

Valeurs toxicologiques de référence - Le dioxyde de titane sous forme nanoparticulaire

Stéphane Binet, Irina Guseva Canu, Claude Emond, Valérie Fessard, Emmanuel Flahaut, Henri Schroeder, Bernard Salles, Olivier Sorg, Lydie Sparfel, Paule Vasseur, et al.

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alimentation, environnement, travail

Connaître, évaluer, protéger

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Saisine n° « 2017-SA-0162 »
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Le directeur général

Maisons-Alfort, le 30 janvier 2019

AVIS
de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

relatif à « la proposition de VTR chronique par voie respiratoire pour le dioxyde de titane sous forme nanométrique (CAS n°13463-67-7) »

L'Anses met en œuvre une expertise scientifique indépendante et pluraliste.
L'Anses contribue principalement à assurer la sécurité sanitaire dans les domaines de l'environnement, du travail et de l'alimentation et à évaluer les risques sanitaires qu'ils peuvent comporter.
Elle contribue également à assurer d'une part la protection de la santé et du bien-être des animaux et de la santé des végétaux et d'autre part à l'évaluation des propriétés nutritionnelles des aliments.

Elle fournit aux autorités compétentes toutes les informations sur ces risques ainsi que l'expertise et l'appui scientifique technique nécessaires à l'élaboration des dispositions législatives et réglementaires et à la mise en œuvre des mesures de gestion du risque (article L.1313-1 du code de la santé publique).

Ses avis sont publiés sur son site internet.

L'Anses a été saisie le 4 juillet 2018 par la Direction générale de la santé (DGS), la Direction générale de la prévention des risques (DGPR) et la Direction générale du travail (DGT) pour la réalisation de l'expertise suivante : définition d'une valeur toxicologique de référence (VTR) concernant le dioxyde de titane sous forme nanométrique (TiO_2).

1. CONTEXTE ET OBJET DE LA SAISINE

Dans le cadre des évaluations de risques réalisées dans le traitement des dossiers relatifs aux installations classées pour la protection de l'environnement (ICPE) ou à la gestion des sites et sols pollués, les Agences régionales de santé (ARS) ou les bureaux d'étude adressent à la DGS des questions relatives au choix des VTR de certaines substances chimiques. Ce choix est effectué suivant les modalités définies dans la note d'information N° DGS/EA1/DGPR/2014/307 du 31 octobre 2014 relative aux modalités de sélection des substances chimiques et de choix des VTR pour mener les évaluations des risques sanitaires dans le cadre des études d'impact et de la gestion des sites et sols pollués. Dans cette note, l'Anses a été désignée comme agence d'expertise pour le choix et la construction des VTR.

L'Anses a ainsi été saisie par la DGS, la DGPR et la DGT le 4 juillet 2017 pour définir une VTR chronique par inhalation pour le dioxyde de titane sous forme nanométrique. Cette demande aux termes de la saisine résulte de « *l'analyse de la base de données R-Nano indiquant que de nombreux sites industriels en France utilisent du dioxyde de titane sous forme nanométrique. Ces manipulations peuvent être à l'origine d'exposition des travailleurs mais également d'exposition des populations via des émissions à l'extérieur des sites* ». La saisine relève que « *le Centre international de recherche sur le cancer (CIRC) a classé le dioxyde de titane sous forme de*

particules respirables en cancérogène possible par inhalation ». L'Anses a par ailleurs porté, en 2015, auprès de l'Agence européenne des produits chimiques (ECHA) une demande de classification pour la cancérogénicité par inhalation du TiO_2 (cancérogène de catégorie 1B) dans le cadre du règlement européen (CLP) n°1272/2008 relatif à la classification, à l'étiquetage et à l'emballage des substances et mélanges dangereux. En 2017, le Comité d'Evaluation des Risques (CER ou RAC pour *risk assessment committee*) de l'ECHA a conclu que le TiO_2 sous toutes ses formes devrait être classé comme cancérogène suspecté pour l'homme de catégorie 2 par inhalation.

Parallèlement à cette saisine, l'Institut national de l'environnement industriel et des risques (INERIS) a demandé à l'Anses dans un courrier reçu le 28 juin 2017 son avis sur une proposition de valeurs repères pour l'exposition des populations riveraines de sites manipulant du dioxyde de titane à l'état nanoparticulaire. Cette proposition de valeurs repères par l'INERIS a conduit la DGS, la DGPR et la DGT à saisir également le Haut Conseil de santé publique (HCSP) le 4 juillet 2017, afin d'établir des recommandations sur les mesures de gestion à mettre en œuvre vis-à-vis des populations riveraines de sites manipulant du TiO_2 à l'état nanoparticulaire, ainsi que des travailleurs.

Aux termes mêmes de la saisine de la DGS adressée à l'Anses : « *les travaux [faisant l'objet du présent avis] ont vocation à compléter à terme l'avis du HCSP sur les mesures de gestion à mettre en place concernant l'exposition de la population et des travailleurs pour tous les sites manipulant du TiO_2 à l'échelle nanométrique* ».

