent. Cell transformation assay (soft-agar colony assay). Conditions tested: 10, 100 and 1000 µg/ml for 3 weeks; 2 independent experiments and 2 replicates; negative control but no positive control.	
Vales et al. 2014	NM 102 Anatase; primary particle size: 21.7±0.6 nm	Nanogenotox protocol: pre-wetted in 0.5% absolute ethanol and afterwards dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water, the nanoparticles in the dispersion medium were sonicated for 16 min. In exposure medium: 575.9 nm; PDI: 0.471 (DLS), ZP: -19.5 mV (LDV)	BEAS-2B cells	Not reported	Positive: Significant dose-dependent increase in the number of colonies growing on soft-agar for medium-large size colonies at 20 mg/mL. Not significant but dose-dependent increase of total colonies. Soft-agar assay (assessment of acquired cancer phenotype). Conditions tested: 1, 10 and 20 µg/ml for 4 weeks of continuous exposure; 3 independent assays but no positive control.	Uptake after 24h to 20 µg/ml (TEM)
Shi et al. 2010	P25 Anatase:rutile crystalline ratio (8:2) Primary particle diameter approx. 25 nm; surface area (BET) of 50 m^2/g.	Nano-TiO_2 was sterilized by heating to 120°C for 2 h, and freshly suspended in distilled water immediately before use. Culture medium: DMEM containing FBS, penicillin G and streptomycin.	Human hepatocytes L-02	Not cytotoxic with the ATP kit. Conditions tested: 0.01-1 µg/ml for 12, 24 or 36h.	Positive at 1 µg/l 8-OH-dG analysis. Conditions tested: 0.01 -1 µg/l for 24 h in the tables but 0.01 -1 µg/ml in the text; triplicate and repeated three times; negative control but no positive control.	Not reported.
	TiO_2-A12 NP					

Jugan et al, 2012	95% anatase, spherical 12 nm; 92 m^2/g; PZS: 6.4	Dispersion in ultrapure sterile water(pH5.5) by sonication for 30 min at 4 C,in pulsed mode(1 s on/1 s off), at the concentration of 10 mg.ml-1 Suspensions were diluted in cell culture medium (DMEM without serum)	A549 human lung carcinoma cells (CCL-185)	Cytotoxic in MTT assay (less than 25% of cell death after 48h). Conditions tested: 1–200 µg/ml for 4–48 h.	Positive for all durations for A12 and A25 – no duration-dependent. Positive for 24 and 48h exposure for R68 – duration dependent. Positive only for 48h exposure for R20 Negative for A140 8-oxo-dG assay. Conditions tested: 100 µg/ml for 4, 24 or 48 h; triplicate; negative control but no positive control	An unambiguous accumulation of the smallest NPs in the cytoplasms and in the nucleus of cells
	TiO_2-R20 NPs 90% rutile, spherical 21 nm; 73 m^2/g					
	TiO_2-A25 (AEROXIDE P25) 86% anatase/14% rutile, spherical 24 nm; 46 m^2/g; PZS: 7.0					
	TiO_2-R68 100% rutile, elongated L: 68 nm d: 9 nm; 118 m^2/g			Cytotoxic (less than 1% of cell death at the highest dose after 48h) in MTT assay (1–200 µg/ml for 4–48 h)		Cytoplasmic accumulation was also observed but not in cell nuclei
	TiO_2-A140 100% anatase, spherical 142 nm; 10 m^2/g; PZS: 5.2			Cytotoxic (less than 10% of cell death at the highest dose after 48h) in MTT assay (1–200 µg/ml for 4–48 h)		
Wan et al. 2012	Nano-TiO_2. 90 % anatase and 10% rutile ; 28 nm, BET = 45 m^2/g	TiO_2 dispersed in physiological saline and ultrasonicated for 30 min. Then added to culture medium (Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) In cell culture medium: size (DLS): 280 nm	A549 human lung epithelial cells	Not cytotoxic with *in vitro* cytotoxicity kit (SBR) and AlamarBlue™ assay (colorimetric/fluorimetric method).at 2.5-40 µg/ml for 24h	Negative Measurement of phosphorylation of ɣ-H2Ax; 8 oxodG level; repair protein Rad51; accumulation of p53 and phosphorylation. Conditions tested (ɣ-H2Ax test): 5 15 µg/ml for 6 h or 15 µg/ml for 1, 3, 6 or 12h. Conditions tested (8 oxodG test): 5 or 15 µg/ml for 12 or 24h . Conditions tested (Rad51 and p53 tests): 5, 10, 15 for 12h. Negative control but no positive control.	The uptake of Nano-TiO_2 in A549 cells was 2.75×10^{-12} and 4.34×10^{-12} (g/cell) with exposure to 5 and 15 µg/ml respectively for 12h - clear dose dependent (ICP-MS).

