ECHA
EUROPEAN CHEMICALS AGENCY

Helsinki, 02 July 2021

Addressees
Registrants of titanium dioxide listed in the last Appendix of this decision

Registered substance subject to this decision ('the Substance')
Substance name: titanium dioxide
EC number: 236-675-5
CAS number: 13463-67-7

Decision/annotation number
Please refer to the REACH-IT message which delivered this communication (in format SEV-D-XXXXXXXXXX-XX-XX/F)

DECISION ON SUBSTANCE EVALUATION

Under Article 46(1) of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below:

Intratracheal instillation study combined with comet assay in rats on 11 forms of Titanium dioxide (TiO_2).

This intratracheal (IT) instillation study must fulfil the following requirements as further detailed in Appendix A:

- For each form tested, the protocol of the intratracheal instillation study in rats must comprise 3 different concentrations, followed by 3 post-exposure observation times (2-6 h, 1 day and 28 day post exposure);
- The lowest concentration must produce little or no evidence of toxicity and the highest concentration must result in a clear level of toxicity but not inducing overload where feasible;
- 2 instillations must be performed at 24 h interval (at 0 and 24 h). The same test protocol must be used for each tested form of the Substance;
- 5 male rats must be included in each group and observation time;
- Observations and examinations at each post-exposure observation times (2-6 h, 1 day and 28 day post exposure, unless otherwise explicity mentioned) will be used to gather data on traditional endpoints together with early and sensitive markers of toxicities as follows:

    1. The pulmonary response, including bronchoalveolar lavage fluid (BALF) and histopathology of the lung must be performed; as required in the OECD Guidance Document n°39 (GD39) on inhalation studies (2018b), the mandatory BALF parameters are lactate dehydrogenase (LDH), total protein or albumin, total leukocyte count, absolute cell counts, and calculated differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils.

ECHA
EUROPEAN CHEMICALS AGENCY

2. Oxidative stress by measuring reactive oxygen species (ROS) and reactive nitrogen species (RNS) by fluorimetric probes (e.g. DCFDA/H2DCFDA, mitoSox and dihydroethidium (DHE)), malondialdehyde (MDA) by HPLC and Haeme oxygenase 1 activity in the lung must be made. These measurements must not be performed at 28 days post-exposure.
3. Histopathology of liver, kidney, testis and brain.
4. Cardiovascular function by measuring at least nitrogen oxide (NO) content, endothelial nitrogen oxide synthase (eNOS) activity and high sensitivity C reactive protein (hs-CRP) content in the serum.
5. Comet assay on lung tissues must be performed according to the OECD TG 489 with and without specific modification to detect oxidative damage.

- The test must be performed on the following 11 forms of the Substance selected to cover the about 300 TiO_2 forms of the registration dossier:
  1. uncoated mixed phase **nano**,
  2. uncoated **nano** anatase (5 nm),
  3. uncoated pigmentary rutile,
  4. pigmentary rutile coated with alumina and 1,1,1-Trimethylolpropane (TMP).
  5. pigmentary rutile coated with alumina, zirconia and TMP,
  6. **nano** rutile coated with alumina and hydrophobic organic,
  7. pigmentary rutile coated with high Specific Surface Area (SSA) silica and alumina ($40m^2/g$),
  8. **nano** rutile coated with silica ($40m^2/g$),
  9. pigmentary rutile coated with aluminium phosphate,
  10. pigmentary uncoated anatase (E171), and
  11. **nano** anatase containing tungsten in its coating[1].

  Note: the physico-chemical characterisation of each test material must be reported (See Appendix A, section 5).

Deadline to submit the requested information

Since the requested study is non-standard and includes a large set of testing materials, a large number of animals to be tested, and may require some development of analytical methods, ECHA has granted additional time.

The information requested must be generated and provided by **30 months**[2] (see also Section A.8 for details).

Conditions to comply with the information requested

To comply with this decision, you must submit the information in an updated registration dossier, by the deadlines indicated above. The information must comply with the IUCLID

[1] In your comments, you proposed to add this form to the test.
[2] The final deadline includes the 90-day period addressed in Article 53(1) of REACH and the seven-day period addressed in point 9(d) of the terms and conditions of REACH-IT.

ECHA
EUROPEAN CHEMICALS AGENCY

robust study summary format. You must also attach the full study report for the corresponding study/ies in the corresponding endpoint of IUCLID.

You must update the chemical safety report, where relevant, including any changes to classification and labelling, based on the newly generated information.

Appendices

The justifications of this decision and any further test specifications of the requirements are set out in Appendix A. The procedural history is described in Appendix B. Further information, observations and technical guidance as appropriate are provided in Appendix C. Appendix D contains a list of the addressees of this decision.

Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Approved[3] under the authority of Christel Schilliger-Musset, Director of Hazard Assessment

[3] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

ECHA
EUROPEAN CHEMICALS AGENCY

Basis for substance evaluation

The objective of substance evaluation under REACH is to allow for the generation of further information on substances suspected of posing a risk to human health or the environment ('potential risk').

ECHA has concluded that further information on the Substance is necessary to enable the evaluating Member State Competent Authority (MSCA) to clarify a potential risk and whether regulatory risk management is required to ensure the safe use of the Substance.

The ECHA decision requesting further information is based on the following:

(1) There is a potential risk to human health via inhalation, based on a combination of hazard and exposure information;
(2) Information is necessary to clarify the potential risk identified; and
(3) There is a realistic possibility that the information requested would allow improved risk management measures to be taken.

The Appendices entitled 'Reasons to request information' describe why the requested information are necessary and appropriate.

ECHA
EUROPEAN CHEMICALS AGENCY

Appendix A – Reasons to request information to clarify the potential risk related to repeated dose toxicity via inhalation

Based on the evaluation of all relevant information submitted in the chemical safety report on titanium dioxide (TiO_2; the Substance) and other relevant information available in the scientific literature, further information is required to enable the evaluating Member State competent authority (eMSCA) to complete the evaluation of whether the Substance, including its nanoforms, constitutes a risk to human health. To answer your comment that the "*DD completely disregards the overall evidence presented in the dossier...*", an effort has been made to identify the information coming from the latest update of the registration dossier (dated 03 March 2020).

A.1 The potential risk – human health

1. Potential exposure

According to information in the registration dossier, the chemical safety report and the scientific literature, the Substance is used as a pigment and an opacifying agent. It has resistance to chemical attack, thermal stability, resistance to UV degradation (UV blocker) and photocatalysis potential. Due to its photocatalytic properties, when the size of the particle is reduced to the nanoscale in one or more dimensions, nano TiO_2 is also used for water and surface treatment. The Substance 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 registration dossier. The uses of the Substance depend on its properties that are determined by the crystallinity, the size, the shape and surface chemistry of the TiO_2 particle. Based on the very wide uses for various purposes, human exposure, including inhalation route, cannot be excluded.

You commented that exposure of general population is not likely to occur, especially when focusing to the unbound respirable TiO_2. Giving the fact that consumers can be exposed to the Substance via many sources, ECHA considers that exposure is likely to occur. Indeed, the Substance is present in food, pharmaceuticals, and cosmetic products, including cosmetics powder, skin protecting cream, natural white moisture protection cream, powder make-up, and various other cosmetic cream products. The Substance has applications in coatings, such as in plastics, foods packing material, and in printing inks. It is used in aerosol paints. In addition, the Substance has also applications in photocatalysts, UV-resistant materials, antibacterial materials, sewage treatment, and self-cleaning glass and ceramics. It is also used in metallurgical, paper, and astronautics industry.
Further, workers and/or consumers can be exposed to unbound powder used in some of the applications mentioned above. In addition, one should also consider that the aging of material is mostly unknown. As demonstrated for sunscreens, the organic coating present at the surface of the nanomaterial may be disaggregated or being removed by mechanical interaction and thus expose humans to the core of the material (Auffan et al., 2010; Labille et al., 2010). The eMSCA acknowledges that reliable data, measurement methods and strategies for the estimation of consumer and environmental exposure are still under development and validation, and precise estimations are not possible. It is apparent that dermal consumer exposure to the Substance occurs. Also, the eMSCA believes that inhalation of different forms of the Substance cannot be excluded and that there is a potential risk for workers and consumers. Under this substance evaluation process, the eMSCA has decided to focus on the inhalation route.

ECHA
EUROPEAN CHEMICALS AGENCY

The Substance is used in cosmetics and personal care products according to the Regulation (EC) No 1223/2009 on cosmetic products. As noted above, there are also uses under REACH. ECHA's factsheet[4] on the interface between REACH and Cosmetics Regulations, developed jointly with the European Commission, provides that Registrants of substances which use the Substance also for non-cosmetic uses (i.e. mixed-use substances) are required to perform as a last resort, studies for all endpoints requiring vertebrate testing. As is apparent from the Commission Communication of 11 March 2013 on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics (COM(2013)135), such testing would not trigger the testing and marketing bans under the Cosmetics Regulation as the testing is to be performed for the purposes of meeting the requirements of REACH.

2. Potential hazard of the Substance after repeated exposure via inhalation.

The available information suggests that different forms of the Substance tested, including nanoforms (TiO_2-NPs), may cause adverse effects and toxicity after repeated exposure via inhalation: pulmonary inflammation and effects on the cardiovascular system , central nervous system , liver, kidney and testis as well as weak genotoxicity (effects are detailed in sections 2.2. and 2.3. below).

A detailed explanation of the available evidence is provided below substantiating the potential risks that the Substance may cause by repeated inhalation exposure:

2.1. Potential pulmonary toxicity
2.2. Potential systemic organ toxicity
2.3. Potential oxidative stress and genotoxicity

2.1. Potential pulmonary toxicity

2.1.1. Evidence for repeated dose toxicity

Six repeated-dose toxicity studies, from 5 days to 13 weeks exposure, performed by inhalation route with several concentrations of the Substance were identified (Bermudez *et al.*, 2004; Ma-Hock *et al.*, 2009; Lansiedel *et al.*, 2014; Yu *et al.*, 2015; Oyabu *et al.*, 2017; BAuA report F2364, 2018). All but one (BAuA report F2364, 2018) were submitted in the registration dossier.

You provided the BAuA report (F2364, 2018) in your comments as additional information on repeated-dose toxicity by inhalation, which the eMSCA has taken into account. In this study, TiO_2-NPs (P25, to which the test material form number 1 as specified in the request seems to be the closest) increased polymorphonuclear (PMN) leukocyte recruitment by 30-45% which persists after 28 days even in the low dose group, increased clearance half-life in the lung with increase in concentration. This was not observed in the case of exposure to microsized-TiO2 Bayertitan T. The above mentioned studies (6) focused mainly on pulmonary response (mainly inflammation) to TiO2-NPs exposure.

In comparison, the OECD TG 413 (subchronic inhalation toxicity study) will provide a list of about 40 organs to be investigated.

Moreover, the six repeated dose toxicity inhalation studies were performed on different forms of the Substance (e.g. different crystal phases, coated or uncoated), with different durations of exposure (see Table 1), rendering the comparison of the results difficult if not

[4] https://echa.europa.eu/documents/10162/13628/reach_cosmetics_factsheet_en.pdf/2fbcf6bf-cc78-4a2c-83fa-43ca87cfb314

impossible. However, all the provided studies indicate adverse pulmonary effects. The results of these studies are presented in Table 1.

Table 1. Description of the existing repeated-dose toxicity studies by inhalation performed with various forms and concentrations of TiO_2-NPs.

Reference	Material tested	Concentrations	Duration of exposure	Characterisation	Results
Bermudez *et al.*, 2004*	P25	0.5, 2.0, or 10 mg/m3	6 h/day, 5 days/week for 13 weeks	21 nm Information from OECD: about 80% anatase/20% rutile; surface area: about 50 m^2/g	Lung toxicity with hyperplasia/ hypertrophy in rats NOAEC = 0.5 mg/m3 Respiratory effects in mice NOAEC = 2.0 mg/m3 No respiratory effect in hamster
Ma-Hock *et al.*, 2009*	Uncoated TiO_2 with hydrophobic surface	2.0, 10 or 50 mg/m3	5 days	95.1% TiO_2; 14% rutile, 86% anatase; 25.1 ± 8.2 nm; surface area: 51.1 ± 0.2 m^2/g	Transient inflammation; lung hypertrophy/ hyperplasia LOAEC = 2 mg/m3
Lansiedel *et al.*, 2014*	T-Lite SF	0.5, 2 et 10 mg/m3	5 days	Dimethicone coated rutile TiO_2 82% TiO_2 and 10% $Al(OH)_3$) 24 nm	Transient pulmonary inflammatory reaction NOAEC = 0.5 mg/m3
Yu *et al.*, 2015*	$nTiO_2$	2.5, 5, 10 mg/m3	4 weeks	19.3±5.4 nm, no information on crystallinity or coating	Lung inflammation, hyperplasia and haemorrhage in the lung, elevations of markers of liver and kidney toxicity LOAEC = 2.5 mg/m3
Oyabu *et al.*, 2017*	MT-150AW	0.50 ± 0.26 and 1.84 ± 0.74 mg/m3	4 weeks	rutile spindle-shaped; 12x55 nm; average agglomerated particle size: 44.9 nm; purity = 99.5%; surface area = 111 m^2/g	Some alveolar macrophages with pigment-like material deposition in the alveoli NOAEC = 1.84 mg/m3
BAuA, 2018	P25 and µ-TiO_2 Bayertitan T	28 and 85 mg/m3 for µTiO_2 (low and high concentrations) and 9.7 and 29.1	6 hours/day, 5 days/week for 2 weeks and sacrifice at	µ-TiO_2: ρ: 4.3 98,17% TiO_2 99,5% Rutile BET: 1,9 m2/g; EGME: 21,7 m2/g µ-TiO_2; MMGD: 1,8 µm	At high concentration, effect on PMN levels at 3 and 28 days for P25, inflammatory effects of the high dose of P25 at day 3 and normalisation at day

ECHA
EUROPEAN CHEMICALS AGENCY

		mg/m3 for P25 (low and high concentrations)	3 and 28 days	MMAD: 0.90 and 1.37 µm P25: ρ: 4.3 - ρAgg: 1.6 BET: 60 m2/g Anatase/Rutile 80%/20% MMAD : 0.57 and 0.58 µm	28.

* Available in the registration dossier

Hazard after inhalation are described but the available studies are not appropriate to characterise it properly.

New studies according to OECD TG 413 should be conducted in the Tier 2 on appropriate forms selected based on the current decision (see Section A.3).

2.1.2. Studies showing that the physicochemical properties of the Substance can affect the potency of the different forms of TiO2-NPs in inducing various pulmonary toxicities

n.b. based on your comments, this title was modified to strenghten the justification that it is important to add different parameters in the initial Tier 1 study: to ensure proper identification of the TiO_2 forms covered by the joint submission to be further tested in Tier 2, and also to allow, later on, prediction of the toxicity of the TiO_2 forms that will not be tested.

- The impact of **crystal phase** on the pulmonary toxicity of TiO_2-NPs was shown by different authors but with contradictory conclusions. For example, Aragao-Santiago *et al.* (2016) noted a distinct inflammatory potential, between anatase and rutile TiO_2-NPs, the latter not inducing inflammatory response. In contrast, Rahman *et al.* (2017) and Numano *et al.* (2014) reported a higher overall biological response of the lung with rutile compared to anatase.

  Roursgaard *et al.* (2011) described a higher inflammatory response for rutile form when compared to anatase and amorphous TiO_2-NPs. In addition, they identified amorphous polymorph TiO_2-NPs as the most potent form in regard to acute tissue damage, based on the level of total protein in bronchoalveolar fluid. Okada *et al.* (2016) found that mixed-crystal phase and amorphous TiO_2-NPs lead to the most severe fibrosis compared to the one resulting from treatment with anatase and rutile forms. Warheit *et al.* (2007) also observed a more pronounced inflammation with mixed-crystal phase.

  ECHA considers that, at least TiO_2 forms 1, 2, 3, 8 and 10 (as specified in the request) will allow to evaluate the influence of crystal phase on pulmonary toxicity and on the early and sensitive markers to toxicity.

- Regarding the **size**, nanoparticles are expected to be more reactive than bulk materials with an increase of the pulmonary response due to a delayed clearance, longer biopersistence and deeper penetration into interstitial regions of alveoli. In particular, in a general study on particles, Drew *et al.* (2017) found that metrics related to the particle size (such as density, surface area and diameter) appeared to be the most predictive for estimating potency of a nanomaterial in eliciting

pulmonary inflammation. A similar conclusion – the smaller the particle size, the greater the inflammatory response - is also reached by several authors when they compared the lung toxicity (gene expression response, examination of bronchoalveolar lavage fluid (BALF), and lung histopathology) after an intratracheal (IT) exposure to various forms of the Substance (Halappanavar *et al.,* 2015; Hashizume *et al.,* 2016; Kobayashi *et al.,* 2009; Rahman *et al.,* 2017). Noel *et al.* (2013) hypothesized that the lower cytotoxicity observed for the larger TiO_2-NPs could possibly be due to their less efficient penetration into cells. Contrasting with these findings, other authors did not evidence a direct association between particle size and inflammatory potential of TiO_2-NPs after intratracheal (Li *et al.,* 2007; Roursgaard *et al.,* 2011) or inhalation administration (Rossi *et al.,* 2009).

ECHA considers that, at least TiO_2 forms 2, 4, 8 and 10 (as specified in the request) will allow to evaluate the influence of particle size on pulmonary toxicity and on the early and sensitive markers to toxicity.

- The presence of a coating can affect the behavior of nanoparticles in the medium, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or interaction with macromolecules. Moreover, the coating may itself also release toxic material (Charles *et al.*, 2018). Even if coated forms of TiO_2-NPs are rarely tested in toxicological studies, some publications emphasize that it is essential to take into account surface coating in risk assessment. Hashizume *et al.* (2016) reported that $Al(OH)_3$-coated TiO_2-NPs induced a greater pulmonary inflammatory response than non-coated particles. Halappanavar *et al.* (2015) also noted that changes in the surface characteristics, such as the addition of positively charged amino groups, can further enhance the inflammatory potential of TiO_2-NP. Similarly, Rahman *et al.* (2017) demonstrated an exacerbation of the pulmonary response when animals were exposed to TiO_2-NPs covered with a hydrophilic coating, compared with no or hydrophobic coating. Among different forms of TiO_2-NPs tested, Rossi *et al.* (2009) found that only Si-coated rutile TiO_2-NPs elicited clear pulmonary inflammation compared to uncoated TiO_2-NP.

  ECHA considers that, at least TiO_2 forms 3, 4, 5, 7, 8 and 9 (as specified in the request) will allow to evaluate the influence of coating on pulmonary toxicity and on the early and sensitive markers to toxicity.

- The **different shapes** of TiO_2-NPs such as nanospheres, nanobelts, nanorods, nanodots, needles, tubes, fibers-like, can influence the lung toxicity. For example, Hamilton *et al.* (2009) demonstrated that alteration of TiO_2 nanomaterial into a fibre structure of greater than 15 µm creates a highly toxic particle and initiates an inflammatory response by alveolar macrophages. Similar conclusion was reached by Porter *et al.* (2013) and Silva *et al.* (2013) who reported more severe pulmonary responses with nanobelts compared to nanospheres. In addition, Danielsen *et al.* (2020), detailed below following your comment on the impossibility to perform the requested assay in the same animal, reported a high inflammatory response with nanotube TiO_2 and a moderate inflammatory response with nano cube-like or nano sphere-like TiO_2 in the lung of mice at the same concentration in an IT instillation study (all anatase, which TiO_2 form 2, as specified in the request seems to be the closest). They also reported a lesser neutrophils recruitment with nano cube than other shapes tested. The most prominent neutrophil influx was observed with the TiO_2 tube. In contrast, Warheit *et al.*(2006a) did not find any significant differences in the pulmonary responses to anatase nanodots versus anatase nanorods, despite a sixfold difference in surface area properties. Sager and Castranova (2009)

propose severe agglomeration as an explanation for these results, but this has to be confirmed in a proper study since these comparisons were not made for all shapes of the Substance.

ECHA considers that all TiO_2 forms, 1–11 (as specified in the request) will allow to evaluate the influence of shapes on pulmonary toxicity and on the early and sensitive markers to toxicity.

Many of the publications cited above are available in the registration dossier (updated in March 2020), although "*disregarded due to major methodological deficiencies*" without further details.

Finally in your comments, you indicated that there is a need to add an additional 11th form of the Substance due to sufficiently different characteristics compared to the other 10 TiO_2 forms you initially proposed to be studied. ECHA agrees with your proposal to test also the additional 11th form of the Substance and amended the decision accordingly. This will help to evaluate the influence of the various parameters of the different TiO_2 forms on toxicity and allow to clarify the concerns raised by the above-mentioned publications.

2.2. Potential for systemic organ toxicity

The study by Yu *et al.* (2015) presented in Table 1 also provides evidence that the toxicity of TiO2-NPs is not restricted to local lung effects but can also include systemic toxicity after repeated exposure. Effects on the cardiovascular system, the central nervous system, the liver and kidney were reported after exposure to the Substance via inhalation route. Thus, inhalation of TiO_2-NPs may induce effects in other organs than lungs. However, from the available database, it is not clear (i) at which the level these effects may occur, compared to the occurrence of lung effects, and (ii) what is the different potency of different forms of TiO_2 causing these effects.

2.2.1. Cardiovascular system

Effects of TiO_2-NPs on cardiovascular system were noted in several studies after a single and repeated exposure, and consist mainly of microvascular dysfunction. Many of these studies are available in the registration dossier, though you have disregarded them due to "*major methodological deficiencies*" without further details. The effects on cardiovascular system were reported in non-pregnant rats after a single exposure to P25 by inhalation (Nurkiewicz *et al.*, 2008 & 2009, LeBlanc *et al.*, 2009 & 2010, Knuckles *et al.*, 2012) but also in pregnant rats and their foetuses when TiO_2-NPs were administered by inhalation during gestation (Stapelton *et al.*, 2013 & 2015).