Par ailleurs, dans le cadre du règlement REACh, l'Anses instruit actuellement un dossier d'évaluation des dangers et des risques du TiO_2 pour la santé humaine et pour l'environnement Dans le cadre de l'instruction de ce dossier, dont la date butoir réglementaire est mars 2019, des données supplémentaires sur les dangers, les usages du TiO_2 pourront être requises par l'Anses auprès des industriels.

Une VTR est un indice toxicologique qui permet de qualifier ou de quantifier un risque pour la santé humaine. Elle établit le lien entre une exposition à une substance toxique et l'occurrence d'un effet sanitaire indésirable. Les VTR sont spécifiques d'une durée d'exposition (aiguë, subchronique ou chronique) et d'une voie d'exposition (orale ou respiratoire). La construction des VTR diffère en fonction des connaissances ou des hypothèses formulées sur les mécanismes d'action des substances. Actuellement, l'hypothèse par défaut est de considérer une relation monotone entre l'exposition, ou la dose, et l'effet, ou la réponse. En l'état actuel des connaissances et par défaut, on considère généralement que, pour les effets non cancérogènes, la toxicité ne s'exprime qu'au-delà d'un seuil de dose (Anses, 2017).

En pratique, la construction de la VTR comprend les étapes suivantes :

- recenser et analyser les données de toxicité disponibles, sur la base d'études épidémiologiques et/ou expérimentales,
- identifier le ou les organes cibles et l'effet critique ;
- identifier l'hypothèse de construction, à seuil ou sans seuil de dose, en fonction du mode d'action de la substance,
- choisir une étude clé de bonne qualité scientifique permettant généralement d'établir une relation dose – réponse ;
- définir une dose critique chez l'Homme ou l'animal à partir de cette étude, éventuellement dans le cas d'une dose critique obtenue chez l'animal, ajuster cette dose à l'Homme ;

- pour une VTR à seuil, appliquer des facteurs d'incertitude à cette dose critique de manière à dériver une VTR applicable à l'ensemble de la population concernée ;
- pour une VTR sans seuil, réaliser une extrapolation linéaire à l'origine afin de déterminer un excès de risque unitaire.

L'élaboration des VTR suit une approche très structurée et exigeante qui implique des évaluations collectives par des groupes de spécialistes.

2. ORGANISATION DE L'EXPERTISE

L'expertise a été réalisée dans le respect de la norme NF X 50-110 « Qualité en expertise – Prescriptions générales de compétence pour une expertise (Mai 2003) ».

Dans le but de répondre à la saisine susmentionnée, l'Anses a nommé des experts rapporteurs issus des comités d'experts spécialisés (CES) « Valeurs sanitaires de référence », « REACH-CLP », et « Evaluation des risques liés aux agents physiques, aux nouvelles technologies et aux grands aménagements » ou disponibles en tant que personnalité compétente de l'Agence.

Le profil toxicologique a été rédigé à partir d'une recherche bibliographique systématique réalisée sur Medline et Scopus en décembre 2017. Cette recherche s'est concentrée sur les études de toxicologie *in vivo* par inhalation réalisées avec du TiO_2 sous forme nanométrique et publiées à partir de 2000. Ainsi, les études n'ayant pas pour but d'évaluer la toxicité sur la santé humaine après une exposition à du TiO_2 sous forme nanométrique seul ont été exclues. Ont également été exclues, les études n'incluant pas une caractérisation suffisante du matériel testé.
Dans un but de transparence et de traçabilité, des fiches de lecture ont été rédigées pour toutes les publications analysées, et présentées au groupe d'experts rapporteurs. Afin d'évaluer la pertinence des études utilisables pour la dérivation de la VTR chronique par inhalation, les critères de cotation de la qualité des études ont été appliqués à l'aide du logiciel ToxRTool.

L'expertise collective a été réalisée par le CES « Valeurs sanitaires de référence ». Les travaux ont été présentés au CES tant sur les aspects méthodologiques que scientifiques. Ils ont été adoptés par le CES « Valeurs sanitaires de référence » réuni le 29 novembre 2018.

L'Anses analyse les liens d'intérêts déclarés par les experts avant leur nomination et tout au long des travaux, afin d'éviter les risques de conflits d'intérêts au regard des points traités dans le cadre de l'expertise.
Les déclarations d'intérêts des experts sont publiées sur le site internet de l'Anses (www.anses.fr).

3. ANALYSE ET CONCLUSIONS DU CES

Introduction

Le TiO_2 existe sous forme micro ou nanométrique. Le présent avis concerne exclusivement le TiO_2 sous forme nanométrique (ci-dessous TiO_2-NP).

Selon la définition de la Commission européenne, « *on entend par « nanomatériau » un matériau naturel, formé accidentellement ou manufacturé contenant des particules libres, sous forme d'agrégat ou sous forme d'agglomérat, dont au moins 50 % des particules, dans la répartition*

numérique par taille, présentent une ou plusieurs dimensions externes se situant entre 1 nm et 100 nm. Dans des cas spécifiques, lorsque cela se justifie pour des raisons tenant à la protection de l'environnement, à la santé publique, à la sécurité ou à la compétitivité, le seuil de 50 % fixé pour la répartition numérique par taille peut être remplacé par un seuil compris entre 1 % et 50 %.[1] » (recommandation de la CE 2011/696/UE). Cette définition est celle utilisée dans le présent avis pour définir le TiO_2-NP.