Msiska et al. 2010	Fine TiO_2 < 5 µm, SSA: 2.28 m^2/g.	Stock solution prepared in RPMI 1640 basal media containing FBS. Working concentrations were prepared immediately before treatment of cells in medium with FBSI and vortexed for 2 min before use.	A549 human pulmonary cells Normal human SAE cells	Not cytotoxic in A549 cells Cytotoxic in SAE cells after 24h only. LDH assay. Conditions tested: 100 µg/ml for 6, 18 and 24h.	Positive in A549 (relative ɣ-H2Ax levels and number of ɣ-H2Ax foci) Positive in SAE cells (only for number of ɣ-H2Ax foci) Phosphorylation of ɣ-H2Ax assay. Conditions tested: 100 µg/ml for 24h; each experiment performed 3 times in triplicates; negative control but no positive control	Not reported.
Toyooka et al. 2012	TiO_2-NP Anatase ; 5 nm Micro-TiO_2 Anatase ; < 5000 nm	Suspended in DMEM and then sonicated for 1 min in a bath-type sonicator. Added to cells growing in DMEM with FBS and penicillin/streptomycin. In DMEM: TiO_2-NP: 250-650 nm Micro-TiO_2 : 600-1050 nm	A549 human pulmonary cells	Cytotoxic from 750 µg/ml for NP and at the highest concentrations for micro-TiO_2. Over 80% of the cells that took up the micro- and nanoparticles survived during culture for 24 h. Tryptan Blue assay. Conditions tested: 50-1000 µg/ml for 24h.	Positive Degree of ɣ-H2AX generation was different between micro and nano- TiO_2. In nanoparticles, a small amount (from 1 µg/ml) could generate ɣ-H2AX. In microparticles, similar generation was detected from over 75 µg/ml. When TiO_2-NP was coated with BSA: γ-H2AX generation not after 1h-exposure but when increasing the time exposure to 8h. Detection of ɣ-H2Ax. Conditions tested : TiO_2-NP at 300 µg/ml for 1h (immunofluorescence); 1-100 µg/ml or 250-1000 µg/ml micro and nano-forms for 1h (Western Blot); 50-250 µg/ml micro and nano-forms for 1h (biased sinusoidal field gel electrophoresis BSFGE); experiments repeated 2 or 3 times. Negative control but no positive control.	TiO_2 dose-dependent changes of SS (side-scattered light) intensity, suggesting that TiO_2-NP were easily incorporated in A549 cells in a dose-dependent manner. Incorporation of NP was more remarkable that that of micro-TiO_2. Incorporation attenuated when TiO_2 was coated with BSA.

ANNEX III: LIST OF ABBREVIATIONS

AA: Ascorbic Acid

AK: Adenylate kinase

ALP: Alkaline phosphatase

ATM: Atomic Force Microscopy

BALf: Bronchoalveolar Fluid

BET: Brunauer, Emmet and Teller calculation method

BNMN: Binucleated cells with micronucleus

BSA: Bovine serum albumin

CA: Chromosome aberrations

CAT: Catalase

CBMN: Cytokinesis -block micronucleus

CBPI: Cytokinesis Block Proliferation Index

CE diameter: Circle Equivalent Diameter

CHL: Chlorophyllin

CI: Confidence interval

CMEM: complete minimum essential medium

SCE: Sister chromatid exchanges

DHPN: N-bis(2-hydroxypropyl)nitrosamine

DI water: Deionized water

DLS: Dynamic light scattering

DMBA: 7,12-dimethylbenz[a]anthracene

DMEM: Dulbecco's Modified Eagle Medium

DMTU: Dimetylthiourea

DSPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

dZ: Z-averaged diameter

FBS: Fetal Bovine Serum

FCS: Fetal Calf Serum

FPG: formamidopyrimidine [fapy] – DNA glycosylase

GGT: ɣ-glutamyltransferase

GPX: Glutathione peroxidase

GSH: Glutathione

HPMC: hydroxypropylmethylcellulose

HPRT gene mutation test: Hypoxanthine-Guanine Phosphoribosyl Transferase gene mutation test

IMEM: incomplete minimum essential medium

LDH: Lactate dehydrogenase

LDV: Laser Doppler Velocimetry

MDA: Malondialdehyde

MEM: Minimal Essential Medium

MMAD: Mass median aerodynamic diameter

MMD: Mass median diameter

MN: Micronucleus

MP: Melting point

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC: N-acetylcysteine

NAG: N-acetyl-b-D-glucosaminidase

NCE: Normochromatic Erythrocytes

NM: Nanomaterial

NP: Nanoparticle

NRU: Neutral Red uptake

OR: Odd ratio

OTM: Olive Tail Moment

PBS: Phosphate-buffered saline

PCE: Polychromatic Erythrocytes

PDI: Polydispersivity index

PI: Proliferation index

PND: Post-natal day

PPS: Primary particle size

PZC: Point of Zero Charge

RCC: Relative cell count

RET: Reticulocytes

RI: Replication Index

RICC: Relative increase in cell count

ROS: Reactive oxygen species

RRs: Risk ratios

SEM: Scanning Electron Microscopy

SMR: Standardized mortality ratio

SOD: Superoxide dismutase

SSA: Specific surface area

T-AOC: total antioxidant capability

TEM: Transmission Electron Microscopy

TPA: 12-o-tetradecanoylphorbol 13-acetate

ZP: Zeta Potential