You commented that "*possible cardiotoxic effects will most likely be manifested after 90 days of exposure, which can then be observed in histopathological examinations of the heart. However, such effects were not observed in sub-chronic inhalation toxicity studies conducted in rats, hamster or mice using the same test material as used by Nurkiewicz and LeBlanc up to concentrations of 10 mg/m3 (Bermudez et al. 2004). According to the study protocol, the heart was histopathologically examined and no effects indicating any kind of cardiotoxicity were reported after 90 days of exposure or up to 52 weeks of recovery*". If you refer to the Bermudez *et al.* (2004) study, the Materials and Methods section clearly states that: "*Histopathology. Paraffin-embedded left lung tissues were sectioned at 5 μm and stained with Masson's trichrome. The trichrome-stained lung sections were evaluated for particle-induced histopathological changes.*" It is therefore clear that the heart was not histologically examined in this study.

Based on your comments, the eMSCA clarified that the cardiovascular function rather than cardiotoxicity should be investigated. In particular NO (nitric oxide), eNOS (endothelial nitric oxide synthase) activity and CRP (C Reactive Protein) have previously been shown to be sensitive biomarkers to TiO_2-NPs in studies by inhalation (Nurkiewicz *et al.* 2008, Nurkiewicz *et al.* 2009, LeBlanc *et al.* 2009, LeBlanc *et al.* 2010, Chen *et al.*, 2013).

Initially, the determination of the amount of SAA3 (serum amyloid A3), another positive acute phase protein, was requested. Following your comment, the request was modified to include the determination of high-sensitivity CRP (hs-CRP) but not SAA3 (see section A5 for rationale).

The above-mentioned markers allow to evaluate risk factors for a cardiovascular disease to occur after inhalation exposure to the Substance. Furthermore, low levels of translocation of TiO_2-NPs to heart were observed after a single IT instillation in mice when analysed at 24 hours post-exposure (Husain et al. 2015).

The eleven forms of the Substance to be tested will allow you to identify whether some parameters impact cardiovascular functioning. Later, it will help you to predict the cardiovascular toxicity of all forms of the Substance included in the registration dossier.

2.2.2. Central nervous system

Some studies also investigated effects of various TiO_2-NPs on the central nervous system and identified deleterious effects on the brain neurochemistry and histopathology (in particular in the hippocampus) after repeated exposure by inhalation (Disdier *et al.*, 2017, four weeks exposure, one high concentration) or after intra-nasal instillation for up to 30 days (Wang *et al.*, 2008 a & b; Zhang *et al.*, 2011). The findings by Disdier *et al.* (2017) indicated dysregulation of blood brain barrier (BBB), neuroinflammation and impact on neuronal acitivity after exposure to nanoforms of the Substance via inhalation in rats. This study did not provide evidence of translocation of TiO_2-NPs to the brain after inhalation exposure. However, Pujalté *et al.* (2017) observed a slight translocation of TiO_2-NPs to the olfactory bulb and the brain after a 6-hr exposure by inhalation in rats.

2.2.3. Liver and kidney

Concerning hepatotoxicity, oedema and loose cytoplasm of liver cells were found in rats exposed to TiO_2-NPs by instillation twice a week, for four consecutive weeks (Chang *et al.*, 2015). After inhalation exposure, translocation of small amounts of TiO2-NPs to liver was observed in rats after acute exposure for 6 hours (Pujalté *et al.* 2017) and after 4 weeks exposure (Gaté *et al.* 2017). In addition, Husain *et al.* (2015) showed low levels of translocation of TiO2-NPs to liver when analysed 24 hours after a single IT instillation in mice. After exposure of female mice to TiO2-NPs by a single IT instillation at a maximum dose of 162 µg/mice, translocation from lung to liver can lead to detection of the Substance in liver 180 days after the end of exposure (Modrzynska *et al.*, 2018a, b).
Renal fibrosis was induced in mice receiving TiO_2-NPs by instillation once per week for four weeks (Huang *et al.*, 2015). Pujalté *et al.* (2017) showed that low-levels of TiO_2-NPs translocate to the kidney after a 6 hour exposure via inhalation in rats.

2.2.4. Testis

The possible effect of the Substance after translocation to testis is illustrated in several experiments available in the open literature. In mice and rats, the Substance was found

ECHA
EUROPEAN CHEMICALS AGENCY

in testis or epididymis after oral exposure. It was associated with several adverse effects such as an effect on seminal vesicle, sperm motility, increase in abnormal and total number of spermatozoid, increase in ROS production, negative effect on tissue histology and decrease in testis weight (NCI, 1979, Song *et al.*, 2017, Tassinari *et al.*, 2014, Jafari *et al.*, 2020).
In your comments on the PfAs, you noticed that the study by Hong *et al.*, 2015 is cited. As indicated under section A7, publication from the [redacted] was not considered and this reference was removed.

Conclusion on potential systemic organ toxicity

The eleven forms of the Substance to be tested will allow you to identify whether some physico-chemical characteristics in TiO_2 forms impact the histopathology of the brain, liver, kidney and testis. Histopathological examination can also provide indirect evidence on the potential translocation of some TiO_2 forms to these organs. Later, it will help you to predict the potential systemic organ effects of all forms of the Substance covered in the registration dossier.

2.3. Potential oxidative stress and genotoxicity

In addition to, or as a consequence of, inflammation, the potential genotoxicity hazard is also identified for the Substance. Some TiO_2-NPs have been shown to be weak genotoxic agents, with positive results mainly obtained in comet and micronucleus assays *in vitro* and *in vivo* (Charles *et al.*, 2018; Larsen *et al.*, 2016; Relier *et al.*, 2017; Wallin *et al.*, 2017).

In your comments you argued that the draft decision disregards the overall evidence presented in the registration dossier. Contrary to your comment, ECHA considers that the analysis as presented below is in line with your registration dossier and literature, where potential genotoxicity was observed as a consequence, or as a concomitant mechanism, of inflammation (Carriere *et al.*, 2020). Among all the publications you reported in your registration dossier or available in the literature, the paragraph below highlights the findings indicating a potential hazard for genotoxicity. This analysis is not conducted to assess genotoxicity in a "*weight of evidence analysis*" as you commented, but rather to point out existing alerts justifying to perform a comet assay.

In your comments on the PfAs, you repeated your concern regarding the analysis of the available data, not considered in a weight of evidence approach according to you. However, your comment is out of scope of the submitted PfAs and is therefore not considered.

2.3.1. In vitro studies

Regarding *in vitro* genotoxicity of TiO2-NPs, most of the published results refer to anatase as well as mixture of anatase and rutile (generally P25). Very few studies assessed the genotoxicity of coated TiO2-NPs or rutile forms. The requested study, in particular when comparing the effects of TiO2 forms 3, 4, 5, 7, 8, 9 and 11 (as specified in the request) will allow assessing if coating affects the potential genotoxicity of the Substance.

According to Charles *et al.* (2018) and in line with the registration dossier, both negative and positive results are reported in the *in vitro* genotoxicity assays. Most of the positive results were found at high doses in micronucleus and comet assays, with a dose-response relationship. Inconsistencies observed among the studies may be the result of differences

ECHA
EUROPEAN CHEMICALS AGENCY

in test materials (e.g. size, crystallinity, coating) as highlighted previously for repeated dose-toxicity by inhalation and after acute instillation exposure. It remains difficult to highlight which parameter(s) could drive these differences. Those inconsistencies could also be explained by the various test conditions used, including dispersion of the material, concentrations and exposure duration, cells/organs examined and parameters assessed. Therefore, it is not easy to compare the studies available so far. In particular, a case study on grouping and read-across for nanomaterial performed by OECD (2018a) on *in vitro* genotoxicity of TiO2-NPs taken as example, demonstrated differences in the genotoxic potential depending on the forms of TiO2-NPs.

2.3.2. In vivo studies

Regarding *in vivo* genotoxicity of TiO_2-NPs, ANSES performed a review of the available *in vivo* studies (2016) with the following statements: *"Several in vivo studies with different protocols, tested materials, routes of exposure are available with TiO_2-NP. Most of the studies referred to the anatase form. Thirty-eight experiments over the 125 identified reported positive results. Most of the positive results were found in comet assays, 8-oxodG tests and H2Ax phosphorylation assays".* Similarly to the *in vitro* data, the results from *in vivo* studies are inconsistent and positive results are provided mainly by comet assays at high doses. It is again not possible to identify the reasoning behind the inconsistencies (e.g. different protocols, different forms of TiO_2-NPs).

Also, and as pointed out in the registration dossier, most of the *in vivo* studies are associated with several methodological limitations (lack of positive control, no proof of target organ exposure, insufficient characterisation of the tested material, non-physiological route of administration, etc.). The data are sufficient to raise a concern but not to conclude on genotoxicity (emphasis added following your comment on the necessity to take into account the existing data).

For example, in your comments you criticised the study by Modrzynska *et al.* (2018a) regarding the relevance of performing a comet assay 28 days after the end of the instillation procedure: in this study, female mice (n= 324, 9 females per group) were exposed to TiO2-NPs by a single IT instillation at maximum dose of 162 µg/mice. At 180 days post-exposure, the comet assay on the lung tissue was positive, but negative in the liver although TiO2-NPs were shown to translocate to this organ. In this study, the test material was NanoAmor (primary particle size of 10.5 nm, DLS (dynamic light scattering) median size of 68 nm, no information in crystallinity was provided) to which the test material form number 2 of the request seems to be the closest. Danielsen *et al.* (2020), a study you also criticised, performed also an IT instillation study in female mice of the same strain and exposed them to a single dose of four types of anatase TiO_2-NPs at maximum dose of 162 µg/mice. The comet assay was performed at various post exposure times on BALF, lung and liver. Some significant increases, but also decreases, in DNA strand breaks were observed. These were considered by the authors to be chance findings.

These two studies performed in the same conditions (same dose, same strain of mice, same type of treatment, same post-exposure time, and comet assay on BALF, lung and liver) but with different TiO2-NPs tested lead to different outcome , indicating the need to perform testing on several forms of the Substance (emphasis added following your comment on the necessity to consider each result in a weight of evidence approach). These uncertainties and variabilities were also observed by Møller *et al.* (2017) in their review of the literature, detailed after your comments on whether the comet assay is able to detect different genotoxic potential of TiO_2 forms. You also discussed the 2 studies by Saber *et*

ECHA
EUROPEAN CHEMICALS AGENCY

al. (2012a, b) and concluded that "*instillation of TiO_2 caused pulmonary inflammation but no genotoxicity*". The first study from Saber *et al.* (2012a) is clearly positive for DNA damage on BALF cells after exposure to 2 out of 3 forms of the Substance tested. The authors explained that "*the magnitude of DNA damaging potency and the inflammatory response did not correlate well for the different materials: The FineTiO_2 and NanoTiO_2 were DNA damaging but only NanoTiO_2 induced inflammation. On the other hand, PhotocatTiO_2 and Printex 90 (Carbon Black), which were the most potent inducers of inflammation, did not induce DNA damage. Similarly, we have previously reported inflammation and DNA damage is unrelated (Saber et al. 2005; Bornholdt et al. 2007). Obviously there is no simple correlation between inflammation and DNA damage.*" On the contrary, in the second study (Saber *et al.* 2012b), the only comet positive result is observed on the liver tissue, at the highest dose, and only one day post-exposure. The discrepancies between these two studies show the difficulty in identifying which form of the Substance should be considered as a worst case for future testing based on the existing knowledge, pleading for additional data to be provided.

In addition, the testing programme you proposed to conduct (see Section A.3.), which is included in this decision, gives the opportunity to compare all forms of the Substance covered in the registration dossier. It is a much broader approach than in the existing programmes such as Nanogenotox (see below for further details) but in line with the responsibilities of the registrant under REACH.

Contrary to what you have commented, this decision does not aim at exhaustively displaying all the scientific literature but to point at the uncertainties arising from some of them. These uncertainties raise a concern.

3. Identification of the potential risk to be clarified

The identification of a potential risk is based on a combination of exposure and hazard information. There is sufficient evidence from the available data to justify that consumers and workers may be exposed by inhalation to the Substance, including TiO_2-NPs. As well there is evidence that different TiO_2-NPs may cause adverse health effects, such as inflammation in the lungs, as well as other systemic organ toxicity after repeated dosing. In addition, there is evidence for potential genotoxicity, direct, indirect or secondary to oxidative stress.

You commented that "*this is in conflict with the RAC opinion in which the mode of action analysis on the carcinogenicity of titanium dioxide in rats describes that "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"*". The eMSCA considers that this remark of the RAC is aligned with its view where "*oxidative stress, formation of reactive oxygen species (ROS) and cytotoxicity"* occur concomitantly to lung overload. Lung overload is not the sole mechanism driving the different toxicities which can be observed in the available data, and this was acknowledged by the RAC in the statement it made.

Furthermore, the information you provided in the registration dossier together with the existing literature do not allow to identify the potency, i.e. which forms of the Substance are the most toxic via inhalation, but also those that would be less toxic.
Therefore, the available data do not allow to ensure a safe use of all the forms of the Substance when the exposure occurs via inhalation.

ECHA
EUROPEAN CHEMICALS AGENCY

The request of this decision is the first step of a tiered approach for clarifying the potency of various forms of the Substance and potential risk on repeated dose toxicity after exposure via inhalation. It is based on the strategy proposed by the TiO_2 Manufacturer Association Consortium (IT instillation study on 11 forms of the Substance) in which also the examination of early and sensitive parameters have been incorporated to:

- choose which forms of the Substance should be further tested in Tier 2
- generate data to predict the toxicity of various forms of the Substance that will not be (further) tested.

A.2 The possible risk management measures – human health

The Substance already has a harmonised classification as Carc. 2 (H351) by inhalation. You commented that "*OECD has identified instillation studies as a rapid and inexpensive way to screen particles for hazard identification for further investigation. However, this technique is not a substitute for inhalation toxicity studies (OECD 2018b). Therefore, the instillation study itself cannot be used to develop improved risk management measures*".

ECHA agrees with your argument. However, the study requested in this decision represents the Tier 1 of a tiered approach. This step will enable to select a smaller number of TiO_2 forms to be tested in the next steps of the tiered approach as per your initial proposal. Based on the early and sensitive markers of toxicity, which have been added to your initial proposal, you will be able to gather information on the forms of the Substance to be further tested, but also on some of the non-tested forms included in the registration dossier. It will allow you to systematically predict the toxicity of all registered forms of the Substance by comparing these early markers of toxicity. Based on the new data generated during the whole process including the other tiers after Tier 1, improved risk management measures are a realistic possibility.

Therefore, only the results obtained *after the entire testing programme* mainly based on a proposal by the TiO_2 Manufacturer Association Consortium (i.e. when having the results requested in the substance evaluation decisions that will be produced later in Tier 2 and possibly in Tier 3) could possibly lead to:

- A lower DNEL for all or some forms of the Substance, possibly resulting in more stringent risk management measures than currently in place.
- Additional/different classifications for some or all forms of the Substance. You commented that the entire testing strategy could possibly lead to a different classification between TiO_2 forms. Indeed, a differentiated classification between some forms, or new classifications (e.g. germ cell mutagen, STOT RE, toxic for the reproduction) of some forms of the Substance is foreseeable. A declassification of certain forms of the Substance is not excluded.
- The possible new classifications will have several impacts according to EU downstream legislation[5], leading to improved protection of workers and the public.
- A need for further regulatory risk management, such as a restriction under REACH.

[5] ECHA guidance, Introductory Guidance on the CLP Regulation, v 2.1, section 22, p. 80-82, August 2015: https://echa.europa.eu/documents/10162/23036412/clp_introductory_en.pdf/b65a97b4-8ef7-4599-b122-7575f6956027

ECHA
EUROPEAN CHEMICALS AGENCY

A.3 Explanation of the testing strategy – human health

In the course of the substance evaluation process, the Titanium Dioxide Manufacturer Association Consortium (TDMA/TDIC) contacted the eMSCA to present a testing programme for a large number of different TiO_2 materials. You proposed that different forms of the Substance will be fully characterised physico-chemically and then assessed in a series of short-term IT instillation studies as a first step. In the next steps subchronic and if necessary chronic studies would follow on some forms of the Substance representing the toxicologically most potent registered forms. The eMSCA has considered the proposed testing programme and agrees that for risk assessment purposes the approach you proposed is appropriate to investigate the potential risk posed by the Substance. In addition, this first step serves as means for you to gather enough sensitive toxicity biomarkers to predict the long-term toxicity of the TiO_2 forms that will not be tested.

The information requested in this decision constitutes the **Tier 1** of a testing strategy to clarify the potential risk of the Substance following repeated exposure via inhalation: an IT instillation screening study aiming at discriminating the parameters driving the potential toxicity of the different forms of the Substance. As emphasized in your comments, this exposure technique offers an easy way to rapidly screen and rank the hazard of solid materials (OECD, 2018b), even if this is not a substitute for inhalation toxicity studies (as the upper respiratory tract is bypassed). In your comments, you agreed with ECHA on the general purpose of the **Tier 1** study.

The 11 forms of the Substance you have proposed to test have been selected by you to cover all the registered forms of the Substance available under the joint submission. These 11 forms of the Substance must be tested under the same test conditions to define a smaller number of forms to be tested in a **Tier 2** (see below). In addition, the number of parameters and endpoints included in this IT instillation study screening programme need to be broad enough to be informative on systemic and long term toxicity. They will also be used as a basis to predict the toxicity of the untested forms of the Substance.

Therefore, the difficulty in the **Tier 1** is to identify endpoints and biomarkers that are sensitive enough to differentiate between the toxicity of the tested forms of the Substance. Indeed, the toxicity profile of different forms of the Substance is expected to significantly vary depending on their physico-chemical characteristics and the endpoints and biomarkers assessed (see section A.1). In addition to local pulmonary effects, the **markers of toxicity requested need to be relevant also for systemic toxicity after a longer repeated exposure**. You proposed "*the instillation study programme as an easy way to screen the lung toxicity of 10 different representative forms of titanium dioxide and use the results to select appropriate candidates for testing in subsequent OECD guideline-conform inhalation toxicity studies on particles demonstrating mild to moderate lung effects as measured by percent increase in polymorphonuclear leukocytes compared to control*". However, solely the effects on the number of polymorphonuclear leukocytes compared to control cannot be a sufficient marker of the parameters driving the toxicity. The additional markers requested in this decision are early markers reflecting the toxicity parameters measured in the studies according to OECD TG 413.

In your comments, you argued that ECHA transforms "*an inexpensive way to screen particles for further testing [...] into an experimental research programme [...] for the sole purpose of testing hypotheses presented by other researchers investigating nanoparticles*". Under substance evaluation, the requested information/studies (or additional parameters) in the decision need to be justified based on the identified concerns

ECHA
EUROPEAN CHEMICALS AGENCY

arising from data available and presented in the registration dossier, as well as in the open literature. The available data (rather than hypotheses as you commented) produced by the scientific community was therefore presented to explain and justify what to request in this screening test in Tier 1. The decision relies on the original screening programme you proposed, which is to test representative forms of the Substance. These are expected to represent the large number of TiO_2 forms covered by the registration dossier (more than 300 chemical compositions).

Therefore, biomarkers are included to inform on the cardiovascular function, and the histopathology of liver, kidney, testis and brain. Effects observed in histopathological examination can also indirectly indicate systemic translocation of the Substance. Examination of local pulmonary response includes the measurement of the production of ROS and RNS to inform on oxidative stress.

These early markers (used as screening and discriminating means) will also allow to refine the design of the **Tier 2** testing with the final goal to better identify differences in the toxicity between the various forms of the Substance covered by the joint submission. Indeed, the foreseen **Tier 2** study is a subchronic inhalation toxicity study (OECD TG 413) in which the additional investigations proposed in the current guideline, *"such as toxicokinetics, and/or systemic toxicity evaluations (e.g., immune, hepatic, neurologic and/or cardiovascular effects evaluations) to better characterise the overall toxicity of a test chemical"* would most probably be warranted.

To further investigate the role of oxidative stress, the comet assay (standard and enzyme-modified) is requested because it is a sensitive and suitable method to detect **oxidative DNA damage**, which can result from the excessive production of ROS and RNS (Charles *et al.*, 2018). Therefore, as there are many parameters potentially driving the adverse effects of the Substance (see section 2.1.), many endpoints need to be included in this screening study as requested. It is the most efficient and targeted way enabling you to later reduce animal testing for long term repeated dose toxicity studies. The strength of the request is to perform a strongly controlled comparison of the different forms of the Substance with a specific test design. The number of parameters and endpoints need to be broad enough to be informative for Tier 2 testing.

The comet assay can easily be combined with the IT instillation study allowing testing multiple parameters in a single setup. You commented that *"The assertion that comet analysis can be combined with IT instillation, biomarker assessment, and histopathology is an over-simplification of a large and complex experiment that requires strict control of variation to produce valid, interpretable results"* or *"The comet assay is frequently described as being a quick, simple, sensitive technique to assess DNA damage. This often leads to the assumption that it can be easily integrated into larger toxicology studies with no adverse impact. However, whilst the assay is in principal simple to perform it requires stringent control of numerous variables to ensure robust and valid data are obtained. The complex nature of the proposed instillation study [...] raises significant concerns. Furthermore, the analysis of 10 different NM, at three dose levels, in at least 2 but possibly 3 tissues, with and without enzyme-modifications is highly likely to generate some spurious increases in DNA strand breaks that are simply chance occurrence, with no biological relevance".*

The addition of the comet assay to an inhalation study (OECD TG 413) is recognised and validated in the OECD TG 489, paragraph 7. In addition, the combination of the two studies has been adopted at least in another ECHA's substance evaluation decision on potassium

ECHA
EUROPEAN CHEMICALS AGENCY

titanium oxide (EC number 432-240-0) (ECHA, 2019). Technically, there is no additional difficulty to add the comet to the IT instillation study instead of an inhalation study. The non-physiological route of exposure (instillation) to the Substance will be the same for all the forms tested and controls. Performing a comet assay with and without enzyme-modification, when animals have already been exposed, treated and sacrificed should not impact the outcome of the other examined endpoints and biomarkers. As they are all separate examinations following the death of the treated animals, ECHA considers that performing the requested analysis will not have an impact on the outcome of each requested endpoints and biomarkers.
You commented that "*given the uncertainties and expected obstacles in this proposed set of studies, there are significant concerns that the primary objectives (i.e. to identify a smaller set of TiO_2 samples to be tested in Tier 2) are unlikely to be achieved*". ECHA emphasises that if any bias, uncertainties and obstacles arise from the design of the study, it would apply to all eleven forms of the Substance, not impairing the goal of the request, i.e. comparing the tested forms.