En plus de la taille, d'autres propriétés physico-chimiques intrinsèques au TiO_2 peuvent également varier et sont supposées influencer sa réactivité, incluant notamment (NIOSH, 2011 ; IARC, 2010) :

- la forme : sphérique, allongée, fibreuse etc.
- la présence d'une modification de surface (revêtement, fonctionnalisation) avec des substances inorganiques (silice, alumine…) ou organiques (siloxane, trimethylolpropane…)
- la cristallinité, 3 polymorphes naturels principaux existent : rutile, anatase et brookite, et au niveau industriel, seules la rutile et l'anatase sont utilisées.

Parmi les études identifiées dans la littérature et retenues pour cette expertise, nombreuses ont été réalisées avec du TiO_2 P25. Le P25 est une forme de référence du TiO_2-NP caractérisée de façon complète par l'OCDE (sous le nom de NM105). Il s'agit d'un mélange 80 % / 20 % anatase/rutile avec une taille primaire d'environ 20-25 nm.

Synthèse des données toxicologiques

- Toxicocinétique

Les études de cinétique relatives à une exposition respiratoire au TiO_2-NP chez le rat se sont majoritairement intéressées à sa distribution et sa biopersistance au niveau pulmonaire. Les particules de TiO_2-NP sont principalement retrouvées dans les macrophages alvéolaires mais aussi, à un niveau moindre, au niveau des pneumocytes (Eydner *et al.* (2012)). Le temps de demi-vie estimé est approximativement de 2 mois chez le rat (Oyabu *et al.* (2017)). En absence de surcharge pulmonaire, la distribution pulmonaire ainsi que le temps de demi-vie ne semblent pas influencés par la durée d'exposition (Zhang *et al.* (2015); Bermudez *et al.* (2004)).
Une translocation[2] vers d'autres organes, tels que le foie, le cœur, les reins, le pancréas, la rate ou encore le cerveau a été rapportée par différents auteurs (Kreyling *et al.* (2017c) ; Pujalte *et al.* (2017) ; Husain *et al.* (2015) ; Eydner *et al.* (2012) ; Gate *et al.* (2017)) même si celle-ci ne semble pas prédominante. En effet, la vitesse de translocation est plus lente que celle de la clairance pulmonaire (Shinohara *et al.* (2014)).
Le TiO_2-NP est principalement excrété dans les fèces (Pujalte *et al.* (2017)), ce qui serait consécutif à une déglutition des particules lors de la clairance mucociliaire au niveau du tractus respiratoire.

- Toxicité aiguë

La plupart des études de toxicité aiguë par voie respiratoire disponibles avec le TiO_2-NP se sont focalisées sur l'étude des effets pulmonaires. Les effets rapportés, que ce soit par inhalation ou par instillation, consistent principalement en une inflammation associée ou non à des modifications histo-pathologiques (inhalation : Grassian *et al.* (2007a & b); Noel *et al.* (2012) ; Leppänen *et al.* (2011); Leppänen *et al.* (2015); Oyabu *et al.* (2016) – instillation : Oberdörster *et al.* (2000), Renwick *et al.* (2004), Chen *et al.* (2006), Nemmar *et al.* (2008), Nemmar *et al.* (2011), Liang *et al.* (2009), Sager and Castranova (2009), Cho *et al.* (2010), Roberts *et al.* (2011), Tang *et al.* (2011),

[1] Le seuil de 50 % précisé dans la définition de la Commission européenne n'a pas été considéré pour la sélection des études qui ne mentionnaient pas cette information.

[2] Translocation : déplacement des particules hors du site de dépôt pulmonaire initial (Handbook on the toxicology of metals, 2015).

Saber *et al.* (2012a), Saber *et al.* (2012b), Hurbankova *et al.* (2013), Husain *et al.* (2013), Husain *et al.* (2015), Lee *et al.* (2014), Choi *et al.* (2014), Yoshiura *et al.* (2015), Kobayashi *et al.* (2016), Wallin *et al.* (2017); Saber *et al.* (2012) ; Oyabu *et al.* (2013)).

Une série d'études réalisée par une même équipe s'est également intéressée aux effets du TiO_2-NP sur le système cardiovasculaire (Nurkiewicz *et al.* (2008), Nurkiewicz *et al.* (2009), LeBlanc *et al.* (2009), LeBlanc *et al.* (2010), Knuckles *et al.* (2012) Stapleton *et al.* (2015b). Les auteurs ont observé qu'une inhalation aiguë au TiO_2 P25 (6 mg/m^3 ; 4 heures) entrainait une altération de la vasodilatation. Ils ont conclu que cette altération serait due à un dysfonctionnement endothélial médié par la production de radicaux libres réduisant ainsi la biodisponibilité du monoxyde d'azote. Ces effets semblent apparaitre à des concentrations pouvant également induire une inflammation pulmonaire.