Your comment reinforces the fact that the 11 forms of the Substance have to be tested under the same experimental conditions in order (i) to remove the uncertainties generally arising from the analysis of the exisiting data (performed in different laboratories, under different protocols and/or experiments, with different animal species/strains, performed with variable equipments and chemicals) and (ii) to test the oxidative potency of each of them to allow a proper comparison and selection of TiO_2 forms for the Tier 2, as many of them have never been tested for the potential to cause oxidative damage. Indeed, performing the comet assay at this stage aims at comparing the different forms of the Substance among each other.

You commented that "*Addition of a comet assay is not appropriate for this requested instillation study. It violates ECHA's mandate to reduce testing of vertebrate animals*". Indeed, by requesting an additional timepoint to your initial proposal, the number of animals to be treated has increased (detailed justification of this additional timepoint provided in section A.5.):

- The combined performance of the IT instillation test with the comet assay will imply the treatment of 15 additional animals compared to an instillation study without comet assay (510 vs 525 animals) for positive control mainly (see Table 3, section A.6.). These 15 animals can be saved if *ex-vivo* positive control generation is performed. This can be performed by taking a piece of lung from negative control, homogenising this tissue and exposing the cells to a known genotoxic substance for generating a positive control. This procedure has already been applied in the substance evaluation decision on potassium titanium oxide (EC number 432-240-0) (ECHA, 2019) for external positive control generation.
- If the standard exposure for positive control generation is performed, the extra 15 animals are a strictly a minimal increase in the number of animals in comparison to performing the IT instillation test and the comet assay in separate experiments: an additional 500 animals would then be needed.
- The enzyme modification procedures for the comet assays, or the performance of a comet assay in combination with the IT instillation tests, do not increase the number of animals needed at all.

Therefore, it is judged to be proportionate and does not violate ECHA's mandate on animal welfare and reduction in animal testing.

ECHA
EUROPEAN CHEMICALS AGENCY

You commented several times that the comet assay appears to be an unnecessary duplication of previously conducted studies, and especially with regards to the Nanogenotox program: "*The eMSCA has already sponsored the conduct of a comet assay on five different nanoforms of titanium dioxide. No statistical effect was noted on genotoxicity. The results were in line with another eMSCA sponsored study where instillation caused pulmonary inflammation but no genotoxicity (Saber et al., 2012). Therefore, the LR deems the Comet Assay an unnecessary repetition of a previously conducted study*"; and "*Since it may be assumed that the Nanogenotox experiments were conducted in compliance or equivalent to accepted guidance, the lead registrant questions for which purpose these experiments require a repetition. In particular as Article 26 (3) of the REACH regulation prohibits the repetition of studies involving vertebrate animals*". Contrary to your comment, the Nanogenotox (2013) program raised a question concerning the genotoxicity of the Substance (scarce data, only NM-105 induced DNA damage in BALF cells; two other forms of the Substance, NM-102 and NM-103, produced equivocal dose reponse in liver, indicative of a potential larger positive outcome: following gavage, some genotoxic effects were also observed with the comet assay with the Substance in spleen, intestine (NM-103), colon (NM-102 and -105)).

Moreover, only few forms of the Substance were tested, far from what you proposed in the testing programme (11 TiO_2 forms representing nano- and non-nano forms, coated and non-coated forms with inorganic and organics coatings, in order to cover the entire compositions of the Substance available in the joint submission). The route of exposure and the number of TiO_2 forms to be tested in the assay as requested in this decision will not be "*a mere repetition of existing studies*".

Concerning the use of the requested parameters for the selection of TiO_2 forms for Tier 2 testing, you commented "*The comet assay extends the initially proposed instillation study beyond the intent to identify particles for further testing. It is unlikely the results could be used to identify a smaller selection of samples to be tested in Tier 2*" or "*It is therefore difficult to understand how ECHA will utilize protein marker data and effects on reactive species and endogenous nitrogen oxide production to facilitate decisions regarding the criteria upon which particles would move forward to a Tier 2 analysis*". This comment contradicts another one stating that "*the LR proposes to defer the suggested Comet assay, extensive organ histopathology, blood and plasma analysis to facilitate during the Tier 2 studies proposed by ECHA to be conducted on particles demonstrating a moderate effect.*" By not performing any of these requested examinations in Tier 1, it is not clear how those "*moderate effects*" used for ranking "*the particles*" for **Tier 2** will be obtained as essential information is lacking, especially with regards to oxidative mecanisms or potential systemic effects indicating possible translocation of the Substance (as detailed in section A.1, point 2.2 and 2.3).

The eMSCA aims to give a priority to the form(s) with a positive result in the (modified) comet assay on lung for further testing in the **Tier 2.** This is based on the consideration of the biological significance of the oxidative stress mode of action for (sub)chronic toxicity and the possible indication of direct contact of the Substance with internal cell components. In addition, the measurement of ROS/RNS by fluorescent probes and HPLC concomitantly with the comet assay will allow the eMSCA to evaluate if potential adverse effects are arising from direct contact of the Substance with cells and/or DNA or they are occurring only after the diffusion of ROS/RNS inside the cells: For example, if ROS/RNS are measured by fluorometric probes while the comet assay is negative, it will provide evidence that the TiO_2-form tested is able to generate oxidative species without impacting DNA. On the other hand, if no ROS/RNS are detected by fluorometric probes but the

ECHA
EUROPEAN CHEMICALS AGENCY

enzyme- modified comet assay is positive, it will indicate that the TiO_2-form is able to generate direct oxidative damage to DNA. This is particularly important as the mechanisms of genotoxicity of the Substance are described in the literature as either primary or secondary, and when primary, mostly indirect, even if a direct contact between the Substance and DNA is not excluded (Carriere *et al.*, 2020). Moreover, measurement of NO content and eNOS activity at the three doses for each form of the Substance in serum will allow to compare effects on the cardiovascular function. Indeed, it is important to rank the different forms of the Substance not only based on their local toxicity but also on their potential systemic toxicity. These examinations will also help to determine which forms will be the most appropriate to be tested in the sub-chronic toxicity studies (**Tier 2**). In addition, the parameters requested will help to compare effects, if any, between acute and long term exposure (when subchronic data will be available for a smaller number of selected TiO_2 forms) with the same form of the Substance, and to fully determine the potential effects of each material on lung and other organs.

Finally, the BALF parameters informing on the potency on the local pulmonary response and the potential histopathological effects as an indication of systemic toxicity (indicating potential of TiO_2 form to translocate) will be balanced with the ability to generate oxidative stress. The forms of the Substance estimated to have the overall most prominent effects will be given the highest priority to be tested in Tier 2.

In your comments on the PfAs, you questioned whether fluorometric probes can differentiate between reactive species generated by the particle itself (as intrinsic property) or by the immune cells. This comment is out of the scope of the submitted PfAs and is therefore not considered.

When the results from the **Tier 1** screening study are available, the eMSCA will review:

- The data generated, taking into account all the parameters investigated. ECHA agrees that "*intratracheal instillation screening should not be utilised to generate hazard data sufficient for a risk assessment*". The requested IT instillation study combined with a comet assay should still be seen as "*an inexpensive way to screen particles for further testing*" avoiding unnecessary animal testing later in **Tier 2**;
- Your proposal for a ranking of the tested forms of the Substance according to the toxicity potential based on the local pulmonary and systemic effects;
- Your reasoned and justified proposal for the selection of a smaller number of toxicologically relevant forms of the Substance to be further tested in **Tier 2**, as they are set to represent the large amount of forms/compositions of the Substance covered by the registration and because it is your responsibility to demonstrate it;
- Your confirmation whether some additional parameters are needed to clarify the identified risk in the **Tier 2** testing (subchronic 90-day toxicity study via inhalation) "*such as toxicokinetics, and/or systemic toxicity evaluations (e.g., immune, hepatic, neurologic and/or cardiovascular effects evaluations) to better characterise the overall toxicity of a test chemical*" if warranted; e.g. if translocation is suggested by systemic effects in histopathological examinations, a toxicokinetics investigation may be warranted.

Thereafter, the eMSCA will review the information from the **Tier 2**, and evaluate if further information should be requested in **Tier 3**.

Regarding all the tests that will be avoided in **Tier 2**, the IT instillation study combined with a comet assay requested by ECHA in the **Tier 1** should still be seen as "*an*

ECHA
EUROPEAN CHEMICALS AGENCY

inexpensive way to screen particles for further testing" avoiding unnecessary animal testing later.

A.4 Why new information is needed

As already explained under section A.3, the requested IT instillation study combined with comet assay is needed as the first step in **Tier 1** to clarify the potential risk because it:

- allows the comparison of the toxicity potential of the various forms of the Substance under the same test conditions in a cost effective way;
- allows to make a justified selection of the TiO_2 forms for the subsequent testing in **Tier 2**, i.e. at least subchronic inhalation toxicity study (OECD TG 413) in which, the additionnal investigations proposed in the current guideline, *"such as toxicokinetics, and/or systemic toxicity evaluations (e.g., immune, hepatic, neurologic and/or cardiovascular effects evaluations) to better characterise the overall toxicity of a test chemical"* could be warranted based on the data gathered in parameters investigated in **Tier 1**. For instance, if systemic effects suggest translocation, a toxicokinetics investigation may be warranted, or if there is a substantial modification of CRP, full histopathology, including heart together with additional parameters informing on cardiovascular effects may be requested in **Tier 2**; and
- may also clarify the potential physico-chemical characteristics driving the genotoxicity of some TiO_2 forms.

Independent studies focusing either on lung, or e.g. cardiovascular system, or neurotoxicity or liver have shown adverse effects. Most of the available studies on the Substance were performed by instillation exposure, and provided useful information. However, alone, they are not sufficient for the purpose of a risk assessment as (i) they are not representative of normal inhalation (the upper respiratory tract is bypassed), (ii) most were performed with only one high concentration (not allowing to establishing a dose-response relationship or to inform on the effects at low concentrations), and (iii) the form of the Substance tested varied (with its specifications not always clearly characterised).

The intrinsic physico-chemical properties of the Substance, such as particle crystallinity, size, surface area and surface modification, can influence its reactivity and behaviour. In the absence of a clear understanding of which property drives the toxicity and how, there is a risk that the toxicity is not properly estimated when extrapolating the results of a study on one form of the Substance to untested forms.

Therefore, and based also on the strategy you proposed, different forms of the Substance must be properly tested under similar experimental conditions as a first step in order to understand the relationship between physico-chemical characteristics and the strength of toxicity (potency) before testing in any longer-term studies and to ensure that the test results can be used for other non-tested forms of the Substance.

To conclude, the lack of proper studies according to OECD TG 413 represents a significant drawback to evaluate different toxicities driven potentially by different forms of the Substance. Therefore, it is not possible to evaluate the potential systemic toxicity of the Substance, including its nano forms, based on the available information and conclude whether the lungs are the only target organ after exposure by inhalation.

ECHA
EUROPEAN CHEMICALS AGENCY

A.5 Considerations on the test method

1. Specifications of the requested study

The protocol you initially envisaged consisted of an acute single-dose IT instillation study in rats exposed to at least 11 forms of the Substance at 3 different concentrations, followed by BALF analysis at 2 different time points (1 day and 28 day post exposure) and histopathology of the lung.

1.1. Study design: **Combination** of an IT instillation and a **comet** assay

The requested study is an IT instillation combined with a comet assay performed on 5 male rats per dose group and observation time.

You commented that "*it is* highly *questionable whether obtaining BAL samples and tissue for histopathology and comet analysis can be achieved in the same animals*". The eMSCA has discussed the feasibility of the request with 2 public research centers and 2 CROs. All confirmed that the performance of the requested assays on a single animal (rat) is possible:

- On the left pulmonary lobe, the BALF can be harvested
- Afterwards, the lung tissue can be used to perform the comet assay and ROS/RNS analysis;
- The right and caudal lobe can be used for histopathological examination
- The rest of the three lobes can be used to characterise deposition and dosing of the Substance.

You can also refer to the study by Gaté *et al.* (2019) on multi-walled carbon nanotubes, where this methodology was applied (*BAL was performed on the left lung [...] The right caudal lung lobe was used for histopathology analysis. For the comet assay, the left lung was dissociated by enzymatic technique [...]*). Further exemples are available in literature (Pothmann *et al.*, 2015; Magnusson *et al.*, 2019).
In your comments on the PfAs, you raised questions on the possibility to perform all requested measurements on one animal/organ. This comment is not in the scope of the submitted PfAs and is therefore not considered.

1.2. Number of forms tested

Different forms of the Substance must be tested under the same test conditions in order to obtain basic information on the relationship between physico-chemical characteristics and the strength of toxicity (potency) and to ensure that the test results can be applied to other non-tested forms of the Substance.
Eleven (11) forms, at least, of the Substance must be selected, representing all the registered forms covered by the boundary composition set out in the registration dossier. Your comment on the number of forms to be tested has been taken into account[6]:

- uncoated mixed phase **nano**,
- uncoated **nano** anatase (5 nm),
- uncoated pigmentary rutile,
- pigmentary rutile coated with alumina and 1,1,1-Trimethylolpropane (TMP),
- pigmentary rutile coated with alumina, zirconia and TMP,
- **nano** rutile coated with alumina and hydrophobic organic,

[6] based on the proposal made by the TiO_2 consortium during Substance Evaluation (TDMA Science programme for titanium dioxide, 2018)

ECHA
EUROPEAN CHEMICALS AGENCY

- pigmentary rutile coated with high specific surface area (SSA) silica and alumina ($40m^2/g$),
- **nano** rutile coated with silica ($40m^2/g$),
- pigmentary rutile coated with aluminium phosphate,
- pigmentary uncoated anatase (E171)
- **nano** anatase containing tungsten in its coating

You have proposed these forms of the Substance because you explained that they represent the best way to investigate the forms of the Substance covered in your TiO_2 registration dossier:

- 5 forms to be tested are TiO2-NPs (and represent around 2% of the total TiO2 market)
- 6 pigmentary (non-nano sized material) forms of the Substance to be tested cover the rest of the forms available on the EU market.

This proposal is not challenged. However, it is your responsibility to ensure that these forms cover the boundary composition of the joint submission.

In your comments on the PfAs, you discussed the test item characterisation. This is out of the scope of the submitted PfAs and is therefore not considered.

Testing on these 11 forms of the Substance is required because they will inform how the intrinsic physicochemical properties, such as crystal structure, particle size, surface area and surface modification (coated, uncoated and type of coating) of the particles can influence the reactivity and behaviour. As indicated in the previous sections, these properties are associated with the differences in toxicity potency between different forms of the Substance. Therefore, different forms of the Substance must be tested under the same test conditions in order to obtain basic information on the relationship between physico-chemical characteristics and the toxicity potency and to ensure that the test results can be applied to other non-tested forms of the Substance.

You must substantiate the representativeness of the selected parameters to cover the different registered forms of the Substance with an explanation on why the tested forms cover all the forms of the REACH registration dossier, and how they cover each form which was not tested. This characterisation must be performed shortly before testing.

The following physico-chemical key parameters must be reported on the 11 forms of the Substance, in order to interpret the toxicological findings:

- Detailed description of the number-based particle size distribution with an indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm, and how it has been measured. To achieve this, a particular attention must be paid for the choice of the dispersing medium. The method and measurement protocol(s) used to characterise the particle size distribution must also be detailed.
- Details on the shape of the particles, their aspect ratio, aggregation and any other morphological characteristics (details of the crystal structure, e.g. rutile, anatase, mixed phase x% of rutile and x% of anatase). The validation of the methods used to measure the size of primary and constituents particles may depend on their shape. Such characteristics indicate also which techniques are preferable for

particle size distribution measurement, especially for deriving a number of particles with size from a mass distribution.

- Details on any surface treatment or functionalization (identity and amount in molar ratio of the agents, weight-by-weight contribution, percentage of coverage, and the manufacturing process used): any functionalization of the surface or any coating process which is part of the manufacturing process of the test materials must be reported.
- Quantitative information on the specific surface-to-volume ratio or the surface-to-weight ratio: As the Substance is practically insoluble in water, its biological activity is essentially due to surface chemistry, and therefore varies with the specific area in contact with the biological fluids and tissues. The specific surface-to-volume ratio or the surface-to-weight ratio (and zeta potential) must be reported as it may cause different toxic effects.
- Quantitative information on the chemical composition of each form of the Substance in the request and not only physical characterisation: As the impurity profile may also contribute to the overall response, and because this parameter may also vary among the chosen test items, a precise and quantitative chemical characterisation must be reported.

1.3. Number of instillations to be performed

ECHA considers that instead of a single-dose instillation as you initially proposed, performing **2 instillations** at 24 hours interval (at 0 and 24 h) at the defined concentrations will increase the sensitivity of the requested study. First, 3 instillations were requested in order to ensure avoiding overload conditions, but following your comments, the protocol was modified and the requested study must be performed with 2 instillations. The same test protocol must be used for each tested form of the Substance. The recommandations provided in the OECD (2018b) guidance document No. 39 on inhalation toxicity studies (Appendix VI. Instillation and aspiration studies) must be considered in the design of this screening test.
You commented on the unnecessary performance of 3 instillations as proposed by the eMSCA. You argued that a three-fold instillation would be highly unusual, would generate a problem with the historical control database, and would induce a stronger method-related background inflammation of lungs. In addition, you argued that instillation as such is a manipulation that causes substantial stress to the animals and therefore unnecessary manipulation of the test animals should be avoided.

You also assured that a two-fold instillation would "*already ensure an adequate distribution of granular particles in lung lobes.*" As indicated in the open literature and after discussion with the eMSCA, there is a need to avoid generation of overloading condition and saturation of the lung by particles with an exposure by IT instillation. Overloading is also the main argument you raised for disregarding many of available studies in the literature. The defined concentrations should be sufficient to ensure that no bolus effect occurs. The same test protocol must be used for each tested form of the Substance. Therefore, **2 instillations must be performed at 24 hours interval (at 0 and 24 h)**.

1.4. Post-exposure observation times

The protocol of the IT instillation study in rats must comprise each form of the Substance tested at 3 different concentrations, followed by 3 post-exposure observation times (2-6 h, 1 day and 28 day post exposure). You questioned the relevance of these three timepoints.

ECHA
EUROPEAN CHEMICALS AGENCY

1.4.1. Comments on post-exposure observation times related to pulmonary effects

You commented that "*based on experience both from the performing laboratory and within industry (*[redacted]* we had foreseen BAL and histopathology to be performed at 3, 14 and 28 days p.a. [...]. The LR would appreciate a reasoning for the choice of [these] observation times, or alternatively an approval of the time points as suggested in the draft protocol by TDMA, in particular because we do not expect any relevant inflammatory response at 2-6h and 1d p.a.*". The laboratory director you consulted also commented on the fact that requesting the 2-6 hr and 24 hr time points will lead to an overlap of the animal handling.

More specifically, concerning inflammation, you noted that a sampling time point at least 24 hr post-exposure (but not 2-6 hr) should be selected to ensure that inflammation caused by the IT instillation procedure does not confound the results, plus a second time point to be foreseen at 28 days post-exposure.

The observation timepoints defined in the current decision were set up in order to link all the investigated parameters, i.e. pulmonary effects, cardiovascular function, oxidative stress, histopathology and (oxidative) DNA damage. Different scientific data tend to prove that 1 day post administration (p.a.) will allow detecting translocation and physiological effects, which may not be the case at 3 days p.a as you proposed in your comments.
Performing 3 observation timepoints at 2-6 hr, 1 day and 28 day post-exposure will allow evaluating acute effects together with longer-term effects in order to select proper forms of the Substance for sub-chronic toxicity testing.

As in the study you provided (Kreyling *et al.*, 2017b), a large amount of the Substance was retained in the lung very early after IT instillation: more than 95% of the Substance in the lungs + BALF at 1 hr p.a. and 99% at 4 hr p.a. These values were consistently increasing from 1 hr to 24 hr p.a. when focusing only on lungs (without BALF; in the BALF it increased from 43% to 67%) and then slightly decreased at 24 hr p.a. (below 2% decreased, reaching 97% of the Substance). Therefore, the timing of the termination at 2-6 hr, 24 hr and 28 days post-exposure seems to be very important. Restricting the post-exposure timepoints here will not ensure a proper evaluation of the toxicity potency of the tested material, thus impairing the choices made on the forms of the Substance for the testing in Tier 2.
In an acute instillation study (therefore comparable to the sreening study here, Saber *et al.*, 2013), the strongest response in increased expression of SAA3 mRNA was seen at early time points (day 1 in this study). In another study by instillation, TiO_2-NPs were already detected in the liver and heart as early as 24 hr post-exposure (Husain *et al.*, 2015). Concerning lung effects, increase in PMN leukocytes was observed as early as 6 hr post exposure after instillation in rats and mice (Oberdörster *et al.*, 2000).