- Toxicité répétée
  - Données humaines

Huit études ont analysé les effets du TiO_2 chez les travailleurs. Trois études ont été réalisées en Chine (Zhen *et al.* (2012); Ichiara *et al.* (2016) and Zhao *et al.* (2018)) et cinq en République tchèque (Pelclova *et al.* (2015, 2016a, b, c et 2017)). Ces études, majoritairement transversales, suggèrent un effet possible sur les fonctions respiratoire et cardiovasculaire. Cependant, aucune de ces études n'a permis de mettre en évidence une relation causale entre une exposition à du TiO_2 sous forme micro- ou nanométrique et l'apparition de ces effets. L'identification de biais de sélection, de classification, notamment sur l'exposition, ainsi que de biais de confusion fortement plausibles ne permettent pas de considérer ces études adéquates pour conclure sur la toxicité du TiO_2-NP chez l'homme.

  - Données animales

Effets pulmonaires

Comme pour les études de toxicité aiguë, la majorité des études de toxicité répétée se sont focalisées sur les effets pulmonaires après une exposition respiratoire au TiO_2-NP. Cinq études de toxicité répétée par inhalation utilisant plusieurs concentrations ont été identifiées dans la littérature.

Une étude de toxicité subchronique réalisée par Bermudez *et al.* (2004) est disponible. Dans cette étude, des femelles de trois espèces (rats, souris et hamsters) ont été exposées nez-seul au P25 pendant 90 jours aux concentrations de 0,5 ; 2,0 ou 10 mg/m^3. Alors que les hamsters ne présentaient aucun effet pulmonaire, une inflammation pulmonaire a été observée chez les souris à la plus forte concentration testée, ainsi que des effets histopathologiques au niveau du poumon chez les rats dès la concentration de 2,0 mg/m^3. Le rat est l'espèce la plus sensible dans cette étude avec l'observation d'effets pré-néoplasiques, tels que des métaplasies, à la plus forte concentration testée. Ainsi, une NOAEC[3] de 10 mg/m^3 peut être dérivée chez le hamster et une NOAEC de 2 mg/m^3 chez la souris. Chez le rat, une NOAEC de 0,5 mg/m^3 peut être dérivée sur la base d'hypertrophies et d'hyperplasies des cellules alvéolaires de type II de sévérité minimale à la concentration de 2 mg/m^3.

Après une exposition de 5 jours au TiO_2-NP (86% / 14% anatase/rutile ; 25 nm) chez le rat mâle, Ma-Hock *et al.* (2009) ont mis en évidence des changements histopathologiques pulmonaires incluant une inflammation à toutes les concentrations testées (2, 10, 50 mg/m^3) et des

[3]NOAEC = No Observed Adverse Effect Concentration (= Concentration maximale n'entraînant pas d'effet néfaste observé)

hypertrophies / hyperplasies des bronches et bronchioles à la plus forte concentration de 50 mg/m^3. Une LOAEC[4] de 2 mg/m^3 a été identifiée par les auteurs. Une inflammation pulmonaire, mise en évidence par des modifications de paramètres dans le liquide de lavage broncho-alvéolaire, a été rapportée chez le rat mâle à cette même concentration par Landsiedel *et al.* (2014) également après une exposition de 5 jours au TiO_2-NP (rutile revêtu en surface par du dimethicone/methicone). Ces derniers résultats permettent de conclure à une NOAEC de 0,5 mg/m^3.

Alors qu'Oyabu *et al.* (2017) ne rapporte pas d'inflammation pulmonaire chez le rat mâle à des concentrations allant jusqu'à 1,84 mg/m^3 après une exposition de 4 semaines à du TiO_2-NP (rutile, 12x55 nm), les paramètres testés étant cependant limités, de nombreux effets pulmonaires ont été notés chez la souris à toutes les doses testées (LOAEC = 2,5 mg/m^3) par Yu *et al.* (2015) avec une forme non caractérisée de TiO_2-NP, et ce, pour une même durée d'exposition. Du fait de l'absence de caractérisation du matériel testé, cette dernière étude ne peut être utilisée pour la construction d'une valeur de référence.

Malgré des durées d'exposition plus courtes et la diversité des protocoles mis en œuvre (TiO_2-NP différent, espèces différentes…), toutes les études par inhalation décrites ci-dessus sont cohérentes avec celle de Bermudez *et al.* (2004).

D'autres études ont été identifiées dans la littérature, mais ont été réalisées par inhalation avec une seule concentration ou par instillation. Elles confirment les résultats précédemment décrits, qualitativement ou quantitativement (Grassian *et al.* (2007b), Eydner *et al.* (2012), Jackson *et al.* (2013), Halappanavar *et al.* (2011) ; Leppänen *et al.* (2011 & 2015); Sun *et al.* (2012a, b), Li *et al.* (2013), Hong *et al.* (2017)).

Effets sur le système cardiovasculaire

Cinq études réalisées par inhalation ou par instillation ont analysé les effets du TiO_2-NP sur le système cardiovasculaire après une exposition répétée. Divers effets, incluant des dysfonctionnements micro-vasculaires ou de l'athérosclérose, ont été rapportés chez le rat ou la souris dans quatre de ces études (Stapleton *et al.* (2013) ; Yu *et al.* (2014) ; Saber *et al.* (2013), Chen *et al.* (2013)). Les concentrations utilisées dans les études par inhalation (10 et 42 mg/m^3) sont cependant bien plus élevées que celles des études évaluant les effets pulmonaires (dès 0,5 mg/m^3), et ne permettent donc pas de comparer quantitativement ces effets avec ceux observés au niveau pulmonaire.

Effets sur le système immunitaire

De nombreuses études évaluant les effets du TiO_2-NP sur le système immunitaire sont disponibles, utilisant différents protocoles, et différentes voies d'exposition (instillation, inhalation).
Deux études ont montré une diminution des cellules CD4+ et CD8+ avec un ratio CD4+/CD8+ augmenté, indiquant une perturbation du système immunitaire chez le rat (Chang *et al.* (2015), Gustafsson *et al.* (2011)). Une augmentation des cellules NK a également été observée suite à une exposition au TiO_2-NP chez le rat (Fu *et al.* (2014), Gustafsson *et al.* (2011)). Il semble néanmoins difficile de conclure quant à l'immunotoxicité du TiO_2-NP au regard des protocoles et des résultats hétérogènes.

Effets sur le système nerveux central

Onze études relatives à la neurotoxicité du TiO_2-NP ont été identifiées dans la littérature.
Des altérations histologiques de l'hippocampe et du cortex cérébral ont été observées chez la souris (Zhang *et al.* (2011), Wang *et al.* (2008a, b)) suite à une administration intranasale avec

[4]LOAEC = Lowest Observed Adverse Effect Concentration (= Concentration minimale entraînant un effet néfaste observé)

différentes formes de TiO_2-NP. Zhang *et al.* (2011) rapporte une toxicité supérieure du TiO_2-NP rutile revêtu en surface par de la silice en comparaison d'une forme rutile non revêtue en surface. Wang *et al.* (2008 a & b) ont noté des effets plus sévères après une exposition à de l'anatase par rapport au rutile.
Une autre équipe a montré une accumulation du TiO_2-NP (anatase ; 6 nm) dans le cerveau de la souris avec une prolifération des cellules gliales, une nécrose et des signes de dégénération cellulaire, ainsi que des dérégulations de gènes liés au stress oxydatif, au développement du cerveau, à la mémoire et l'apprentissage.... Ces résultats suggèrent une toxicité dose dépendante du TiO_2-NP sur le cerveau, l'hippocampe étant identifié comme une région cérébrale plus particulièrement sensible (Ze *et al.* (2013), (2014a, b, c)).

Hépatotoxicité
Alors qu'une analyse transcriptomique n'a pas rapporté d'effet hépatique du TiO2-NP après une exposition gestationnelle de 10 jours par inhalation à la concentration de 42 mg/m3 chez la souris (Halappanavar *et al.*, 2011), des œdèmes et perte cytoplasmique des cellules hépatiques ont été observés après une exposition par instillation pendant 4 semaines à du P25 chez le rat (Chang *et al.* (2015)).

Effets sur les reins
Une seule étude, réalisée par instillation, portant sur les effets rénaux du TiO2-NP a été identifiée dans la littérature. Des modifications histopathologiques, incluant une dilatation tubulaire et une nécrose, en présence d'une augmentation du stress oxydatif, ont été rapportées dès la dose de 0,5 mg/semaine pendant 4 semaines d'exposition au P25 chez la souris (Huang *et al.* (2015)).

- Reprotoxicité et effets sur le développement

Plusieurs équipes ont analysé les effets sur le développement du TiO_2-NP suite à une exposition pré- ou péri-natale par inhalation ou par instillation. Ces travaux n'avaient pas pour but d'étudier la survenue de malformations mais plutôt d'effets mutagènes ou d'effets en lien avec une atteinte pulmonaire, cardiovasculaire ou neurocomportementale.

La première équipe (Hougaard *et al.* (2010), Boisen *et al.* (2012); Kyjovskaa *et al.* (2013), Jackson *et al.* (2013)) a exposé des souris femelles du $8^{ème}$ au $18^{ème}$ jour de gestation à du TiO_2-NP sous forme rutile avec un revêtement de surface organique (UV-Titan L181) à la concentration de 40 mg/m^3 chez la souris. A cette concentration, les mères présentaient une inflammation pulmonaire. Chez les petits, les effets rapportés incluaient des changements neurocomportementaux modérés (Hougaard *et al.* (2010)), ainsi qu'une altération de l'expression génique dans le foie des femelles (Jackson *et al.* (2013)). Une tendance à la réduction du nombre de spermatozoïdes associée à un allongement du délai d'obtention de la première portée a également été rapporté par Kyjovska *et al.* (2013). A contrario, il n'a pas été observé d'augmentation des mutations (Boisen *et al.* (2012)), ni d'effet sur la viabilité ou le sexe-ratio des portées.