The data from Oberdörster *et al.*, (2000) and Kreyling *et al.*, (2017b) described above show that it is possible to perform sampling and dosing at 24 hr interval and that this delay in time point is sufficient to ensure that no overlapping in sampling and handling occurs. Moreover, it is important to remind that the 24 hr time point is recommended in different OECD TGs (e.g. 489 and 413, although not requested in this decision) that are internationally validated in inter-laboratory comparison. It shows that this type of combined design can be conducted with the requested time points. As indicated in the website of the chosen test laboratory facility [7], the laboratory is able to perform studies

[7] https://www.item.fraunhofer.de/en/services-expertise/chemical-safety-assessment/toxicological_testing/genetische-toxikologie.html#cop1)

ECHA
EUROPEAN CHEMICALS AGENCY

according to OECD TG 489 and OECD TG 413. This shows that the facility chosen has the ability to perform more intensive and complicated combination that the study requested in this decision. Therefore, the chosen facility will be able to perform the requested Tier 1 study. More generally, the chosen testing facility advertises that they know how to manage animals to ensure quality of the study according to the requested requirements ensuring animal welfare, international regulation and GLP guidelines. The handling of the animals is related to the IT instillation study you initially proposed. Procedures are commonly used in stages in such facilities to ensure that there is no overlapping in handling of animals (e.g. treatment and sampling).

1.4.2. Specific comments on post-exposure observation times related to comet assay

According to you, the only relevant timepoint for performing the comet assay is after 2-6 hr post exposure. You consider that subsequent comet assays at 24 hr and 28 days post exposure cannot be expected to yield any meaningful information and therefore should be omitted.
The OECD TG 489 states that "*a suitable compromise for the measurement of genotoxicity is to sample at 2-6 h after the last treatment for 2 or more treatments*", as it is the case here. Therefore, time points at 2-6 hr p.a. and at 24 hr p.a. (as justified above) are added to the 28 days p.a. observation time usually used in IT instillation assays.

The OECD TG 489 also indicates that depending on the administration route, kinetics of the substance and target tissue, necropsy time point could be adjusted. Moreover, as detailed in the study by Hartman *et al.*, 2003, providing recommendation for the realisation of the *in vivo* comet assay "*In relation to the time of test substance administration, tissue/organ samples are obtained at 2–6 and 16–26 h after dosing. The shorter sampling time should be sufficient to detect rapidly absorbed as well as unstable or direct acting compounds; the late sampling time is to detect compounds which require time to be absorbed, distributed and metabolized*" (study in which one of your consulted expert participated).

Moreover, you commented that it seems acceptable to perform a comet assay in BALF instead of lung tissue at 2-6 hrs. This proposal seems contradictory with your claim that the comet assay is not useful and that there is no interest in performing this assay at this sampling time. Wallin *et al.* (2017) have shown that after a single instillation exposure, comet assay on lung tissue was positive at 28 days p.a. Moreover, Modrzynska *et al.* (2018a) have shown that comet assay was positive as far as 180 days p.a. Therefore, regarding these two studies, requiring the comet assay also at 28 days p.a. in the requested study appears justified and relevant.

The timepoints for the comet assay are therefore compatible with detecting effects informative for the identification of the most (and the least) potent forms of the Substance in relation to the other endpoints to be evaluated and the evolution of the parameters tested along different timepoints.

After considering all your comments on post-exposure observation times for comet assay, the request is unchanged.

In you comments on the PfAs, you questioned the time point for the IT instillation and comet assay at 2 – 6 h post exposure. Your comment is out of the scope of the submitted PfAs and is therefore not considered.

ECHA
EUROPEAN CHEMICALS AGENCY

1.5. Considerations on the doses of each form of the Substance

The lowest concentration must produce little, or no evidence of toxicity and the highest concentration must result in a clear level of toxicity but not inducing overload where feasible. The doses must be expressed in mass as well as surface area metrics. How the doses are calculated and measured when performing the assays must be substantiated in the results.

In your comments on the PfAs, you made a remark on dosage selection. This comment is out of the scope of the submitted PfAs and is therefore not considered.

1.6.Considerations on the sex of the animals tested

Five (5) male rats must be included in each group and observation time.

You commented on the gender of animals used, and that you prefer to perform the assays on female (huge historical database at Fraunhofer institute, more sensitive sex: tumours elicited by overload of inert particles of the Substance observed only in female rats in studies quoted by ECHA's RAC). However, male rats are more suitable for this screening program, which does not aim at evaluating tumor formation. Indeed, as you mentioned male rats will allow a higher deposition of material in the lung. Moreover, using male rats will allow to have a bigger lung size, weight, respiratory capacity to perform all requested assays in one animal only. It is not uncommon to perform instillation study on male rats (Yokohira *et al.*, 2008, Warheit *et al.*, 2006a, b), as developed and detailed in the Nanogenotox project, available in open literature. The use of male rats will also allow to examine testis histopathology (see below).

1.7. Considerations on the number of the animals tested

You commented that 30 animals per group would be necessary instead of the 5 to obtain the required sensitivity to detect elevated lesions sought by the enzyme modification procedures (i.e. 7,8-dihydro-8-oxoguanine (8-oxoGua)) of the comet assay. You apparently based this estimation on the work published by Møller *et al.* (2015). This is a review investigating the measurement of oxidative damage to DNA in nanomaterial exposed cells and animals. The authors compiled the available data generated with nanomaterials and comet assay with regards to the 8-oxo-7,8-dihydroguanine-2'-deoxyguanosine (8-oxodG) as a DNA nucleobase oxidation product. In that context, they reassessed the difference between baseline level of oxidatively damaged DNA in unexposed cells or animals, agreed by the ESCODD (the European Standards Committee on Oxidative DNA Damage) of being approximately 1 lesion per million unaltered guanines and that normal values were typically between 1 and 5 lesions/10^6 dG in cultured cells. In their review, they concluded that "*the level of 8-oxodG in unexposed cells or animals should be less than five lesions/10^6 dG. Measurement of 8-oxodG that are higher than this threshold in unexposed cells or tissues are likely to be flawed because of spurious oxidation of DNA during processing of samples [ESCODD 2003a; Collins et al., 2004]. The same applies to other types of oxidatively damaged DNA nucleobase lesions because 8-oxodG is one of the most abundant lesions in cellular DNA. Measurement of more than five lesions/10^6 dG of 8-oxodG in unexposed cells or tissues has been considered not informative in the present review*". In relation to this observation, they concluded that "*Based on this assessment, it would be statistically unlikely to detect elevated levels of 8-oxodG with baseline levels above 10 lesions/10^6 dG in any of the published studies because it would require approximately 30 animals/group or 30 independent experiments in cell culture studies.*"

This estimate is statistical, mainly based on *in vitro* data. Moreover, as the basal level of oxidative lesions will be below the maximal threshold evocated in this review (between 1-5 lesions/10^6 dG, less oxidative damage generated in *in vivo* than in *in vitro* experiment), the optimal need highlighted in this review is not necessary and envisaged. Also, the requested study will be a comparative study and do not require the quantififcation of lesions to be detected.

This comment allows ECHA to restate that all quality criteria and advice provided along this decision and in the corresponding guideline should be followed to ensure the high quality of the requested assays. Johansson *et al.* (2010) also demonstrated that enzyme-modified comet assay, in a calibration exercice, is able to detect as low as 0.2 lesions/10^6 base pairs (gamma radiation-induced lesions in A549 cells treated with Ro198022 + light), corresponding to 0.29 strand breaks/10^9 Daltons DNA. Therefore the enzyme-modified comet assay will be sensitive enough to detect the potential oxidising adverse effect of the Substance. Actually, the enzyme-modified comet assay has been performed with several test materials *in vivo* as detailed by Collins *et al.* (2020).

According to your comment and if you wish to increase the animal number to ensure that low signals are detected, the eMSCA does not object to this proposal, but **still believes that 5 animals per group is sufficient in the framework of this screening study**. Increasing the number of animals per group always increases the sensitivity of a study.

However, it should be balanced with the feasibility of handling a higher number of animals and the principle of animal testing reduction. Again, it should be kept in mind that the requested screening study will allow to minimise animal testing in longer-term studies by testing representative forms of the Substance covering the entire registration dossier. You also commented that "*We can properly process approx. 10 animals per day for the comet analysis of two organs (liver and lung)*", which is also a main argument for you in the impossibility for performing the requested study. As the comet assay on liver and gonads was removed, the limitation highlighted in your comment is considered obsolete as only the lung will have to be processed at each sampling timepoint.

1.8. Considerations on the inclusion of several parameters at each post-exposure time (2-6 hr, 1 day and 28 days) unless otherwise explicitly mentioned

1.8.1. Examination of the pulmonary response, including bronchoalveolar lavage fluid (BALF) and histopathology of the lung

As required in the OECD GD39 on inhalation studies, the mandatory BALF parameters are lactate dehydrogenase (LDH), total protein or albumin, total leukocyte count, absolute cell counts, and calculated differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils. Those were the parameters you initially proposed, as standard parameters of the IT instillation study. The rationale of these parameters are developed in section A.1.2.1.

1.8.2. Measurement of oxidative stress by measuring reactive oxygen species (ROS) and reactive nitrogen species (RNS) by fluorimetric probes, malondialdehyde (MDA) by HPLC and Haeme oxygenase 1 activity in the lung

Different oxidative stress markers must be measured in the lung as it is a suspected mode of action of toxicity of the Substance. This will enable to get information on the toxicity

potency of different forms of the Substance. To measure oxidative stress and based also on your comments, the markers above have been selected as relevant markers as they are well defined, sensitive and methodologies are easily available. However, you proposed also glutathione as a marker. It was not selected because it appears to have no added value compared to the other requested markers. Regarding your comment concerning the necessity of measuring these parameters at all doses/animals, cell preparations from lung tissue for the various requested examinations can be prepared at the same time.

These cell preparations could be used for determination of ROS/RNS by fluorometric probes (e.g. DCFDA/H2DCFDA, mitoSox and dihydroethidium (DHE)), and malondialdehyde (MDA) by HPLC and measurement of Haeme oxygenase 1 activity, which all must be measured in the lung. However, these measurements must not be performed at 28 days post exposure.
Therefore, this approach does not induce any extra preparation steps for tissue preparation. After that each protocol can be proceed separately. Finally, you commented that it was not specified why "*ROS/RNS shall be quantified in this testing programme - no reliable scientific evidence was provided that conclusively shows that titanium dioxide exposure is the primary cause for an increase of reactive oxygen species [...] It is therefore unclear how the additional investigation for reactive oxygen and nitrogen species is justified*". Therefore, you suggest to omit these measurements due to the lack of scientific relevance.

As detailed in Section A.1.2, the Substance shows photocatalytic properties, which implies that it releases reactive oxygen species when exposed to light – even laboratory light – (Carriere *et al.*, 2020). Surface reactivity with production of free radicals under UV-light, natural light and also in the dark are reported among scientific literature. Fenoglio *et al.* (2009) showed direct evidence that TiO_2-NPs are active in the generation of free radicals, including oxygenated free radicals and carbon-centered radicals, causing cleavage of C-H bonds in a model organic molecules. These reactions can occur even in the dark and could serve as the first step of oxidative damage of biological molecules.

With regards to mammalian/human cell studies, it was shown that, even in the dark, TiO_2-NPs induce oxidative stress-mediated toxicity in many cell types (Gurr *et al.*, 2005; Wang *et al.*, 2007; Hussain *et al.*, 2010; Shukla *et al.*, 2011; Meena *et al.*, 2012; Saquib *et al.*, 2012) and lipid peroxidation. In human bronchial epithelial cell line (BEAS-2B), Gurr *et al.* (2005) reported that in the absence of photoactivation, the anatase TiO_2-NPs (10 and 20 nm) can induce an increase in the cellular MDA level, oxidative DNA damage, and hydrogen peroxide levels. Shukla *et al.* (2011) showed that TiO_2-NPs can induce micronucleus formation, significant reduction in glutathione with a concomitant increase in lipid hydroperoxide and ROS generation in exposed human epidermal cells. Indeed, TiO_2-NPs are photocatalytic when exposed to UV light (Brezová *et al.*, 2005; Reddy *et al.*, 2007) and can cause oxidative stress and cell damage in the ambient environment.

In your comments on the PfAs, you raised discussion on technical point regarding oxidative biomarkers. These comments are out of the scope of the submitted PfAs and are therefore not considered.

1.8.3. Histopathology of liver, kidney, testis and brain to inform on the systemic availability

ECHA
EUROPEAN CHEMICALS AGENCY

Based on your comments discussing the "*extensive multiorgan histopathology*", it is reminded that these parameters are requested because they have been shown to be sensitive to exposure to the Substance (see A1.2. above for further details).

You commented on the impossibility to ascertain the performance of comet assay on frozen germ cells, together with the risk you pointed out "*of obtaining a mixture of somatic and germ cells*". Therefore, the request for the comet assay on the male gonadal cells was removed but histopathology on testis is requested instead. Consequently, you must store the testis and perform histopathological examination on it. Potential effects could be regarded as an indication of the presence of the Substance in this organ. This type of evidence may be relevant for the overall assessment of possible organ damage.

In your comments on the PfAs, you made some remarks on the fact that "*to verify the presence of TiO2 in this organ, we would think that instead the analytical verification of titanium content in testes tissue should be sufficient.*" However, the potential histopathological adverse effects in the testes would be missed if only analytical verification of the presence of the Substance is performed. Due to these limitations, losses of information and for animal welfare, and having the most relevant information after animal testing, the histopathological examination of the testis is requested.

In your comments on the PfAs, you made some remarks on the "*choice of type of study and route of administration for hazard identification*" and "*histopathological examination*". You raised questions that studies where the Substance was administrated by oral route (intragastric administration) were used to raise concern regarding translocation and effects to organs including testis in male rat and that no inhalation study was used instead. As explained in the decision, when possible, oral route should not be used for hazard identification after inhalation but it raises concerns whether the adverse effects observed after oral exposure would occur also after inhalation. Indeed, based on the data obtained by different routes, it is relevant to question if the Substance will also translocate and cause systemic effects while exposed via other routes than oral. This is particularly relevant for testes.

In relation to this comment, you emphasised that "*the validity of our comments and referenced studies arguing on the absence of systemic toxicity in an oral 90d-toxicity study ([redacted] 2011) and two chronic toxicity studies (NCI, 1979; Bernard, 1989)*" should be considered. Furthermore, you referred to the toxicokinetic investigation of TiO_2-NPs performed by Kreyling *et al.* (2017 a and b) that compared the absorption and translocation to various organs (including liver, spleen, heart, brain, kidney) via IT instillation and oral administration by gavage where the resulting organ levels of the Substance were actually quite similar.
Regarding the two studies by Bernard *et al.* (1990) and NCI (1979), you scored them as not reliable (Klimisch score 3) in the registration dossier. No information is available in the registration dossier on effects on the testis in Bernard *et al.* (1990). In the study by NCI (1979), some indications of effects on the testis are observed in exposed male rats compared to control. Indeed, alterations in seminal vesicles (SV) were observed: incidence 12% (low dose) and 20% (high dose) compared to 0% in controls. Also indication of testis atrophy was observed: incidence 10% (low dose) and 14% (high dose) compared to 6% in controls. Even if the effects in the testes are within the historical control (HC) range in the low dose group and only slightly above the HC data in the high dose group, these findings indicate potential effects of the Substance in the testes (reference reported in section A.1, point 2.2.1 after your comment).

ECHA
EUROPEAN CHEMICALS AGENCY

In respect to the lack of data on effects of the Substance in rodents via inhalation, the available data after oral administration were investigated in a detailed manner. The alerts arising after oral administration cannot be ignored especially when the aim is to identify the most relevant forms of the Substance to be tested in a tiered approach to represent all the forms covered by the registration dossier. Moreover, adverse effects in the testis, such as effects on seminal vesicles, sperm motility, increase in abnormal and total number of spermatozoid, increase in ROS production, negative effect on tissue histology and decrease in testis weight were observed in male mice and rats after oral exposure to the Substance (see section A.1 point 2.2.4 and Jafari *et al.*, 2020, Song *et al.*, 2017, Jia *et al.*, 2014, Tassinari *et al.*, 2014 and Shahin and Mhamed, 2017). Based on these observations and the fact that adverse effects have been identified in the testis after oral administration, the histopathological assessment of the testis after IT instillation is requested. The potential effects of the Substance in the testes can depend at least on the physico-chemical characteristics of TiO_2 forms. This substantiates the need for a ranking study.

1.8.4. Examination of cardiovascular function by measuring at least NO concentration, eNOS activity and hs-CRP content in the serum

These are standard parameters requiring non-invasive methods to be determined. Proper justification must be provided on the selected methodology.

Initially, the measurement of SAA3 protein was requested. You questioned the relevance of the SAA3 protein as a marker in rat. The eMSCA acknowledges that there are uncertainties about the expression of this isoform in the rat, although ELISA kits are available to measure this isoform in rats. Therefore, you must measure another acute phase protein, the high sensitivity C-reactive protein (hs-CRP) instead of SAA3. Indeed, recent studies have shown that an intraperitoneal (IP) exposure (Rizk *et al.*, 2020) and instillation exposure (Chen *et al.*, 2013) to TiO_2-NPs increased the CRP in mice. Similar observations were made in male rats after oral exposure (Fadda *et al.*, 2018). The hs-CRP is a well-known and described factor of atherosclerosis in humans also (Koenig *et al.*, 2006). ELISA kits exist and are available to determine the rat hs-CRP.

You commented that "*nitric oxide (NO) and endothelial nitrogen oxide synthase (eNOS) activity in serum should be measured as well. However, it is unclear why the NO content and eNOS activity should be measured in serum when the examinations done by Nurkiewicz and LeBlanc (referenced by ECHA) were performed using the exteriorized spinotrapezius. The complex operative procedure can obviously not be performed on the same animals used for all other parameters, which is probably why ECHA wants us to measure the parameters in serum, but it raises the question whether the methods and the results will be comparable to those of Nurkiewicz and LeBlanc.*"

Nurkiewicz *et al.* (2008) and Leblanc *et al.* (2009) were cited for demonstrating the interest of measuring NO content and eNOS activity after an acute exposure. However, ECHA agrees that an identical protocol cannot be performed in the **Tier 1**. These results are supported by the findings Chen *et al.* (2013) observed in mice after repeated exposure. In this study, the results (amount of NO and activity of eNOS in the serum) were comparable with those of Nurkiewicz *et al.* (2008) and LeBlanc *et al.* (2009). Therefore, you must measure the NO content and eNOS activity in the serum.

You commented on the need to measure the amount of NO and activity of eNOS simultaneously: *"it is questionable why the activity of eNOS, which produces NO, and the NO content should be measured at the same time. The activity of eNOS should be reflected*

ECHA
EUROPEAN CHEMICALS AGENCY

by the presence of NO. [...] This clearly shows that a simultaneous measurement of both parameters, eNOS activity and nitric oxide, is definitely not necessary". Csonka *et al.* (2015) summarised the methods available, direct and indirect, and they discussed the advantages and limitations of all of them:

- First, the authors confirmed that "*measurement of NO is technically difficult, due to its rapid chemical reactions with a wide range of biomolecules and its very short half-life of approximately a few seconds".* Contrary to your claim "*it should be considered that NO is highly reactive and can only be measured by using an indirect method",* Csonka *et al.* (2015) confirmed that a direct measurement is possible, citing 3 different methods, and even concluding that "*the use of direct NO detection methods as first choice should be considered*".
- Secondly, according to the authors, a limitation of the measurement of NOS activity is that it does not take into account the non-enzymatic NO formation.
- Finally, in line with the request, they concluded that "*a series of methods to follow NO synthesis, NO content, molecular targets and reaction products of NO are recommended to get meaningful insights into the role of NO in a certain physiological or pathological process*".

Therefore, it is proportionate to request both the measurement of the amount of NO and the activity of eNOS.

In your comments on the PfAs, you expressed that "*cardiovascular function is commonly assessed by measuring either (i) physiological parameters such as heart rate, arterial pressure, cardiac output, stroke volume etc., or (ii) electrocardiographic parameters such as duration p-wave/T-wave, P-wave amplitude etc."* Although they are not requested in this decision, you may consider measuring them.

You also commented on the relevance of measuring serum hs-CRP. This protein seems to be sensitive to the Substance. The request is based on the studies with Substance (as described above) where the markers, including NO and eNOS, were used to highlight cardiovascular/microvascular function impairment as a consequence of the exposure to the Substance. In addition, they are well known and well established parameters, particularly in humans, as detailed in the literature. CRP for instance is considered as "*one of the strongest predictors of risk of cardiovascular disease via inflammation*" (Zoltani, 2019). The hs-CRP, an inflammatory biomarker, is involved in the development of cardiovascular disease (Ridker, 2001). It can promote leukocyte adhesion and transendothelial migration to the vascular wall, and may therefore exert effects on atherosclerosis, plaque angiogenesis and deposition, and cause the infiltration of macrophages and development of atherosclerotic lesions (Hubbard and Rothlein, 2000; Yousuf *et al.*, 2013; Golia *et al.*, 2014).

You also expressed some concerns in your comments on the PfAs regarding the fact that CRP can react to a "*wide variety of conditions, from infection to cancer*". The rat models used in the requested short-term study are not expected to develop neither cancer nor any infection. Therefore, effects on CRP will indicate the potential inflammation induced by the treatment. We agree with you that the potential effects in the CRP levels will not indicate specifically cardiovascular impairment yet, but could predict the capability of the tested forms of the Substance to induce inflammation. If the exposure becomes chronic, it could lead to cardiovascular effects. Again, the aim of measuring the hs-CRP, in addition to NO content and eNOS activity, is to anticipate longer-term toxicity. You also questioned whether any relevant changes in CRP could be seen in the requested short-term study as it "*is known to rise in response to chronic systemic inflammation*".