La deuxième équipe (Stapleton *et al.* (2013, 2015, 2018), Engler-Chiurazzi *et al.* (2016), Hathaway *et al.* (2017)) a testé une exposition des femelles gestantes à du P25 pendant environ 8 jours à partir de l'implantation, à la concentration de 10 mg/m^3 chez le rat. Stapleton *et al.* (2013) a observé une diminution de la taille et du poids des portées lors d'une exposition de 11 jours durant la période de gestation, contrairement à une durée d'exposition de 7 jours. Ces effets n'ont pas non plus été observés dans les études consécutives avec une durée d'exposition de 7-8 jours. Des altérations microvasculaires et cardiaques ont été observées chez les petits (Stapleton *et al.* (2013, 2015, 2018), Hathaway *et al.* (2017)) ainsi que des effets sur les fonctions cognitives et comportementales (Engler-Chiurazzi *et al.* (2016).

Enfin, deux études par instillation se sont intéressées aux effets sur le développement pulmonaire chez la souris. Ambalavanan *et al.* (2013) suggèrent une augmentation du risque de survenue de

maladies respiratoires après une administration unique d'anatase (6 nm) au 4ème, 7ème ou 10ème jour après la naissance. Une altération pulmonaire a également été notée par Paul *et al.* (2017), chez des fœtus après une exposition au 2ème, 9ème et 16ème jour de gestation. Cet effet n'était pas associé à une réponse inflammatoire pulmonaire chez les mères ou les fœtus, ni au niveau placentaire.

Ainsi, les études décrites ci-dessus suggèrent un effet possible sur le développement après une exposition à du TiO_2-NP. Cependant, ces études, réalisées à une seule concentration, ne permettent pas d'identifier une NOAEC.

- Génotoxicité

De nombreuses publications ont analysé les propriétés mutagènes du TiO_2-NP, principalement sous la forme anatase ou anatase/rutile.

Les études *in vitro* et *in vivo* rapportent des résultats contradictoires, avec des résultats positifs observés principalement à fortes doses dans des tests des comètes et des études de micronoyaux. Cette disparité des résultats pourrait s'expliquer par des différences dans les protocoles et/ou dans les formes de TiO_2-NP testées. Cependant, à ce jour, et malgré la quantité de données disponibles, il n'est pas possible d'identifier un paramètre clé relié aux effets génotoxiques identifiés (Magdolenova *et al.* (2014); Chen *et al.* (2014); ANSES (2016); Charles *et al.* (2018)).

D'un point de vue mécanistique, les données tendent à montrer que les effets génotoxiques seraient liés à un mécanisme secondaire, via la production de radicaux libres. Cependant, d'autres mécanismes d'action ne peuvent être totalement écartés en l'absence d'outils spécifiques pour les investiguer (Charles *et al.* (2018)).

Sur la base de ces résultats et considérant que les effets cancérogènes apparaissent uniquement à de fortes concentrations, induisant une réponse inflammatoire et une altération de la clairance, la conclusion est que le TiO_2-NP présente une faible génotoxicité. Ces conclusions sont similaires à celles du CIRC (2010), du NIOSH[5] (2011), de l'Anses (2016) et de l'OCDE[6] (2018).

- Cancérogénicité

  - Données humaines

Sept études épidémiologiques, dont cinq études de cohorte rétrospectives (Chen & Fayerweather (1988); Fryzek *et al.* (2003) ; Boffetta *et al.* (2004) ; Ellis *et al.* (2010 & 2013)) et deux études cas-témoin (Boffetta *et al.* (2001), Ramanakumar *et al.* (2008)) ont analysé le lien entre une exposition au TiO_2 et l'apparition de cancers. Le TiO_2 n'étant pas caractérisé dans les publications, il ne peut pas être exclu que les populations analysées soit exposées, au moins en partie, à du TiO_2 sous forme nanoparticulaire.

La plupart de ces études rapportent une augmentation (significative ou non) de la mortalité par cancer pulmonaire. Cependant, aucune n'a permis de mettre en évidence une relation causale entre une exposition à du TiO_2 et l'apparition de cet effet. L'identification de biais de sélection, de classification, notamment sur l'exposition ainsi que de biais de confusion, ne permettent pas de considérer ces études adéquates pour conclure à l'absence ou l'existence d'effet cancérogène chez l'Homme.

[5] National Institute for Occupational Safety and Health

[6] Organisation de Coopération et de développement économiques

- Données animales

Les effets cancérogènes du TiO_2 (sous toutes ses formes) ont été analysés par différentes instances nationales ou internationales d'experts, incluant l'Anses en 2015.

Concernant le TiO_2-NP, une seule étude par inhalation est disponible (Heinrich *et al.* (1995)). Dans cette étude, une augmentation de l'incidence de tumeurs pulmonaires bénignes et malignes (tumeurs cystiques des cellules squameuses, carcinomes des cellules squameuses et adénomes/adénocarcinomes bronchioalvéolaires) a été observée chez des rats exposés par inhalation corps entier à du P25 (7,2 mg/m^3 pendant 4 mois, puis 14,8 mg/m^3 pendant 4 mois et enfin 9,4 mg/m^3 pendant 16 mois). Des tumeurs similaires ont également été rapportées chez des rats après une instillation répétée de P25 (Pott *et al.* (2005)).

A contrario, aucun effet promoteur n'a été identifié dans deux études réalisées par instillation (Xu *et al.* (2010); Yokohira *et al.* (2009)). Cependant, les protocoles de ces différentes études présentaient des biais méthodologiques (pas d'information sur la cristallinité du TiO_2-NP, peu d'expérience avec le modèle utilisé, pas de justification du choix des marqueurs et des critères d'évaluation des tumeurs etc…) ne permettant pas d'utiliser ces études pour la construction d'une valeur de référence.

En conclusion, le TiO_2-NP est considéré comme un agent cancérogène chez le rat à des concentrations induisant une inflammation pulmonaire et une altération de la clairance pulmonaire. Les données épidémiologiques sont inadéquates pour conclure à la pertinence de cet effet chez l'Homme. Ces conclusions sont en accord avec celle du CIRC (2010), qui a classé le TiO_2 comme cancérogène possible pour l'Homme (groupe 2B) et du RAC[7] (2017) concluant que le TiO_2 doit être classé comme cancérogène suspecté (catégorie 2) selon le règlement CLP n°1272/2008.

- Population sensible

Seules quelques études ont évalué la sensibilité de certains groupes au TiO_2-NP.
Après induction d'emphysème chez des rats, des auteurs ont montré que l'exposition par instillation au TiO_2-NP n'aggravait pas l'inflammation pulmonaire et l'emphysème. Ces résultats suggèrent que les personnes souffrant de pathologie pulmonaire ne seraient pas plus sensibles aux TiO_2-NP (Roulet *et al.*, 2012), mais doivent être confirmés par d'autres études.
Deux auteurs ont montré que les rats âgés (19 mois) étaient plus susceptibles que les jeunes (12-13 semaines) que ce soit dans une étude évaluant la translocation et la biopersistance des particules (Gate *et al.*, 2017) ou dans une étude de neurotoxicité (Disdier *et al.*, 2017). Au contraire, Scuri *et al.* (2010) ont observé une sensibilité plus forte des jeunes rats (nouveaux nés et 2 semaines) en comparaison des adultes après 3 jours d'exposition par inhalation.

■ Proposition d'une VTR chronique par inhalation

- Analyse des VTR chroniques existantes

Seul l'INERIS a proposé des valeurs toxicologiques pour des expositions environnementales par voies respiratoire et orale (INERIS, 2016). L'INERIS souligne dans son document qu'il s'agit de repères toxicologiques, qui sont des valeurs indicatives, provisoires, ne permettant pas de couvrir l'ensemble des potentiels effets induits par les différentes formes de TiO_2-NP, et pouvant être révisées en fonction de l'évolution des connaissances.

[7] Risk Assessment Committee (ou CER : Comité d'évaluation des risques) de l'ECHA (European Chemicals Agency)

Tableau 1 : Valeur repère toxicologique chronique par voie respiratoire

Type de valeur	Organisme	Effet critique (étude clé)	Concentration critique	UF[8]	VTR
Repère toxicologique	INERIS	Altérations fibroprolifératives et progressives de l'épithélium et bronchiolisation alvéolaire Bermudez *et al.* (2004)	NOAEC = 0,5 mg/m^3 Ajustement temporel Ajustement allométrique 0.089 mg/m^3	900 UF_A = 3 UF_H = 10 UF_S = 3 UF_D = 10	0,1 µg/m^3

La construction de cette valeur repère a été analysée par le CES « VSR ».
Les experts du CES ont souligné la qualité du travail réalisé compte tenu notamment du temps imparti et de la complexité du sujet. Néanmoins, les experts ont décidé de ne pas retenir les valeurs repères élaborées par l'INERIS pour les raisons suivantes :

- Le CES estime qu'il est fondamental de remettre à jour la bibliographie pour pouvoir prendre en compte les avancées les plus récentes sur le sujet. En effet, le CES note qu'il y a une avancée rapide des connaissances sur les nanomatériaux et en particulier sur le dioxyde de titane et que de très nombreuses études scientifiques sont publiées régulièrement sur le sujet. De plus, il est indiqué que la bibliographie, arrêtée en 2016, n'est pas exhaustive ;
- Le choix et la qualité des études clé ainsi que les points de départ retenus pour la construction des valeurs repères ont été remis en cause par le CES ;
- L'INERIS n'a pas pris en compte dans son rapport les études épidémiologiques existantes et le CES estime qu'il est difficile de passer outre ces informations ;
- De nombreuses questions se posent également sur la caractérisation des formes de dioxyde de titane utilisées dans les études et sa prise en compte dans la dérivation de valeurs repères. Le CES estime également que le rapport de l'INERIS ne détaille pas suffisamment ce point qui est fondamental pour la construction d'une valeur de référence.

- Choix de l'effet critique

Sur la base des données disponibles chez l'animal, le TiO_2-NP induit des effets au niveau pulmonaire (à la fois néoplasiques et non-néoplasiques), du système cardiovasculaire, du cerveau, du foie et des reins. Des effets sur le développement ont également été rapportés après une exposition gestationnelle chez l'animal.

L'analyse de l'ensemble des études de toxicité répétée réalisées par inhalation identifie l'inflammation pulmonaire comme étant l'effet critique, c'est-à-dire l'effet apparaissant aux concentrations les plus faibles. L'inflammation pulmonaire est rapportée à des concentrations supérieures ou égales à 2 mg/m^3 chez le rat. Des atteintes pulmonaires plus sévères, incluant une tumorigenèse, apparaissent chez le rat à des concentrations plus élevées (≥ 10 mg/m^3).