ECHA
EUROPEAN CHEMICALS AGENCY

Although CRP has been described to be modified after repeated dose studies only (but in different publications after various routes and in two species, showing its sensitivity), SAA3, another positive acute phase protein, was significantly increased in an acute instillation study in mice (Saber *et al.* 2013). SAA3 was initially requested but for the reasons explained above, CRP was finally requested. Taken together, these elements lead the eMSCA to believe that CRP could be modified also after short-term exposure via IT instillation. Taking on board your reservation, it also justifies the measurement to take place at the 28 day timepoint as well. To counterbalance the short exposure duration, it emphasizes the need to have doses high enough to produce toxicity without lung overload.

In your comments on the PfAs, you also stated that transcriptional induction of the CRP gene is mediated by inflammatory cytokines,interleukin 6 (IL-6) in particular, in hepatocytes in liver, and therefore, an increase in this cytokine is expected. You argued that some publications show no modification of the level of inflammatory cytokines after exposure to the Substance (e.g. Elgrabli *et al.* (2015)). According to you, this raises the question why CRP should be increased to any relevant degree after acute instillation of the Substance. However, other studies show increase in cytokines, such as the study by Park *et al.* (2009): mice were exposed by a single IT instillation with 3 doses of TiO_2-NPs (5 mg/kg, 20 mg/kg, and 50 mg/kg), and the level of blood IL-6 was significantly increased in a dose dependant manner 1, 3, 7 and 14 days after the exposure. In this study, tumour necrosis factor alpha (TNF-α) was also significantly increased. If you believe this is relevant to strengthen the conclusions regarding the capability of each form to induce inflammation, you could also add cytokines measurement, such as IL-6, TNF-α, interferon gamma (IFN-γ, but this is not requested in the decision.

In your comments on the PfAs, you also commented on the relevance of measuring serum NO and eNOS, and raised a concern regarding your capacity to measure NO and eNOS. These comments are out the scope of the submitted PfAs and are therefore not considered. However, as you pointed out, nitric oxide plays a pivotal role in physiopathology of the cardiovascular system.

2. Specifications for the comet assay

A comet assay must be performed at each post-exposure timepoint on lung tissues according to the OECD TG 489 including the following:

2.1. Conduct of the comet with and without the addition of DNA repair enzymes

Regarding your comment on the relevance of the enzyme-modified comet assay, using Fpg (formamidopyrimidine [fapy]-DNA glycosylase) or human oxoguanine DNA glycosylase (hOGG1), in order to investigate a mode of action linked to direct genotoxic damage or oxidative DNA damage secondary to oxidative stress, DiBucchianio *et al.* (2017) highlighted that the enzyme-modified comet assay is able to detect an increase in Fpg-sensitive sites (markers of oxidative damage) *in vitro* in BEAS-2B cells after 3 or 24 h exposure to the Substance (NM100, NM101 or NM103) dispersions at concentrations of 1, 5 or 15 µg/ml (0.2–3.15 µg/cm2). The review by Collins *et al.* (2020) detailed the use of the enzyme-modified comet assay to detect oxidative lesion *in vivo* in various organs following exposure to a large variety of chemicals.

You commented that that "*There is currently a shortage for the hOGG1 enzyme supply, which may hamper the execution of these experiments considering the extreme large*

ECHA
EUROPEAN CHEMICALS AGENCY

number of samples. It is not recommended to use different batches of the enzyme, as this would complicate comparability between samples. The use of Fpg is not recommended, as this method is not very specific for oxidised bases and also has endonuclease activity and thereby generates strand breaks in undamaged DNA (see Speit et al. 2015)."

It is at your discretion to choose between hOGG1 and Fpg enzymes, but you must justify the selection having in mind that these enzymes have their own specificities. Generally, both enzymes are suitable to detect potential oxidising effects of the Substance on DNA in lung cells. Moreover, as detailed below, the Fpg enzyme is considered a valid choice. We strongly encourage you to use this enzyme as the preferred option in the performance of the enzyme-modified comet assay taking into account the potential shortage of hOGG1 enzyme supply.

2.2. Inclusion of a positive control (e.g. KBrO3 or Ro19-8022 + light for enzyme-modified comet assay, and KBrO3, MMS or EMS for standard comet assay).

You questioned the use of some positive control for the requested assay. Positive controls can be obtained by treating the comet assay slides prepared by agarose embedding of the cells isolated from negative control animals prior to lysis. The treatment can be performed e.g. with (R)-1-((10-Chloro-4-oxo-3-phenyl-4H-benzo(a)quinolizin-1-yl)carbonyl)-2-pyrrolidinemethanol (RO 19-8022) followed by light induction (Collins *et al.,* 2014). This would also decrease the total number of the treated animals in the whole study. For more details, you can see Nanogenotox project (2013), Di Bucchianico *et al.*(2017), Kain *et al.*(2012), Asare *et al.*(2016) and Møller *et al.*(2015, 2017 a and b).

You commented on the use of potassium bromate ($KBrO_3$) as a positive control. When performing the comet assay, alkylating agents are frequently used for the Fpg-modified comet assay or even for hOGG1-modified assay because these agents are recommended in the OECD TG 489 as positive controls for DNA strand breaks. However, alkylating agents are not the best choice for enzyme-modified comet assays due to the variation in lesions that they can cause. $KBrO_3$ has been shown to induce both 8-oxodG and Fpg-sites in Chinese hamster (V79) lung cells, in peripheral blood mononuclear cells (PBMCs), in mouse lymphoma cells, in human bronchoalveolar cells, in the kidney of mice and rats, with little concurrent generation of DNA strand breaks.

$KBrO_3$ appears to be a suitable positive control for the Fpg- and hOGG1-modified comet assay because it produces high levels of oxidatively damaged DNA (Møller *et al.*, 2018). $KBrO_3$ is genotoxic via a mechanism whereby glutathione activates bromate to bromine radicals or oxides, which cause oxidation of guanines in the DNA. Fpg concentration and incubation time are critical in the Fpg-modified comet assay. The concentration of Fpg should be high enough to detect the maximum amount of lesions present in the cells (ideally all of them) without exhibiting non-specific breakage activity. The time of incubation is also critical. The ESCODD protocol recommends to expose samples for a maximum of 30 min in order to achieve the expected DNA repair enzyme activity (Møller *et al.*, 2018).

For the standard comet assay *in vivo*, the recommended positive assay control, for example ethyl methanesulfonate (EMS) or methyl methanesulfonate (MMS), can be used as they are recommended for the lung tissue.

ECHA
EUROPEAN CHEMICALS AGENCY

2.3. Considerations regarding the request to evaluate the effects of each parameter that can affect the outcome of the comet assay

You must describe in details the procedure and steps used during the performance of the comet assay.

2.3.1. Controls (positive, negative and assay controls)

When performing the comet assay on lung tissue, you have to test proper positive and negative controls, and if possible assay controls, along the Substance tested. You commented on the number of control groups by indicating that it will be difficult to carry out the assays concurrently. Consequently, the number of animals in the control group was adjusted. This modification is emphasized in Table 3 (below).

You also commented on the necessity to assess the day-to-day variability of the comet assay "*In case the testing programme is executed with staggered exposure and sacrifice, this would require an additional 60 negative control groups, i.e. additional 300 animals. Additional control groups are required to control for the day-to-day variability of the comet assay*". ECHA acknowledges that if you decide to use staggered exposure of the animals, an increase in the number of animals will probably occur. Nevertheless, this increase is not caused by the combination of instillation study and the comet assay, but by the high number of TiO_2 forms to be examined in order to represent all the forms of the Substance covered by the registration dossier. Taking this into account, the eMSCA estimated that if an additional group (corresponding to a control group for each form of the Substance) is needed, it would lead to an increase of approximatively 150 animals and not 300 as per your estimate. This increase will also occur to ensure the estimation of the day-to day variability, even if the comet assay is not requested contrary to what you argued.

In your comments on the PfAs, you made some remarks on the day-to-day variability when performing the comet assay. Your comment is out of the scope of the submitted PfAs and is therefore not considered.

You requested additional information on the assay control. It is defined as 'Samples that are included in every comet assay experiment within the same laboratory (preferably cryopreserved samples that have been exposed to a DNA damaging agent)' (Møller *et al.*, 2018). Assay controls are prepared from a single batch of cells (or pooled samples), either untreated (negative standard) or treated with an appropriate DNA damaging agent (positive standard), frozen slowly as a large number of aliquots in freezing medium and stored at -80°C. Assay controls must be included in each electrophoresis run during the whole study and may be combined with the tested samples if they can be distinguished from each other during the scoring to even better control the performance of the assay. Assay controls will guarantee that intralaboratory variability is properly controlled and are also essential for inter-laboratory comparison. The implementation of these assay controls enables the detection of possible abnormal values (excessively low or high) if compared to historical controls, and also gives the opportunity to normalize data in order to minimize technique-associated variability and consequently allow a meaningful comparison of data (Esteves *et al.*, 2020).

If the addition of this type of assay control is feasible, it will (i) help to improve the data analysis, and (ii) address your comment that "*large and complex experiment [...] requires strict control of variation to produce valid, interpretable results.*" Indeed, the assay control is a valid indicator of the DNA damage measurement, although it does not reveal flawed experimental procedures related to particle exposure or sampling of cells for analysis of

ECHA
EUROPEAN CHEMICALS AGENCY

DNA damage. This type of assay control differs from a positive control. However, the same substance can be used as both an assay control and a positive control. Data from such assay controls may also serve to explain differences in response levels and sensitivity between different studies (Møller *et al.*, 2015 and 2017a and b).

2.3.2. Lung tissue

You also commented that lung tissue is uncommon in the majority of the comet assays performed with the Substance, and considered that it is difficult to collect and process lung tissue. Therefore, high background damage is likely due to required tissue processing.

However, in the recent studies mentioned earlier in this decision and notably the one from Wallin *et al.*(2017) and Danielsen *et al.*(2020), the usefulness of analysing the lung tissue is demonstrated. Moreover, Collins *et al.* (2020) identified 15 comet assays performed *in vivo* with different nanomaterials. The lung was tested more than 10 times. Therefore, the selection of the lung as a target for evaluating toxicity of the Substance after inhalation is considered appropriate, and the lung tissue is relevant also in an IT instillation study. You considered that the comet assay should preferentially be performed on BAL cells, rather than on the lung tissue. You provided comments and references on the comet assay performed on BALF, which are partly irrelevant for the present decision. However, as the respiratory tract and lung are the site of contacts of the Substance after instillation they are also first targets of the possible adverse effects. Therefore, the lung tissue would be more sensitive and more appropriate to perform the comet assay.

Moreover, as defensive mechanisms, macrophages may be mobilised from elsewhere in the body. Performing a comet assay on the cells in the BALF may introduce a bias by performing the assay on unexposed cells.

However, if you still consider that "*alveolar macrophages are instead considered the cell/tissue type with the highest exposure due to their phagocytic capacity and are also identified as target tissue/cell type for the tumorigenicity of titanium dioxide in rats*" it is left at your discretion to perform the comet assay on BAL cells **as well**.

2.4. Further considerations

You mainly questioned the relevance or raised uncertainties related to (1) the use of frozen tissues, (2) the use of DNA repair enzymes and related uncertainties, (3) the possible interaction of nanomaterial with the DNA repair enzymes, (4) the time involved for the comet assay analysis, (5) the non validation in interlaboratory assays of the requested use of DNA repair enzymes and (6) the use of Fpg and hOGG1 enzyme and their sensitivity, (7) performing a comet assay 28 days after IT instillation, and (8) performing a comet assay on inorganic poorly soluble particles. You also (9) preferred to use immunohistochemistry to detect potential oxidative DNA damage (10) and contest the justification of the comet assay.

1) You commented that "*The studies cited by ECHA/eMSCA to support the comet assay requirement use frozen tissue. OECD guideline 489 confirms no agreement exists on freeze/thaw methods. Therefore, it is not possible to know the reliability or reproducibility of results of using frozen tissue*".

   First, is should be noted that the exact wording of the OECD TG 489 on the use of frozen material is "*Tissues or cell nuclei have been successfully frozen for later analysis. This usually results in a measurable effect on the response to the vehicle*

ECHA
EUROPEAN CHEMICALS AGENCY

and positive control (Recio at al., 2010; Recio at al., 2012; Jackson at al., 2013). If used, the laboratory should demonstrate competency in freezing methodologies and confirm acceptable low ranges of % tail DNA in target tissues of vehicle treated animals, and that positive responses can still be detected. In the literature, the freezing of tissues has been described using different methods. However, currently there is no agreement on how to best freeze and thaw tissues, and how to assess whether a potentially altered response may affect the sensitivity of the test." As a consequence, the use of frozen tissue by itself does not question the results obtained. Moreover, Aztequa and colleagues (2019) have recently demonstrated that performing enzymes-modified comet assays on frozen material is suitable to detect oxidative DNA lesions.

Second, the requested study design does not suggest to use frozen tissues. Some studies referred to in this decision used such a frozen-tissue protocol and were able to raise concerns on the genotoxic potential of the Substance. Therefore, you may use frozen tissue to perform the comet assay as long as your competence in doing so is demonstrated, but this is not a part of the request.

2) Regarding the enzyme-modified comet assay, the use of enzyme modification does not imply to treat and use more animals at all. The protocols of the standard comet assay and the enzyme-modified comet assay are the same with an exception to include a single additional step in the protocol during the tissue processing part (consisting generally of nine steps): The first step consists of collection and homogenisation of tissues to proceed to obtain a single-cell suspension. The second step consists of embedding single cells in agarose gels and lysing them in step 3 to remove membranes and other cellular material. This third step will leave protein-depleted nuclei with a supercoiled DNA (called "nucleoids"). The modification of the standard alkaline comet assay occurs at the fourth step, where the prepared slides undergo incubation of the nucleoids with lesion-specific enzyme including Fpg, hOGG1, endonuclease III (Endo III) or T4 endonuclease V (T4 Endo V). This step generally has a duration of 30 min to a maximum of 2 hours. In the fifth step, the standard alkaline comet assay protocol is followed, and samples are treated in alkaline solution to convert alkali-labile sites to strand breaks and allow DNA to unwind. The samples are then subjected to alkaline electrophoresis, resulting in the formation of single-cell comets (corresponding to the sixth step). After electrophoresis the samples are rinsed in neutralizing solution (corresponding to seventh step). The eight step includes staining and visualization of the comets by fluorescence microscopy. Finally, the last ninth step corresponds to the scoring of the comets and data analysis. Your comment on the number of animals for reaching sensitivity in enzyme-modifed comet assay is addressed above.

3) You raised the possibility that NPs may interact with DNA repair enzymes. This hypothesis cannot be invalidated. However, as the enzymes are used in excess, there will be sufficient amount of enzymes to react properly with DNA. Thus, as for any methodology with fluorescence and imaging detection, calibration must be performed and confounding factors assessed. This will lower the uncertainties and ensure a proper assessment when interpreting the assay results. Interference of Fpg with NPs, when using the Fpg-modified comet assay, has indeed been described in the literature (Kain *et al.*, 2012). However, Kain *et al.* (2012) mixed the NPs with the Fpg leading to the creation of a protein corona effect, which greatly impacted the ability of the enzymes to repair DNA lesions and affected the enzyme activity. Other authors have claimed that such interference is unlikely to occur when

ECHA
EUROPEAN CHEMICALS AGENCY

performing the assay correctly (Magdolenova *et al.*, 2012, Azqueta and Dusinska, 2015, Di Bucchianico *et al.*, 2017). The applicability of the Fpg-modified comet assay *in vitro* and *in vivo* to detect the effects of NPs has been demonstrated with several nanomaterials (El Yamani *et al.*, 2017; Iglesias *et al.* 2017a, b; Azqueta *et al.*, 2019; Asare *et al.* 2016; Jalili *et al.*, 2020). The hOGG1 has also been used to detect the genotoxic potential of NPs *in vitro* and *in vivo* (Fernandez-Bertolez *et al.*, 2019; Pfuhler *et al.*, 2017).

4) You commented the effort and time needed to perform the requested comet assay "*it would necessitate the evaluation of 5760 comet slides (12 slides per animal as described below), corresponding to approx. 264 working days for the slide evaluation only (!). This does not include the control animals or any other work associated with the comet assay, such as cell isolation, lysis, electrophoresis. It is highlighted that the presence of particulate matter interferes significantly with the automatic slide evaluation so that especially in the high dose animals the comet slides need to be evaluated manually*".

However,:

- following your comment, the comet assays on liver and testis were removed. This will greatly decrease the number of slides and assays to be performed
- automatisation is largely developed and imaging assisted detection is routinely applied in laboratories;
- the OECD TG 489 aknowledges that comet assay can be performed in 96 well microplates. The use of automatic reader and 96-well microplates (such as the system provided e.g. by CometChip and Norgenotech) can drastically reduce the time to read and assess the comet assay data. Even if some slides have to be read again due to particles interference or other reasons, your estimate on time needed for the whole reading of the data seems to be overestimated (264 working days);
- the enzyme-modified comet assay can be performed in a high-throughput way using the 12 minigels/slide format, or different numbers of minigels (following the standard 24, 48 or 96 well formats) or the CometChip technology to significantly reduce the time to perform these analyses;
- the reading can be performed all along the experiments, allowing a longer delay for performing this task. The possibility to use the automated reader or the use minigels/slides was evidenced and recognized by the institute group contacted by you in a recent publication (Kohl *et al.*, 2020). The authors recognize that "*12 mini-gels per slide, 96 mini-gels on a GelBond film a special 96-well multi chamber plate (MCP) -allow increased throughput and analysis of numerous types or modifications of NMs in a time- and cost-effective manner.*" Moreover, this publication acknowledeged that the use of automatic system allows to decrease the reading from hours to minute-range "*To improve the very time-consuming DNA damage evaluation by manual microscopic fluorescence-analysis, several automatic systems for image analysis have been developed to help increase the throughput of the assay for high screening capacity; for instance the fully automated slide-scanning platform Metafer and the Meta Cyte Comet Scan software. Using such automated evaluating systems, the analysis duration is reduced from hour-range to minute-range.*" This type of platform, and the one cited by the authors, CometScan can automatically find, acquire, and analyze Comet assay cells - fully unattended and in up to 800 slides. The CometAssay software has been used by the identified laboratory (i.e.

ECHA
EUROPEAN CHEMICALS AGENCY

Fraunhofer Institute) in a previously published study (Ziemann *et al.*, 2009).

This software is able to perform the analysis and extraction of a slide in two minutes[8], drastically decreasing the estimated time claimed by you, especially as being aleady in place at the selected laboratory. The requested study will generate large amount of data to be processed but this only results from the high number of TiO_2 forms to be tested. Accordingly, the number of slides, which must be assessed will not be as time-consuming as you indicated. Moreover, information is available regarding the storage of gels containing treated cells allowing to perform the reading all along the allowed time "*It is often impracticable to score slides immediately after performing the comet assay. If the gels are prepared on ordinary glass slides, they can be dried and stored indefinitely*" (Collins, 2004).

5) Regarding your comment concerning the lack of inter-laboratory ring tests (validation) on the hOGG1, the eMSCA acknowledges that the requested study is not *per* se a full guideline compliant study. However, it has already been requested in another ECHA's substance evaluation decision on potassium titanium oxide (EC 432-240-0) (ECHA, 2019). There is still the possibility for you to choose to use the Fpg enzyme, which is more often used. Fpg is able to detect oxidative lesions and the protocol and related data are described in the literature (See above).

   In your comments on the PfAs, you expressed surprise that "*a particular study design has been requested by another ECHA decision recursively validates a study design as valid.*" This refers to ECHA's substance evaluation decision on potassium titanium oxide (EC 432-240-0) (ECHA, 2019). You misinterpreted our argument. Indeed, requesting a non guideline compliant study in a previous decision does not validate the study design. This reference indicates that non-validated studies can be and have been requested when necessary.

6) You do not recommend to use Fpg based on the argument that "*this method being not very specific for oxidised bases and also has endonuclease activity and thereby generates strand breaks in undamaged DNA*". The choice of the DNA repair enzyme is left at your discretion even if the Fpg DNA repair enzyme is the preferred one. hOGG1 is the eukaryotic counterpart of Fpg. hOGG1 can repair oxidized bases only when the damaged base is paired with cytosine. Therefore, it is more specific than Fpg, which recognizes 8-oxoGua paired with cytosine, guanine or thymidine. Nevertheless, hOGG1 appears to be less efficient than Fpg (David and Williams, 1998). These two enzymes can increase the sensitivity of the comet assay. Indeed, Fpg presents some advantages compared to hOGG1. It has been much widely used and is more easily available. Fpg also allows the detection of more types of DNA lesions than hOGG1, even though, as you commented, Fpg is less sensitive than hOGG1 to oxidised damage recognition. On the other hand, it is more sensitive as more Fpg sensitive sites (specific lesions recognised) are produced when cells are treated with damaging agents. This allows the detection of indirect effects of some non-alkylating agents, such as bulky adducts. Furthermore, hOGG1 does not recognize AP (apurinic/ apyrimidinic) sites produced from spontaneous depurination of methylated guanines (Speit *et al.*, 2004, Smith *et al.*, 2006, Muruzabal *et al.*, 2020). However, the final choice of one or the other enzyme will rely on the expertise of the laboratory conducting the study. Both enzymes are

[8] https://www.instem.com/solutions/genetic-toxicology/comet-assay.php

ECHA
EUROPEAN CHEMICALS AGENCY

suitable to detect potential oxidising effects of the Substance (especially to measure 8-oxoGua in DNA, Collins *et al.*, 2014) on DNA in lung cells. You claimed that there is variation between laboratories when using Fpg. This is a common feature for many experimental studies in laboratories corresponding to variations commonly described in literature. To decrease these variations as much as possible, the recommendations provided in this decision and in the studies describing the method should be followed (e.g. Collins *et al.*, 2014, Møller *et al.*, 2015, Møller *et al.*, 2017a, b, Azqueta *et al.*, 2019; recommendation drafted by the participants of the ECVAG (European Comet assay Validation Group) and ESCODD).

7) You questionned the relevance of performing a comet assay 28 days after the end of the IT instillation procedure, in particular because you claimed that "*any positive results in the comet assay would be secondary to the sustained inflammation and not primary (direct and indirect) to the particle exposure*".

   You cited the Annex VIII, Section 8.6.1, Column 2 of REACH and stated that secondary genotoxicity is not a concern. Neither the reference nor the statement is completely clear. Actually, the specific section at Annex VIII you referred to does not discuss the secondary toxicity but states, as an example, the possibility for the Agency to request specific toxicological studies in accordance with Article 40 or 41 in case of indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. So far, the available data do not allow a proper hazard characterisation. In particular, the 28-day request is justified when considering that oxidative stress leading to DNA damage can occur also after a long time after the end of exposure (see Modrzynska *et al.*, 2018a). Indeed, the 28-day timepoint will allow clearance to occur in the lung and to see if the potential adverse effects persist, and can provide indirect evidence whether translocation of the Substance from the lung to other organs occurs.

   The 3 observation timepoints defined in the current decision were set up in order to link all the investigated parameters, i.e. pulmonary effects, cardiovascular function, oxidative stress, histopathology and (oxidative) DNA damage. Restricting the post-exposure timepoints here will not ensure a proper evaluation of the potency of the tested forms of the Substance. This could impair the subsequent choice for the forms of the Substance to be tested in the Tier 2.

8) The OECD TG 489 clearly stipulates on page 7, paragraph 26 that "*Solid test chemicals should be dissolved or suspended in appropriate vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test chemicals can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties*". This provides indications on how to test inorganic poorly soluble particles.

9) As hOGG1 is specific for the pre-mutagenic oxidative DNA lesion 8-oxo-dG, one of your advisors (Dr. C. Ziemann, study director, Fraunhofer, ITEM) recommended to assess this type of lesion via immunohistochemistry (IHC). The reason why this alternative technic to the enzyme-modified comet assay is judged not acceptable is addressed under section A.6.

10) A comet assay is requested because it can detect also oxidative DNA damage, it can be performed on a larger number of samples and consequently many problems

ECHA
EUROPEAN CHEMICALS AGENCY

linked to the presence of nanomaterials can be avoided (e.g the scoring of positive outcome which can be challenging for some tests when aggregates and agglomerates are present). Moreover, the inclusion of the comet assay in the IT instillation test will not exert additional manipulations of the animals as it will be performed post-mortem, avoiding generation of stress. The comet assay will allow to identify whether some of the TiO_2 forms tested are able to generate oxidative stress leading to genotoxicity by causing oxidative DNA damage. Excessive production of ROS/RNS resulting in oxidative stress is known to be a common mechanisim of toxicity for many compounds (Auten and Davis, 2009; Fu *et al.*, 2014; Abdal Dayem *et al.*, 2017, Modrzynska *et al.*, 2018). Therefore, indication of oxidative stress after acute exposure in an IT instillation study may predict also other adverse effects in addition to oxidative DNA damage after longer repeated exposure via inhalation. As translocation of the Substance to the liver was observed in one study by instillation (Husain *et al.*, 2015), performing histopathological assessment of the liver, the kidney, the brain and the testis concomitantly to the lung may allow the eMSCA to confirm which forms of the Substance can translocate if histopathological changes or presence of TiO_2 particles are observed in these organs after instillation exposure. This information is essential to select the forms of the Substance for the Tier 2 testing to obtain more extensive data on local and systemic toxicity after subchronic exposure via inhalation.

3. Summary of test protocol

Table 2 describes the protocol of the IT instillation study.

Table 2 : Summary of the protocol of the IT instillation study in Tier 1 when performed concomitantly for all forms.

Treatment	2 instillations must be performed at 24 hr interval (at 0 and 24 hr)
Post-exposure observation	2-6 hours - 1 day - 28 days
Test Performed	Histopathology of lung, liver, kidney, brain and testis Cardiovascular function BALF analysis Oxidative stress in lung (not measured at 28 days) Comet assay (standard and enzyme-modified protocol) on lung tissue
Negative control[9]	Yes
Low dose	Yes
Mid dose	Yes
High dose	Yes
Positive control (for comet assay)[10]	Yes
Number of groups	5

You must submit full study reports of the **Tier 1** study (IT instillation study combined with the comet assay). Considering the complexity, a complete rationale of the test design and interpretation of results, as well as access to all information available in the full study

[9] One single negative control for all forms tested
[10] One single positive control for all forms tested

ECHA
EUROPEAN CHEMICALS AGENCY

reports (implemented method, raw data collected, interpretations and calculations including the statistical analysis, consideration of uncertainties, argumentation, etc.) are needed.

This will allow the eMSCA to fully assess all provided information, and to make an informed decision on the next steps of the proposed testing strategy.

A.6 Alternative approaches and proportionality of the request

The request for this study by the IT route is suitable and necessary to obtain preliminary information on the toxicity and relative potency of different forms of the Substance under the same test conditions. Therefore, this test is essential to define, which forms of the Substance shall be tested in the **Tier 2** of the testing strategy. It will also allow you to explain how you compare the non-tested forms with the tested ones while defining their toxicity via inhalation.

A possible alternative could have been to request directly a subchronic repeated dose toxicity study and/or chronic study, should it have been clear what are the most suitable test materials to be tested. Various physico-chemical properties of different forms of the Substance are reported to impact toxicological properties. Therefore, it is appropriate to collect information on these forms in a shorter term test first in a test performed under the same conditions for all the forms tested. The IT instillation test is a pragmatic approach allowing an efficient comparison of the toxicity of different forms of the Substance. Finally, ECHA notes that there are no *in vitro* or *in silico* experimental methods available at this stage that will generate the necessary comparable information without vertebrate testing.

Inclusion of additional parameters to be investigated, in comparison to the test initially proposed, also allows providing more certainty for the adequate selection of the test materials to be further tested and a proper comparison to predict toxicity of the untested ones. Moreover, it will allow to identify the potential adverse effects occurring from oxidative mechanisms, will allow to observe the potential ability of the Substance to translocate to other organs based on histopathological observations or an impact on cardiovascular function, which all will help designing the next studies appropriately.

You commented on the difficulty to integrate the comet assay to the IT study without compromising the reliability of both results. For your clarification, the comet assay must be integrated to the IT instillation study as it is intended especially to screen the effects of 11 forms of the Substance on the oxidative stress potentially leading to genotoxicity. IT instillation study alone is not sufficient for the selection of the forms to be tested in the **Tier 2** as the parameters initially proposed by you may not be sensitive enough for selecting the most appropriate forms of the Substance for further testing.

As 5 male rats/group are required, the eMSCA estimated that this combined test protocol will save up to 500 animals compared to performing the IT instillation study and comet assay separately (See Table 3).

Table 3 : Calculation of animal saving by combining comet assay and IT instillation study

	Combined toxicity test + comet assay	Toxicity test only	Comet assay
Number of treated groups per time point	3	3	3

ECHA
EUROPEAN CHEMICALS AGENCY

Number of treated groups for 3 time points	9	9	9
Number of treated groups for 3 time points and 11 forms of the Substance (A)	99	99	99
Number of control groups per time point	2	1	2
Number of control groups for 3 time points (B)	6	3	6
Total number of groups (A+B)	105	102	105
Number of animals per group	5	5	5
Total number of animals	**525**	510	525
		1035	

Instead of using the the enzyme-modified comet assay, you proposed to assess the pre-mutagenic oxidative DNA lesion 8-oxo-dG by immunohistochemistry (IHC) to "*allow a localisation within the lung slides and facilitate the tasks to be conducted at necropsy*". While this technique is a promising tool to assess this premutagnic DNA lesion, especially the possibility to localize the lesion in cell or tissue, this technique suffers some major drawbacks detailed below, such as imprecision:

- Each step of IHC might lead to deviations and new challenges.
- This method may lead to rather strong background, since cytoplasmic RNA staining interferes with the DNA-specific quantitation of 8-OHdG.
- IHC is not very quantitative as the antibodies may not be totally specific for the lesions of interest: they may cross-react with lesions of similar structure leading to an overestimation of DNA damages. The specificity of this technique therefore highly depends on the antibodies used and is a major source of uncertainties in the outcome of the assay (Korkmaz *et al.*, 2018). Møller *et al.* (2015) also considered antibody-based methods for detecting 8-oxodG or other oxidatively damaged DNA nucleobase lesions as non-informative measurements, because of the lack of specificity of antibodies.
- To the eMSCA's knowledge, IHC has only been used a few times with nanomaterials, and even less in the case of the Substance. Armand *et al.* (2016) demonstrated that IHC was able to detect slighty more DNA damage than classical comet assay when using 53BP1 foci antibodies, but that the sensitivity of the assay is much lower than the enzyme-modified comet assay using Fpg.
- This semi-quantitative assay requires complex signal density analysis with extensive operator time in comparison to the standard and enzyme-modified comet assay.

Therefore, despite the interest of this technique, there is not sufficient data to validate this approach so far in this specific case. In addition, the confounding factors may not allow proper interpretation for selecting the forms of the Substance for further testing.

The eMSCA acknowledges that the enzyme-modified comet assay has not been validated for regulatory purpose. However, it differs from the standard OECD TG 489 protocol by only one additional step.

On this basis, the request to investigate oxidative damage using the standard comet assay and enzyme-modified comet assay is proportionate and the best approach. The enzyme-modified comet assay is a quite simple, sensitive and specific technique, requiring very small sample size. The enzyme-modified comet assay is also a robust method with many years of experience. It has been evaluated and improved by many European groups including ESCODD and ECVAG.

ECHA
EUROPEAN CHEMICALS AGENCY

A.7 Additional considerations of your comments on the draft decision

Your comments have led to substantial changes in the initial request and to numerous clarifications (see sections A.1 to A.6). Consequently, the decision was amended accordingly to reflect your comments. Some specific comments are also addressed below:

You commented that existing data show that one (and maybe more) form of the Substance does not elicit toxicity below alveolar macrophage overload, requesting to modify the request to reach "*clear level of toxicity (highest test concentration) but not including overload*". As stated in the the request "*where feasible*", it has not been modified.

You suggested to perform a new range-finding study. However, such a range-finding study seems to exist already in the study report F2336 (BauA, 2017), which you need to consider to avoid unnecessary duplication of an (existing) testing. Indeed, this very detailed study, where the rats were sacrified at 3, 28 and 90 days, will allow you to define the appropriate dosing for the Tier 1 tesing requested in this decision. It would be disproportionate to request such a new range-finding study. You commented that the existing range-finding does not cover all the forms of the Substance to be tested. It is left to your discretion to consider the need to perform such extra range-finding study to ensure that the information required in this decision fulfils the requests taking into consideration that this part of the protocol is the one initially proposed by you. The details on the inhalation study are described under section A.1, while the details on the IT study can be found in the study report F2336 (BauA, 2017).

You highlighted several sources of data in your comments. The eMSCA has the following considerations on these data:

- Data from the [redacted] have no longer been considered (Ze *et al.*, 2013, 2014 a, b, c & 2016, Yu *et al.*, 2014, Hong *et al.* 2015) as uncertainties were raised regarding possible fraud and quality problems. However, this does not change the general conclusions as evaluation of publications from other universities/ laboratories highlight the same concerns.
- The concern that triggers this decision (Tier 1) is the repeated dose toxicity by inhalation. You proposed in your comments to consider data generated by oral route (NCI, 1979; Bernard *et al.*, 1990; [redacted], 2011), and based on results of a study comparing translocation after instillation and oral exposure (Kreyling *et al.* 2017 a,b). In absence of sufficient quantitative and qualitative comparative data regarding route to route extrapolation, studies by oral routes were only considered for raising a concern on hazard potential but these data cannot be used for the assessment of hazard properties of the Substance after exposure through inhalation.
- Your comments emphasised also that "*at this point in time TiO_2 is not formally classified as a Cat2 carcinogen. In addition, the RAC opinion explicitly states that this is a particle effect, and not a substance-specific toxicity*". This decision aims at requesting additional data for clarifying the potential risk after inhalation exposure to different forms of the Substance and does not aim at discussing existing or future harmonised classifications. The Substance is classified as Cat.2 carcinogen (14th adaptation to technical progress (ATP) to the CLP Regulation)[11]. This current classification covers the Substance "in powder form containing 1 % or more of particles with aerodynamic diameter ≤ 10 µm" as Carc. 2 by inhalation. This is a minimal classification and, depending on the forthcoming information, a more stringent classification may be needed for some forms of the Substance.

ECHA
EUROPEAN CHEMICALS AGENCY

A.8 Consideration of the time needed to perform the requested study

You commented on the effort and time needed to perform the requested study design. You stated that is not possible to handle 160 animals per day. The eMSCA acknowledges that performing the study on all 11 forms of the Substance at one time is a challenge. Staggered approach in exposure, necropsy, sample processing and analysis is recommended in order to maintain the quality of the study as high as possible. How to schedule the testing process for the 11 forms of the Substance (e.g. the exposure and sampling time) is left at your discretion. Indeed, this difficulty is inherent to the IT instillation study and not to the addition of the comet assay.

The timeline to provide the requested data takes into account the time that you may need to agree on the name of the registrant(s) that will perform the required test (according to Article 50(3))) and includes the time required for developing an analytical method, conduct of the study, prepare the study report and report the results in IUCLID.

In your comments, you mentioned that the designated test facility will have to apply for permission with the local animal welfare committee. You anticipated that it will require a minimum of 3-4 months. You also emphasized on the extent of the testing requested, and especially the time needed for performing the comet assay slides reading (see section A.5). Consequently, the deadline was extended accordingly from 24 months to 30 months.

ECHA considers that this deadline is appropriate to conduct and report the requested studies.

ECHA
EUROPEAN CHEMICALS AGENCY

A.9 References

Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S. G. 2017. "The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles." International journal of molecular sciences, 18(1), 120. https://doi.org/10.3390/ijms18010120

ANSES (2016). CLH Report Proposal for Titanium Dioxide, Based on Regulation (EC) No 1272/2008 Annex VI, Part 2.

Aragao-Santiago, L., H. Hillaireau, N. Grabowski, S. Mura, T. L. Nascimento, S. Dufort, J. L. Coll, N. Tsapis, and E. Fattal. 2016. "Compared in vivo toxicity in mice of lung delivered biodegradable and non-biodegradable nanoparticles." *Nanotoxicology* 10 (3):292-302. doi: 10.3109/17435390.2015.1054908.

Armand L, Tarantini A, Beal D, Biola-Clier M, Bobyk L, Sorieul S, Pernet-Gallay K, Marie-Desvergne C, Lynch I, Herlin-Boime N, Carriere M. Long-term exposure of A549 cells to titanium dioxide nanoparticles induces DNA damage and sensitizes cells towards genotoxic agents. Nanotoxicology. 2016 Sep;10(7):913-23. doi: 10.3109/17435390.2016.1141338. Epub 2016 Feb 22. PMID: 26785166

Asare, Nana, Nur Duale, Hege H. Slagsvold, Birgitte Lindeman, Ann Karin Olsen, Joanna Gromadzka-Ostrowska, Sylwia Meczynska-Wielgosz, Marcin Kruszewski, Gunnar Brunborg, and Christine Instanes. 2016. "Genotoxicity and gene expression modulation of silver and titanium dioxide nanoparticles in mice." Nanotoxicology 10 (3):312-321. doi: 10.3109/17435390.2015.1071443.

Auffan, M., M. Pedeutour, J. Rose, A. Masion, F. Ziarelli, et al.. Structural Degradation at the Surface of a TiO2-Based Nanomaterial Used in Cosmetics. Environmental Science and Technology, American Chemical Society, 2010, 44 (7), pp.2689-2694. (10.1021/es903757q).

Auten, R., Davis, J. 2009. "Oxygen Toxicity and Reactive Oxygen Species: The Devil Is in the Details." Pediatr Res 66, 121–127. https://doi.org/10.1203/PDR.0b013e3181a9eafb

Azqueta, A., & Dusinska, M. 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in genetics, 6, 239.

Azqueta A, Enciso JM, Pastor L, López de Cerain A, Vettorazzi A. 2019. "Applying the comet assay to fresh vs frozen animal solid tissues: A technical approach." Food Chem Toxicol. 132:110671. doi: 10.1016/j.fct.2019.110671. Epub 2019 Jul 10. PMID: 31301324.

BAuA, 2017, Methodology for the Identification of Granular Biopersistent Particles (GBP) at Workplaces" – Project F 2336, doi:10.21934/baua:bericht20170419

BAuA, 2018, Comparison of Inhalation and Intratracheal Instillation as Testing Methods for Characterisation of Granular Biopersistent Particles (GBP)" – Project F 2364, doi:10.21934/baua:bericht20180823

Bermudez, E., J. B. Mangum, B. A. Wong, B. Asgharian, P. M. Hext, D. B. Warheit, and J. I. Everitt. 2004. "Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles." *Toxicological Sciences* 77 (2):347-357. doi: 10.1093/toxsci/kfh019.

Bernard BK, Osheroff MR, Hofmann A, Mennear JH (1990): Toxicology and Carcinogenesis Studies of Dietary Titanium Dioxide-coated Mica in Male and Female Fischer 344 Rats, Journal of Toxicology and Environmental Health, 29:417-429. doi: 10.1080/15287399009531402. PMID: 2325155.

Bornholdt J, Saber AT, Sharma AK, Savolainen K, Vogel U, Wallin H. 2007. Inflammatory response and genotoxicity of seven wood dusts in the human epithelial cell line A549. Mutat Res 632:78–88.

Brezová V, Gabcová S, Dvoranová D, Stasko A. Reactive oxygen species produced upon photoexcitation of sunscreens containing titanium dioxide (an EPR study). J Photochem Photobiol B. 2005 May 13;79(2):121-34. doi: 10.1016/j.jphotobiol.2004.12.006. PMID: 15878117.

Carriere M, Arnal ME, Douki T. TiO2 genotoxicity: An update of the results published over the last six years. Mutat Res. 2020 Jun-Jul;854-855:503198. doi: 10.1016/j.mrgentox.2020.503198. Epub 2020 May 15. PMID: 32660822.

Chang, X., Y. Xie, J. Wu, M. Tang, and B. Wang. 2015. "Toxicological Characteristics of Titanium Dioxide Nanoparticle in Rats." J Nanosci Nanotechnol 15 (2):1135-42.

Charles, S., S. Jomini, V. Fessard, E. Bigorgne-Vizade, C. Rousselle, and C. Michel. 2018. "Assessment of the in vitro genotoxicity of TiO2 nanoparticles in a regulatory context." Nanotoxicology 12 (4):357-374. doi: 10.1080/17435390.2018.1451567.

Chen, T., J. Hu, C. Chen, J. Pu, X. Cui, and G. Jia. 2013. "Cardiovascular effects of pulmonary exposure to titanium dioxide nanoparticles in ApoE knockout mice." J Nanosci Nanotechnol 13 (5):3214-22.

Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 2004 Mar;26(3):249-61. doi: 10.1385/MB:26:3:249. PMID: 15004294.

Collins AR, El Yamani N, Lorenzo Y, Shaposhnikov S, Brunborg G, Azqueta A. Controlling variation in the comet assay. Front Genet. 2014;5:359. Published 2014 Oct 20. doi:10.3389/fgene.2014.00359

Collins, A., Vettorazzi, A., Azqueta, A. 2020. The role of the enzyme-modified comet assay in *in vivo* studies. Toxicology Letters 327:58-68.

Csonka, C. Páli, T. Bencsik, P. Görbe, A. Ferdinandy, P. Csont, T. 2015. Measurement of NO in biological samples. British Journal of Pharmacology, Volume 172, Issue 6, March 2015, Pages 1620-1632. DOI:10.1111/bph.12832

Danielsen, P. H., Knudsen, K. B., Štrancar, J., Umek, P., Koklič, T., Garvas, M., Vanhala, E., Savukoski, S., Ding, Y., Madsen, A. M., Jacobsen, N. R., Weydahl, I. K., Berthing, T., Poulsen, S. S., Schmid, O., Wolff, H., & Vogel, U. 2020. "Effects of physicochemical properties of TiO2 nanomaterials for pulmonary inflammation, acute phase response and alveolar proteinosis in intratracheally exposed mice." Toxicology and applied pharmacology, 386, 114830. https://doi.org/10.1016/j.taap.2019.114830

David SS, Williams SD. Chemistry of Glycosylases and Endonucleases Involved in Base-Excision Repair. Chem Rev. 1998 May 7;98(3):1221-1262. doi: 10.1021/cr980321h. PMID: 11848931.

Di Bucchianico S, Cappellini F, Le Bihanic F, Zhang Y, Dreij K, Karlsson HL 2017. Genotoxicity of TiO2 Nanoparticles Assessed by Mini-Gel Comet Assay and Micronucleus Scoring with Flow Cytometry. Mutagenesis 32 (1): 127–37. doi:10.1093/mutage/gew030.

Disdier, C., M. Chalansonnet, F. Gagnaire, L. Gaté, F. Cosnier, J. Devoy, W. Saba, A. K. Lund, E. Brun, and A. Mabondzo. 2017. "Brain Inflammation, Blood Brain Barrier dysfunction and Neuronal Synaptophysin Decrease after Inhalation Exposure to Titanium Dioxide Nano-aerosol in Aging Rats." *Scientific Reports* 7 (1). doi: 10.1038/s41598-017-12404-5

ECHA
EUROPEAN CHEMICALS AGENCY

Drew, Nathan M., Eileen D. Kuempel, Ying Pei, and Feng Yang. 2017. "A quantitative framework to group nanoscale and microscale particles by hazard potency to derive occupational exposure limits: Proof of concept evaluation." *Regulatory Toxicology and Pharmacology* 89:253-267. doi:

[redacted] 2011: [redacted]: Subchronic Toxicity, 90-Day Gavage Study in Rats, [redacted]

ECHA, 2019, Decision On Substance Evaluation on potassium titanium oxide (EC 432-240-0), https://echa.europa.eu/documents/10162/a0861425-bee5-8d29-f2cf-c2f6bdd8b4ea.

Elgrabli D, Beaudouin R, Jbilou N, Floriani M, Pery A, Rogerieux F, et al. (2015) Biodistribution and Clearance of TiO2 Nanoparticles in Rats after Intravenous Injection. PLoS ONE 10(4): e0124490. doi:10.1371/journal.pone.0124490

El Yamani N, Collins AR, Rundén-Pran E, Fjellsbø LM, Shaposhnikov S, Zienolddiny S, Dusinska M. In vitro genotoxicity testing of four reference metal nanomaterials, titanium dioxide, zinc oxide, cerium oxide and silver: towards reliable hazard assessment. Mutagenesis. 2017 Jan; 32(1):117-126. doi: 10.1093/mutage/gew060. Epub 2016 Nov 12. Erratum in: Mutagenesis. 2017 May 1;32(3):409. PMID: 27838631.

Esteves, F., Amaro, R., Silva, S., Sánchez-Flores, M., Teixeira, J.P., Costa, C. 2020. "The impact of comet assay data normalization in human biomonitoring studies outcomes." Toxicology letters. Volume 332, Pages 56-64

Fadda L. M., Hagar H., Mohamed A. M., and Ali H. M. (2018). Quercetin and Idebenone Ameliorate Oxidative Stress, Inflammation, DNA damage, and Apoptosis Induced by Titanium Dioxide Nanoparticles in Rat Liver. Dose-Response: An International Journal October-December 2018:1-9

Fenoglio, I., Greco, G., Livraghi, S., Fubini, B., 2009. Non-UV-induced radical reactions at the surface of TiO2 nanoparticles that may trigger toxic responses. Chemistry 15, 4614–4621

Fernández-Bertólez N, Costa C, Bessa MJ, Park M, Carriere M, Dussert F, Teixeira JP, Pásaro E, Laffon B, Valdiglesias V. Assessment of oxidative damage induced by iron oxide nanoparticles on different nervous system cells. Mutat Res. 2019 Sep;845:402989. doi: 10.1016/j.mrgentox.2018.11.013. Epub 2018 Nov 30. PMID: 31561889.

Fu, P. P., Xia, Q., Hwang, H. M., Ray, P. C., & Yu, H. 2014. "Mechanisms of nanotoxicity: generation of reactive oxygen species." Journal of food and drug analysis, 22(1), 64-75.

Gaté, L., Disdier, C., Cosnier, F., Gagnaire, F., Devoy, J., Saba, W., Brun, E., Chalansonnet, M., & Mabondzo, A. 2017. "Biopersistence and translocation to extrapulmonary organs of titanium dioxide nanoparticles after subacute inhalation exposure to aerosol in adult and elderly rats." Toxicology letters, 265, 61–69. https://doi.org/10.1016/j.toxlet.2016.11.009

Gaté L., Knudsen K.B., Seidel C., Berthing T., Chézeau L., Jacobsen N.R., Valentino S., Wallin H., Bau S., Wolff H., Sébillaud S., Lorcin M., Grossmann S., Viton S., Nunge H., Darne C., Vogel U., Cosnier F., 2019. Pulmonary toxicity of two different multi-walled carbon nanotubes in rat: Comparison between intratracheal instillation and inhalation exposure. Toxicol Appl Pharmacol. 2019 Jul 15; 375:17-31. Spanish. doi: 10.1016/j.taap.2019.05.001. Epub 2019 May 7. PMID: 31075343.

Golia, E., Limongelli, G., Natale, F., Fimiani, F., Maddaloni, V., Pariggiano, I., Bianchi, R., Crisci, M., D'Acierno, L., Giordano, R., Di Palma, G., Conte, M., Golino, P., Russo, M.G., Calabrò, R. and Calabrò, P. 2014. Inflammation and cardiovascular dis- ease: from pathogenesis to therapeutic target. Curr. Atheroscler. Rep., 16, 435.

Gurr, J., Wang, A.S.S., Chen, C., Jan, K., 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 213, 66–73

Halappanavar, S., A. T. Saber, N. Decan, K. A. Jensen, D. Wu, N. R. Jacobsen, C. Guo, J. Rogowski, I. K. Koponen, M. Levin, A. M. Madsen, R. Atluri, V. Snitka, R. K. Birkedal, D. Rickerby, A. Williams, H. Wallin, C. L. Yauk, and U. Vogel. 2015. "Transcriptional profiling identifies physicochemical properties of nanomaterials that are determinants of the in vivo pulmonary response." *Environ Mol Mutagen* 56 (2):245-64. doi: 10.1002/em.21936.

Hamilton, R. F., N. Wu, D. Porter, M. Buford, M. Wolfarth, and A. Holian. 2009. "Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity." Part Fibre Toxicol 6:35. doi: 10.1186/1743-8977-6-35.

Hartmann A., Agurell E., Beevers C., Brendler-Schwaab S., Burlinson B., Clay P., Collins A., Smith A., Speit G., Thybaud V., Tice, 2003 R.R.. Recommendations for conducting the in vivo alkaline Comet assay, Mutagenesis, Volume 18, Issue 1, January 2003, Pages 45–51, https://doi.org/10.1093/mutage/18.1.45

Hashizume, N., Y. Oshima, M. Nakai, T. Kobayashi, T. Sasaki, K. Kawaguchi, K. Honda, M. Gamo, K. Yamamoto, Y. Tsubokura, S. Ajimi, Y. Inoue, and N. Imatanaka. 2016. "Categorization of nano-structured titanium dioxide according to physicochemical characteristics and pulmonary toxicity." Toxicol Rep 3:490-500. doi: 10.1016/j.toxrep.2016.05.005.

Hong, F.; Zhao, X.; Si, W.; Ze, Y.; Wang, L.; Zhou, Y.; Hong, J.; Yu, X.; Sheng, L.; Liu, D.; Xu, B.; Zhang, J. 2015. "Decreased Spermatogenesis Led to Alterations of Testis-Specific Gene Expression in Male Mice Following NanoTiO2 Exposure." J. Hazard. Mater. 300, 718–728.

Huang, K. T., C. T. Wu, K. H. Huang, W. C. Lin, C. M. Chen, S. S. Guan, C. K. Chiang, and S. H. Liu. 2015. "Titanium nanoparticle inhalation induces renal fibrosis in mice via an oxidative stress upregulated transforming growth factor-beta pathway." *Chem Res Toxicol* 28 (3):354-64. doi: 10.1021/tx500287f.

Hubbard, A.K. and Rothlein, R. 2000. Intercellular adhesion mol- ecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic. Biol. Med., 28, 1379-1386.

Husain, M., Wu, D., Saber, A. T., Decan, N., Jacobsen, N. R., Williams, A., Yauk, C. L., Wallin, H., Vogel, U., & Halappanavar, S. 2015. "Intratracheally instilled titanium dioxide nanoparticles translocate to heart and liver and activate complement cascade in the heart of C57BL/6 mice." Nanotoxicology, 9(8), 1013–1022. https://doi.org/10.3109/17435390.2014.996192

Hussain, S., Thomassen, L.C., Ferecatu, I., Borot, M.C., Andreau, K., Martens, J.A., Fleury, J., Baeza-Squiban, A., Marano, F., Boland, S., 2010. Carbon black and titanium dioxide nanoparticles elicit distinct apoptotic pathways in bronchial epithelial cells. Particle and Fibre Toxicology 7, 10.

Iglesias, T., Irache, J. M., Butinar, M., Turk, B., de Cerain, A. L., & Azqueta, A. 2017a. Genotoxic evaluation of poly (anhydride) nanoparticles in the gastrointestinal tract of mice. International journal of pharmaceutics, 530(1-2), 187-194.

Iglesias, T., Dusinska, M., El Yamani, N., Irache, J. M., Azqueta, A., & de Cerain, A. L. 2017b. In vitro evaluation of the genotoxicity of poly (anhydride) nanoparticles

designed for oral drug delivery. International journal of pharmaceutics, 523(1), 418-426.

Jackson P, Pedersen LM, Kyjovoska ZO, Jackobsen NR, Saber AT, Hougaard KS, Vogel U, Wallin H (2013). Validation of freezing tissues and cells for analysis of DNA strand break levels by comet assay. *Mutagenesis*, Vol. 28(6): 699-707.

Jafari, A.; Karimipour, M.; Khaksar, M. R.; Ghasemnejad-Berenji, M. 2020. "Protective Effects of Orally Administered Thymol against Titanium Dioxide Nanoparticle-Induced Testicular Damage." Environ Sci Pollut Res Int, 27 (2), 2353–2360.

Jalili, P., Huet, S., Lanceleur, R., Jarry, G., Le Hegarat, L., Nesslany, F., ... & Fessard, V. 2020. Genotoxicity of aluminum and aluminum oxide nanomaterials in rats following oral exposure. Nanomaterials, 10(2), 305.

Jia F, Sun Z, Yan X, Zhou B, Wang J. Effect of pubertal nano-TiO2 exposure on testosterone synthesis and spermatogenesis in mice. Arch Toxicol. 2014 Mar;88(3):781-8. doi: 10.1007/s00204-013-1167-5. Epub 2013 Nov 19. PMID: 24241477.

Johansson C, Møller P, Forchhammer L, Loft S, Godschalk RW, Langie SA, Lumeij S, Jones GD, Kwok RW, Azqueta A, Phillips DH, Sozeri O, Routledge MN, Charlton AJ, Riso P, Porrini M, Allione A, Matullo G, Palus J, Stepnik M, Collins AR, Möller L. An ECVAG trial on assessment of oxidative damage to DNA measured by the comet assay. Mutagenesis. 2010 Mar;25(2):125-32. doi: 10.1093/mutage/gep055. Epub 2009 Nov 30. PMID: 19948595; PMCID: PMC2825342.

Kain, J., H. L. Karlsson, and L. Möller. 2012. "DNA Damage Induced by Micro- and Nanoparticles—interaction with FPG Influences the Detection of DNA Oxidation in the Comet Assay." Mutagenesis 27 (4): 491–500. doi:10.1093/mutage/ges010.

Knuckles, T. L., J. Yi, D. G. Frazer, H. D. Leonard, B. T. Chen, V. Castranova, and T. R. Nurkiewicz. 2012. "Nanoparticle inhalation alters systemic arteriolar vasoreactivity through sympathetic and cyclooxygenase-mediated pathways." *Nanotoxicology* 6 (7):724-35. doi: 10.3109/17435390.2011.606926.

Kobayashi, N., M. Naya, S. Endoh, J. Maru, K. Yamamoto, and J. Nakanishi. 2009. "Comparative pulmonary toxicity study of nano-TiO(2) particles of different sizes and agglomerations in rats: different short- and long-term post-instillation results." *Toxicology* 264 (1-2):110-8. doi: 10.1016/j.tox.2009.08.002.

Koenig W, Khuseyinova N, Baumert J, Thorand B, Loewel H, Chambless L, Meisinger C, Schneider A, Martin S, Kolb H, Herder C. Increased concentrations of C-reactive protein and IL-6 but not IL-18 are independently associated with incident coronary events in middle-aged men and women: results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. Arterioscler Thromb Vasc Biol. 2006 Dec;26(12):2745-51. doi: 10.1161/01.ATV.0000248096.62495.73. Epub 2006 Sep 28. PMID: 17008587.

Kohl, Y., Rundén-Pran, E., Mariussen, E., Hesler, M., El Yamani, N., Longhin, E. M., & Dusinska, M., 2020. Genotoxicity of Nanomaterials: Advanced In Vitro Models and High Throughput Methods for Human Hazard Assessment—A Review. *Nanomaterials*, *10*(10), 1911.

Korkmaz K. S., Butuner B. D., Roggenbuck D. 2018. Detection of 8-OHdG as a diagnostic biomarker. J Lab Precis Med;3:95

Kreyling et al. (2017a). Quantitative biokinetics of titanium dioxide nanoparticles after oral application in rats: Part 2, NANOTOXICOLOGY, 2017, VOL. 11, NO. 4, 443–453 http://dx.doi.org/10.1080/17435390.2017.1306893

ECHA
EUROPEAN CHEMICALS AGENCY

Kreyling et al (2017b): Quantitative biokinetics of titanium dioxide nanoparticles after intratracheal instillation in rats: Part 3, NANOTOXICOLOGY, 2017 http://dx.doi.org/10.1080/17435390.2017.1306894

Labille, J., J. Feng, C. Botta, D. Borschneck, M. Sammut, et al.. Aging of TiO2 nanocomposites used in sunscreen. Dispersion and fate of the degradation products in aqueous environment. Environmental Pollution, Elsevier, 2010, 158 (12), pp.3482-3489. ⟨10.1016/j.envpol.2010.02.012⟩. ⟨hal-01519536⟩

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

Larsen, S. T., P. Jackson, S. S. Poulsen, M. Levin, K. A. Jensen, H. Wallin, G. D. Nielsen, and I. K. Koponen. 2016. "Airway irritation, inflammation, and toxicity in mice following inhalation of metal oxide nanoparticles." Nanotoxicology 10 (9):1254-1262. doi: 10.1080/17435390.2016.1202350.

LeBlanc, A. J., J. L. Cumpston, B. T. Chen, D. Frazer, V. Castranova, and T. R. Nurkiewicz. 2009. "Nanoparticle inhalation impairs endothelium-dependent vasodilation in subepicardial arterioles." *J Toxicol Environ Health A* 72 (24):1576-84. doi: 10.1080/15287390903232467.

LeBlanc, A. J., A. M. Moseley, B. T. Chen, D. Frazer, V. Castranova, and T. R. Nurkiewicz. 2010. "Nanoparticle inhalation impairs coronary microvascular reactivity via a local reactive oxygen species-dependent mechanism." *Cardiovasc Toxicol* 10 (1):27-36. doi: 10.1007/s12012-009-9060-4.

Li, J., Q. Li, J. Xu, J. Li, X. Cai, R. Liu, Y. Li, J. Ma, and W. Li. 2007. "Comparative study on the acute pulmonary toxicity induced by 3 and 20 nm TiO2 primary particles in mice." Environmental Toxicology and Pharmacology 24 (3):239-244. doi: 10.1016/j.etap.2007.06.004.

Magdolenova, Z., Lorenzo, Y., Collins, A., & Dusinska, M. 2012. Can standard genotoxicity tests be applied to nanoparticles?. Journal of Toxicology and Environmental Health, Part A, 75(13-15), 800-806.

Magnusson P, Dziendzikowska K, Oczkowski M, Øvrevik J, Eide DM, Brunborg G, Gutzkow KB, Instanes C, Gajewska M, Wilczak J, Sapierzynski R, Kamola D, Królikowski T, Kruszewski M, Lankoff A, Mruk R, Duale N, Gromadzka-Ostrowska J, Myhre O. Lung effects of 7- and 28-day inhalation exposure of rats to emissions from 1st and 2nd generation biodiesel fuels with and without particle filter - The FuelHealth project. Environ Toxicol Pharmacol. 2019 Apr;67:8-20. doi: 10.1016/j.etap.2019.01.005. Epub 2019 Jan 18. PMID: 30685595.

Ma-Hock, L., S. Burkhardt, V. Strauss, A. O. Gamer, K. Wiench, B. van Ravenzwaay, and R. Landsiedel. 2009. "Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance." *Inhal Toxicol* 21 (2):102-18. doi: 10.1080/08958370802361057.

Meena, R., Rani, M., Pal, R. et al. Nano-TiO2-Induced Apoptosis by Oxidative Stress-Mediated DNA Damage and Activation of p53 in Human Embryonic Kidney Cells. Appl Biochem Biotechnol 167, 791–808 (2012). https://doi.org/10.1007/s12010-012-9699-3 Journal of Biomedical Nanotechnology 7, 100–101.

Modrzynska, J., Berthing, T., Ravn-Haren, G., Jacobsen, N. R., Weydahl, I. K., Loeschner, K., Mortensen, A., Saber, A. T., & Vogel, U. 2018a. Primary genotoxicity in the liver following pulmonary exposure to carbon black nanoparticles in mice. Particle and fibre toxicology, 15(1), 2. https://doi.org/10.1186/s12989-017-0238-9

ECHA
EUROPEAN CHEMICALS AGENCY

Modrzynska, J., Berthing, T., Ravn-Haren, G., Kling, K., Mortensen, A., Rasmussen, R. R., Larsen, E. H., Saber, A. T., Vogel, U., and Loeschner, K. 2018b. "In vivo-induced size transformation of cerium oxide nanoparticles in both lung and liver does not affect long-term hepatic accumulation following pulmonary exposure." PloS one, 13(8), e0202477. https://doi.org/10.1371/journal.pone.0202477

Møller, P., D. M. Jensen, D. V. Christophersen, A. Kermanizadeh, N. R. Jacobsen, J. G. Hemmingsen, P. H. Danielsen, D. G. Karottki, M. Roursgaard, Y. Cao, K. Jantzen, H. Klingberg, L. G. Hersoug, and S. Loft. 2015. "Measurement of oxidative damage to DNA in nanomaterial exposed cells and animals." Environ Mol Mutagen 56 (2):97-110. doi: 10.1002/em.21899.

Møller, P., D. M. Jensen, R. S. Wils, M. H. G. Andersen, P. H. Danielsen, and M. Roursgaard. 2017a. "Assessment of evidence for nanosized titanium dioxide-generated DNA strand breaks and oxidatively damaged DNA in cells and animal models." Nanotoxicology 11 (9-10):1237-1256. doi: 10.1080/17435390.2017.1406549.

Møller P, Jantzen K, Løhr M, Andersen MH, Jensen DM, Roursgaard M, Høgh Danielsen P, Jensen A, Loft S. 2018. "Searching for assay controls for the Fpg- and hOGG1-modified comet assay." Mutagenesis 33 (1):9-19. doi: 10.1093/mutage/gex015.

Muruzabal, D., Collins, A., & Azqueta, A. 2020. "The enzyme-modified comet assay: past, present and future." Food and Chemical Toxicology, 111865.

Nanogenotox. 2013. https://www.anses.fr/en/content/nanogenotox-final-report

NCI (1979): Bioassay of Titanium Dioxide for possible Carcinogenicity, CAS No. 13463-67-7, NCI-CG-TR-9, National Cancer Institute, Technical Report Series No. 97

Noel, A., M. Charbonneau, Y. Cloutier, R. Tardif, and G. Truchon. 2013. "Rat pulmonary responses to inhaled nano-TiO(2): effect of primary particle size and agglomeration state." Part Fibre Toxicol 10:48. doi: 10.1186/1743-8977-10-48.

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

Nurkiewicz, T. R., D. W. Porter, A. F. Hubbs, J. L. Cumpston, B. T. Chen, D. G. Frazer, and V. Castranova. 2008. "Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction." *Particle and Fibre Toxicology* 5. doi: 10.1186/1743-8977-5-1.

Nurkiewicz, T. R., D. W. Porter, A. F. Hubbs, S. Stone, B. T. Chen, D. G. Frazer, M. A. Boegehold, and V. Castranova. 2009. "Pulmonary nanoparticle exposure disrupts systemic microvascular nitric oxide signaling." Toxicol Sci 110 (1):191-203. doi: 10.1093/toxsci/kfp051.

Oberdörster G, Finkelstein JN, Johnston C, Gelein R, Cox C, Baggs R, Elder ACP. 2000. Acute Pulmonary Effects of Ultrafine Paricles in Rats and Mice. Research Report 96. Health Effects Institute, Cambridge MA.

OECD. 2018a. CASE STUDY ON GROUPING AND READ-ACROSS FOR NANOMATERIALS GENOTOXICITY OF NANO- TiO2.

OECD. 2018b. Guidance document on inhalation toxicity studies. In Series on Testing and Assessment

Okada, T., A. Ogami, B. W. Lee, C. Kadoya, T. Oyabu, and T. Myojo. 2016. "Pulmonary responses in rat lungs after intratracheal instillation of 4 crystal forms of titanium dioxide nanoparticles." *J Occup Health* 58 (6):602-611. doi: 10.1539/joh.16-0094-OA.

ECHA
EUROPEAN CHEMICALS AGENCY

Oyabu, T., T. Myojo, B. W. Lee, T. Okada, H. Izumi, Y. Yoshiura, T. Tomonaga, Y. S. Li, K. Kawai, M. Shimada, M. Kubo, K. Yamamoto, K. Kawaguchi, T. Sasaki, and Y. Morimoto. 2017. "Biopersistence of NiO and TiO2 nanoparticles following intratracheal instillation and inhalation." *International Journal of Molecular Sciences* 18 (12). doi: 10.3390/ijms18122757.

Park, E.J., Yoon, J., Choi, K., Yi, J. and Park, K. 2009. Induction of chronic inflammation in mice treated with titanium dioxide nanoparticles by intratracheal instillation. Toxicology, 260, 37- 46.

Pfuhler, S., Downs, T. R., Allemang, A. J., Shan, Y., & Crosby, M. E. 2017. Weak silica nanomaterial-induced genotoxicity can be explained by indirect DNA damage as shown by the OGG1-modified comet assay and genomic analysis. Mutagenesis, 32(1), 5-12.

Porter, D. W., N. Wu, A. F. Hubbs, R. R. Mercer, K. Funk, F. Meng, J. Li, M. G. Wolfarth, L. Battelli, S. Friend, M. Andrew, R. Hamilton, Jr., K. Sriram, F. Yang, V. Castranova, and A. Holian. 2013. "Differential mouse pulmonary dose and time course responses to titanium dioxide nanospheres and nanobelts." Toxicol Sci 131 (1):179-93. doi: 10.1093/toxsci/kfs261.

Pothmann, D., Simar, S., Schuler, D. *et al.* Lung inflammation and lack of genotoxicity in the comet and micronucleus assays of industrial multiwalled carbon nanotubes Graphistrength© C100 after a 90-day nose-only inhalation exposure of rats. *Part Fibre Toxicol* **12,** 21 (2015). https://doi.org/10.1186/s12989-015-0096-2

Pujalté, I., Dieme, D., Haddad, S., Serventi, A. M., & Bouchard, M. 2017. "Toxicokinetics of titanium dioxide (TiO2) nanoparticles after inhalation in rats." Toxicology letters, 265, 77–85. https://doi.org/10.1016/j.toxlet.2016.11.014

Rahman, L., D. Wu, M. Johnston, A. William, and S. Halappanavar. 2017. "Toxicogenomics analysis of mouse lung responses following exposure to titanium dioxide nanomaterials reveal their disease potential at high doses." Mutagenesis 32 (1):59-76. doi: 10.1093/mutage/gew048.

Recio L, Hobbs C, Caspary W, Witt KL (2010). Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol. J Toxicol Sci. 35(2):149-62. doi: 10.2131/jts.35.149. PMID: 20371966; PMCID: PMC3520611.

Recio L, Kissling GE, Hobbs CA, Witt KL. (2012). Comparison of Comet assay dose-response for ethyl methanesulfonate using freshly prepared versus cryopreserved tissues. *Environmental and molecular mutagenesis*, *53*(2), 101-113.

Reddy, M. P., Venugopal, A., & Subrahmanyam, M. (2007). Hydroxyapatite-supported Ag–TiO2 as Escherichia coli disinfection photocatalyst. *Water research*, *41*(2), 379-386.

Relier, C., M. Dubreuil, O. Lozano Garcia, E. Cordelli, J. Mejia, P. Eleuteri, F. Robidel, T. Loret, F. Pacchierotti, S. Lucas, G. Lacroix, and B. Trouiller. 2017. "Study of TiO2 P25 Nanoparticles Genotoxicity on Lung, Blood, and Liver Cells in Lung Overload and Non-Overload Conditions After Repeated Respiratory Exposure in Rats." Toxicol Sci 156 (2):527-537. doi: 10.1093/toxsci/kfx006.

Ridker, P. M. 2001. High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. Circulation, 103(13), 1813-1818.

Rizk MZ, Ali SA, Kadry MO, Fouad GI, Kamel NN, Younis EA, Gouda SM. C-reactive Protein Signaling and Chromosomal Abnormalities in Nanotoxicity Induced via Different Doses of TiO2 (80 nm) Boost Liver Function. Biol Trace Elem Res. 2020

Nov;198(1):157-167. doi: 10.1007/s12011-020-02030-0. Epub 2020 Feb 3. PMID: 32016825.

Rossi, E. M., L. Pylkkänen, A. J. Koivisto, M. Vippola, K. A. Jensen, M. Miettinen, K. Sirola, H. Nykäsenoja, P. Karisola, T. Stjernvall, E. Vanhala, M. Kiilunen, P. Pasanen, M. Mäkinen, K. Hämeri, J. Joutsensaari, T. Tuomi, J. Jokiniemi, H. Wolff, K. Savolainen, S. Matikainen, and H. Alenius. 2009. "Airway exposure to silica-coated TiO2 nanoparticles induces pulmonary neutrophilia in mice." Toxicological Sciences 113 (2):422-433. doi: 10.1093/toxsci/kfp254.

Roursgaard, M., K. A. Jensen, S. S. Poulsen, N. E. Jensen, L. K. Poulsen, M. Hammer, G. D. Nielsen, and S. T. Larsen. 2011. "Acute and subchronic airway inflammation after intratracheal instillation of quartz and titanium dioxide agglomerates in mice." ScientificWorldJournal 11:801-25. doi: 10.1100/tsw.2011.67.

Saber AT, Bornholdt J, Dybdahl M, Sharma AK, Loft S, Vogel U, Wallin H. 2005. Tumor necrosis factor is not required for particle-induced genotoxicity and pulmonary inflammation. Arch Toxicol 79:177–182.

Saber, A. T., Jensen, K. A., Jacobsen, N. R., Birkedal, R., Mikkelsen, L., Møller, P., ... & Vogel, U., 2012a. Inflammatory and genotoxic effects of nanoparticles designed for inclusion in paints and lacquers. Nanotoxicology, 6(5), 453-471.

Saber, A. T., Koponen, I. K., Jensen, K. A., Jacobsen, N. R., Mikkelsen, L., Møller, P., ... & Wallin, H., 2012b. Inflammatory and genotoxic effects of sanding dust generated from nanoparticle-containing paints and lacquers. Nanotoxicology, 6(7), 776-788.

Saber, Anne Thoustrup, Jacob Stuart Lamson, Nicklas Raun Jacobsen, Gitte Ravn-Haren, Karin Sørig Hougaard, Allen Njimeri Nyendi, Pia Wahlberg, Anne Mette Madsen, Petra Jackson, Håkan Wallin, and Ulla Vogel. 2013. "Particle-Induced Pulmonary Acute Phase Response Correlates with Neutrophil Influx Linking Inhaled Particles and Cardiovascular Risk." PLOS ONE 8 (7):e69020. doi: 10.1371/journal.pone.0069020.

Sager, T. M., and V. Castranova. 2009. "Surface area of particle administered versus mass in determining the pulmonary toxicity of ultrafine and fine carbon black: comparison to ultrafine titanium dioxide." Part Fibre Toxicol 6:15. doi: 10.1186/1743-8977-6-15.

Saquib, Q., Al-Khedhairy, A.A., Siddiqui, M.A., Abou-Tarboush, F.M., Azam, A., Musarrat, J., 2012. Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. Toxicology In Vitro 26, 351–361.

Shahin NN, Mohamed MM. Nano-sized titanium dioxide toxicity in rat prostate and testis: Possible ameliorative effect of morin. Toxicol Appl Pharmacol. 2017 Nov 1;334:129-141. doi: 10.1016/j.taap.2017.08.014. Epub 2017 Aug 26. PMID: 28844848.

Shukla, R.K., Kumar, A., Pandey, A.K., Singh, S.S., Dhawan, A., 2011. Titanium dioxide nanoparticles induce oxidative stress-mediated apoptosis in human keratinocyte cells.

Silva, R. M., C. Teesy, L. Franzi, A. Weir, P. Westerhoff, J. E. Evans, and K. E. Pinkerton. 2013. "Biological response to nano-scale titanium dioxide (TiO2): role of particle dose, shape, and retention." J Toxicol Environ Health A 76 (16):953-72. doi: 10.1080/15287394.2013.826567.

Smith CC, O'Donovan MR, Martin EA., 2006. "hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII." Mutagenesis. 21(3):185-90. doi: 10.1093/mutage/gel019. Epub 2006 Apr 5. PMID: 16597659.

Song, G.; Lin, L.; Liu, L.; Wang, K.; Ding, Y.; Niu, Q.; Mu, L.; Wang, H.; Shen, H.; Guo, S., 2017. "Toxic Effects of Anatase Titanium Dioxide Nanoparticles on Spermatogenesis and Testicles in Male Mice." Polish Journal of Environmental Studies. 26 (6), 2739–2746.

Song, G., Lin, L., Liu, L., Wang, K., Ding, Y., Niu, Q., Mu, L., Wang, H., Shen, H., and Guo, S. 2017. Toxic Effects of Anatase Titanium Dioxide Nanoparticles on Spermatogenesis and Testicles in Male Mice. Polish Journal of Environmental Studies, 26(6), pp.2739-2745. https://doi.org/10.15244/pjoes/70807

Speit G, Kojima H, Burlinson B, Collins AR, Kasper P, Plappert-Helbig U et al. (2015). Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT); Mutation Research 783: 6–12.

Speit, G., Schütz, P., Bonzheim, I., Trenz, K., & Hoffmann, H. 2004. "Sensitivity of the FPG protein towards alkylation damage in the comet assay." Toxicology letters, 146(2), 151-158.

Stapleton, P. A., V. C. Minarchick, J. Yi, K. Engels, C. R. McBride, and T. R. Nurkiewicz. 2013. "Maternal engineered nanomaterial exposure and fetal microvascular function: does the Barker hypothesis apply?" *Am J Obstet Gynecol* 209 (3):227.e1-11. doi: 10.1016/j.ajog.2013.04.036.

Stapleton, P. A., C. E. Nichols, J. Yi, C. R. McBride, V. C. Minarchick, D. L. Shepherd, J. M. Hollander, and T. R. Nurkiewicz. 2015. "Microvascular and mitochondrial dysfunction in the female F1 generation after gestational TiO2 nanoparticle exposure." Nanotoxicology 9 (8):941-51. doi: 10.3109/17435390.2014.984251.

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. 2014. "Oral, Short-Term Exposure to Titanium Dioxide Nanoparticles in Sprague-Dawley Rat: Focus on Reproductive and Endocrine Systems and Spleen." Nanotoxicology. 8 (6), 654–662.

Wallin, H., Z. O. Kyjovska, S. S. Poulsen, N. R. Jacobsen, A. T. Saber, S. Bengtson, P. Jackson, and U. Vogel. 2017. "Surface modification does not influence the genotoxic and inflammatory effects of TiO2 nanoparticles after pulmonary exposure by instillation in mice." Mutagenesis 32 (1):47-57. doi: 10.1093/mutage/gew046.

Wang, J.J., Sanderson, B.J.S., Wang, H., 2007. Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. Mutat. Res. Toxicol. Environ. Mutagen. 628, 99–106. doi:10.1016/j.mrgentox.2006.12.003

Wang, J., C. Chen, Y. Liu, F. Jiao, W. Li, F. Lao, Y. Li, B. Li, C. Ge, G. Zhou, Y. Gao, Y. Zhao, and Z. Chai. 2008a. "Potential neurological lesion after nasal instillation of TiO(2) nanoparticles in the anatase and rutile crystal phases." Toxicol *Lett* 183 (1-3):72-80. doi: 10.1016/j.toxlet.2008.10.001.

Wang, J., Y. Liu, F. Jiao, F. Lao, W. Li, Y. Gu, Y. Li, C. Ge, G. Zhou, B. Li, Y. Zhao, Z. Chai, and C. Chen. 2008b. "Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO(2) nanoparticles." *Toxicology* 254 (1-2):82-90. doi: 10.1016/j.tox.2008.09.014.

Warheit, D. B., T. R. Webb, C. M. Sayes, V. L. Colvin, and K. L. Reed. 2006a. "Pulmonary instillation studies with nanoscale TiO2 rods and dots in rats: toxicity is not dependent upon particle size and surface area." Toxicol Sci 91 (1):227-36. doi: 10.1093/toxsci/kfj140.

Warheit D.B., Webb T.R., Reed K.L. 2006b. Pulmonary toxicity screening studies in male rats with TiO2 particulates substantially encapsulated with pyrogenically deposited,

amorphous silica. Part Fibre Toxicol. 2006;3:3. Published 2006 Jan 26. doi:10.1186/1743-8977-3-3

Warheit, D. B., T. R. Webb, K. L. Reed, S. Frerichs, and C. M. Sayes. 2007. "Pulmonary toxicity study in rats with three forms of ultrafine-TiO2 particles: differential responses related to surface properties." *Toxicology* 230 (1):90-104. doi: 10.1016/j.tox.2006.11.002.

Yokohira M, Kuno T, Yamakawa K, Hosokawa K, Matsuda Y, Hashimoto N, Suzuki S, Saoo K, Imaida K. Lung toxicity of 16 fine particles on intratracheal instillation in a bioassay model using f344 male rats. Toxicol Pathol. 2008 Jun;36(4):620-31. doi: 10.1177/0192623308318214. Epub 2008 May 8. PMID: 18467675.

Yousuf, O., Mohanty, B.D., Martin, S.S., Joshi, P.H., Blaha, M.J., Nasir, K., Blumenthal, R.S. and Budoff, M.J. 2013. High-sen- sitivity C-reactive protein and cardiovascular disease: a resolute belief or an elusive link? J. Am. Coll. Cardiol., 62, 397-408.

Yu KN, Chang SH, Park SJ, Lim J, Lee J, Yoon TJ, Kim JS, Cho MH. Titanium Dioxide Nanoparticles Induce Endoplasmic Reticulum Stress-Mediated Autophagic Cell Death via Mitochondria-Associated Endoplasmic Reticulum Membrane Disruption in Normal Lung Cells. PLoS One. 2015 Jun 29;10(6):e0131208. doi: 10.1371/journal.pone.0131208. PMID: 26121477; PMCID: PMC4485469.

Yu, X., X. Zhao, Y. Ze, L. Wang, D. Liu, J. Hong, B. Xu, A. Lin, C. Zhang, Y. Zhao, B. Li, and F. Hong. 2014. "Changes of serum parameters of TiO(2) nanoparticle-induced atherosclerosis in mice." *J Hazard Mater* 280:364-71. doi: 10.1016/j.jhazmat.2014.08.015.

Ze, X., M. Su, X. Zhao, H. Jiang, J. Hong, X. Yu, D. Liu, B. Xu, L. Sheng, Q. Zhou, J. Zhou, J. Cui, K. Li, L. Wang, Y. Ze, and F. Hong. 2016. "TiO2 nanoparticle-induced neurotoxicity may be involved in dysfunction of glutamate metabolism and its receptor expression in mice." *Environ Toxicol* 31 (6):655-62. doi: 10.1002/tox.22077.

Ze, Y., R. Hu, X. Wang, X. Sang, X. Ze, B. Li, J. Su, Y. Wang, N. Guan, X. Zhao, S. Gui, L. Zhu, Z. Cheng, J. Cheng, L. Sheng, Q. Sun, L. Wang, and F. Hong. 2014a. "Neurotoxicity and gene-expressed profile in brain-injured mice caused by exposure to titanium dioxide nanoparticles." *J Biomed Mater Res A* 102 (2):470-8. doi: 10.1002/jbm.a.34705.

Ze, Y., L. Sheng, X. Zhao, J. Hong, X. Ze, X. Yu, X. Pan, A. Lin, Y. Zhao, C. Zhang, Q. Zhou, L. Wang, and F. Hong. 2014b. "TiO2 nanoparticles induced hippocampal neuroinflammation in mice." *PLoS One* 9 (3):e92230. doi: 10.1371/journal.pone.0092230.

Ze, Y., L. Sheng, X. Zhao, X. Ze, X. Wang, Q. Zhou, J. Liu, Y. Yuan, S. Gui, X. Sang, Q. Sun, J. Hong, X. Yu, L. Wang, B. Li, and F. Hong. 2014c. "Neurotoxic characteristics of spatial recognition damage of the hippocampus in mice following subchronic peroral exposure to TiO2 nanoparticles." *J Hazard Mater* 264:219-29. doi: 10.1016/j.jhazmat.2013.10.072.

Ze, Y., L. Zheng, X. Zhao, S. Gui, X. Sang, J. Su, N. Guan, L. Zhu, L. Sheng, R. Hu, J. Cheng, Z. Cheng, Q. Sun, L. Wang, and F. Hong. 2013. "Molecular mechanism of titanium dioxide nanoparticles-induced oxidative injury in the brain of mice." *Chemosphere* 92 (9):1183-9. doi: 10.1016/j.chemosphere.2013.01.094.

Zhang, L., R. Bai, B. Li, C. Ge, J. Du, Y. Liu, L. Le Guyader, Y. Zhao, Y. Wu, S. He, Y. Ma, and C. Chen. 2011. "Rutile TiO(2) particles exert size and surface coating dependent retention and lesions on the murine brain." Toxicol Lett 207 (1):73-81. doi: 10.1016/j.toxlet.2011.08.001.

ECHA
EUROPEAN CHEMICALS AGENCY

Ziemann, C., Jackson, P., Brown, R., Attik, G., Rihn, B. H., & Creutzenberg, O. (2009, February). Quartz-containing ceramic dusts: in vitro screening of the cytotoxic, genotoxic and pro-inflammatory potential of 5 factory samples. In Journal of Physics: Conference Series (Vol. 151, No. 1, p. 012022). IOP Publishing.

Zoltani Csaba K., 2019. Chapter 12 - Cardiovascular Toxicity Biomarkers, Editor(s): Ramesh C. Gupta, Biomarkers in Toxicology (Second Edition), Academic Press, 2019, Pages 209-228, ISBN 9780128146552, https://doi.org/10.1016/B978-0-12-814655-2.00012-8.

ECHA
EUROPEAN CHEMICALS AGENCY

Appendix B: Procedure

This decision does not imply that the information you submitted in your registration dossier(s) are in compliance with the REACH requirements. ECHA may still initiate a compliance check on your dossiers.

Titanium dioxide (EC No 236-675-5, CAS RN 13463-67-7,) was first included in the CoRAP list published on 20 March 2013 based on concerns regarding its properties as a suspected CMR, sensitiser, PBT/vPvB and due to its wide dispersive use, consumer use, high aggregated tonnage and exposure of sensitive populations.

The lack of information available on the nanoforms of titanium dioxide reinforced the need of evaluating this Substance. ECHA performed a compliance check on the Substance dossier, initiated on 13 November 2013. The scope of the compliance check was limited to the standard information requirements of Annex VI of REACH. The compliance check decision was adopted on 17 June 2014. You appealed on this decision on 16 September 2014 and the substance evaluation was postponed. The Board of Appeal annulled the contested decision on 2 March 2017.

The substance evaluation was updated to start on 20 March 2018. The Competent Authority of France (the evaluating MSCA) was appointed to carry out the evaluation.

In accordance with Article 45(4) of REACH, the eMSCA carried out the evaluation based on the information in your registration(s) and other relevant and available information.

The eMSCA considered that further information was required to clarify the potential risk identified during the evaluation for repeated dose toxicity via inhalation. Therefore, it prepared a draft decision under Article 46(1) of REACH to request further information. It subsequently submitted the draft decision to ECHA on 20 March 2019.

Registrant(s)' commenting phase

ECHA notified you of the draft decision and invited you to provide comments. You provided comments which were submitted to eMSCA in late July 2019. As these comments led to a substantial modification of the draft decision and it may contain some new elements, ECHA exceptionally notified you of the modified draft decision and invited you to provide comments for a second time in June 2020.

ECHA received your comments and forwarded them to the eMSCA without delay. Due to the current situation with regards to the pandemic, some delays occur in the assessments of these comments. The eMSCA took the comments, which were sent within the commenting period, into account and they are reflected in the reasons (Appendix A).

For the purpose of this decision-making, dossier updates made after the date the modified draft of this decision was notified to you (Article 50(1) of REACH) were not taken into account.

(i) Proposals for amendment by other MSCAs and ECHA and referral to the Member State Committee

The evaluating MSCA notified the draft decision to the competent authorities of the other Member States and ECHA for proposal(s) for amendment.

ECHA
EUROPEAN CHEMICALS AGENCY

Subsequently, the evaluating MSCA received proposal(s) for amendment to the draft decision and did not modify the draft decision apart from the following considerations:

The proposal for amendments (PfA) were related to the removal of some initially requested assays based on your comments and addition of new requests (histopathology of testis and measurement of hs-CRP). Based on a PfA, a reference to the Substance evaluation decision on Potassium Titanium Oxide (EC number 432-240-0, ECHA, 2019) was added. Based on the last PfA, the deadline to provide the requested information was set to 30 months to take into account your comment on the necessity for test development to perform the requested study on 11 forms of the Substance.

ECHA referred the draft decision, together with your comments, to the Member State Committee.

ECHA invited you to comment on the proposed amendment(s). Your comments on the proposed amendment(s) were taken into account by the Member State Committee.

In addition you provided comments on the draft decision. Your comments were not taken into account by the Member State Committee as they were considered to be outside of the scope of Article 52(2) and Article 51(5).

Most of these comments were already raised during the initial commenting phase and have been addressed previously (your comments: number 1.1 on study design, 1.2 on dose selection and dose metrics, 1.3 Markers of oxidative stress, 1.6 Influence of total number of parameters on the feasibility of gaining all information from one animal, 2.3 Measurement of ROS/RNS/oxidative DNA lesions, 2.4 Technical feasibility of performing multiple analyses on a single organ/tissue, 2.5 Timepoints for administration, 2.6 Number of test animals required, 3 Test Materials).

In the above-mentioned comments, you also requested some further clarifications. As these were out of the scope of the submitted PfAs, they were not considered. However, if needed, the eMSCA is willing to provide further clarification following the adoption of the ECHA decision and would be happy to set up exchanges with you.

(ii) MSC agreement seeking stage

The Member State Committee reached a unanimous agreement in its MSC-74 written procedure and ECHA took the decision according to Article 52(2) and Article 51(6) of REACH.

After the deadline set in this decision has passed, the evaluating MSCA will review the information you have submitted and will evaluate whether further information is still needed to clarify the potential risk, according to Article 46(3) of REACH.

ECHA
EUROPEAN CHEMICALS AGENCY

Appendix C: Further information, observations and technical guidance

1. This decision does not imply that the information provided by you in the registration(s) is in compliance with the REACH requirements. The decision neither prevents ECHA from initiating compliance checks on your dossier(s) at a later stage, nor does it prevent a subsequent decision under the current substance evaluation or a new substance evaluation process once the present substance evaluation has been completed.

2. Failure to comply with the request(s) in this decision, or to otherwise fulfil the information request (s) with a valid and documented adaptation, will result in a notification to the enforcement authorities of your Member State.

3. In relation to the required experimental study/ies, the sample of the substance to be used ('test material') has to have a composition that is within the specifications of the substance composition that are given by all registrant(s). It is the responsibility of all the registrant(s) to agree on the tested material to be subjected to the test(s) subject to this decision and to document the necessary information on the composition of the test material. The substance identity information of the Substance and of the sample tested must enable the evaluating MSCA and ECHA to confirm the relevance of the testing for the substance subject to substance evaluation.