Les études visant à l'identification d'autres organes cibles n'ont été réalisées qu'à une seule concentration, souvent bien supérieure à 2 mg/m^3. Ainsi, les effets sur le système cardiovasculaire ont été rapportés à la concentration de 6 mg/m^3, les effets sur le cerveau et sur le développement à la concentration de 10 mg/m^3, et les effets sur le foie à la concentration de 42 mg/m^3.

[8] Voir signification des facteurs d'incertitudes dans la partie « Choix des facteurs d'incertitude ».

Concernant les effets sur les reins, la seule étude identifiée a été réalisée par instillation, qui n'est pas une voie d'administration physiologique.

- Extrapolation de l'animal à l'Homme

Les données expérimentales suggèrent que le rat est particulièrement sensible à la toxicité pulmonaire du TiO_2-NP en comparaison à d'autres rongeurs. En effet, des différences inter-espèces claires ont été observées dans l'étude de Bermudez *et al.* (2004) réalisée avec du P25 pendant 13 semaines chez des femelles de trois espèces : rats, souris et hamsters. Les lésions pulmonaires étaient plus sévères et apparaissaient à des concentrations plus faibles chez le rat, qui était la seule espèce à développer des lésions fibro-prolifératives. Ces différences inter-espèce pourraient être expliquées, au moins en partie, par des différences dans leur système de détoxification. En effet, une augmentation des niveaux de concentration de certains antioxydants a été observée dans les tissus pulmonaires chez les souris par rapport aux rats après une exposition particulaire (Oberdörster, 1995). Par ailleurs il est reconnu que les hamsters ont un système de clairance pulmonaire très efficace, ceci étant démontré par un temps de demi-vie pour la rétention pulmonaire très inférieur dans cette espèce par rapport aux rats ou souris (Bermudez *et al.* (2004)).

Il existe des différences de distribution/dépôt entre les poumons des rats et de l'Homme qui résultent d'importantes différences anatomiques au niveau des bifurcations bronchiques. En conséquence, chez l'Homme, les particules se déposent massivement dans le tissu interstitiel au niveau des aires proches des bifurcations bronchiques. Chez le rat, un dépôt plus intense et plus uniforme au niveau des alvéoles est observé dans la périphérie pulmonaire, bronchioles terminales et zones alvéolaires immédiatement adjacentes, avec une clairance pulmonaire plus rapide que chez l'Homme. Malgré ces différences, l'Homme et le rat présentent des réactions physiopathologiques comparables consécutivement à une exposition particulaire incluant une fibrose interstitielle diffuse, une lipoprotéinose, une fibrose et une hyperplasie des alvéoles et des bronchioles. Ainsi, les effets pulmonaires rapportés chez le rat sont considérés extrapolables à l'Homme (NIOSH, 2011).

Le CES retient donc l'inflammation pulmonaire comme effet critique.

- Choix de l'étude clé

Les données humaines ont toutes été considérées comme inadéquates pour l'établissement de la VTR.

Parmi les études expérimentales de toxicité répétée, la majorité a été réalisée par instillation, ce qui ne permet pas de les utiliser pour l'élaboration de la VTR. En effet, en induisant un effet bolus, et en passant outre le passage des voies aériennes supérieures, ce mode d'administration n'est pas jugé représentatif d'une exposition par inhalation.

Parmi les quelques études de toxicité par inhalation disponibles (Ma-Hock *et al.* (2009); Landsiedel *et al.* (2014); Yu *et al.* (2015); Oyabu *et al.* (2017), Bermudez *et al.* (2004)), l'étude de Bermudez *et al.* (2004) a été retenue comme étude clé. En effet, il s'agit d'une étude de toxicité sub-chronique réalisée avec plusieurs concentrations et sur une forme de TiO_2-NP bien caractérisée par l'OCDE (P25). La caractérisation du matériel testé est jugée essentielle considérant la diversité des formes de TiO_2-NP disponibles sur le marché, présentant des propriétés physicochimiques différentes pouvant impacter sa réactivité et sa cinétique. Néanmoins, certaines limites méthodologiques ont été identifiées :

- Comme seule la toxicité pulmonaire a été analysée dans cette étude, il n'est pas possible de savoir si l'inflammation pulmonaire est réellement l'effet le plus sensible. C'est cependant le cas dans la majorité des études par inhalation disponibles.
- Seules des femelles ont été utilisées dans cette étude. Ce point n'est pas jugé critique, car il n'est pas attendu de forte variabilité inter-sexe quant à la réponse inflammatoire.
- Les rats ont été exposés corps entier, alors qu'actuellement l'exposition par nez seul est privilégiée par l'OCDE. Cependant, au regard de l'effet critique, il n'est pas attendu d'impact majeur de cette voie d'administration. Ceci a été confirmé par Oyabu *et al.* (2016) qui ont comparé les réponses inflammatoires pulmonaires après une exposition au TiO_2-NP par ces deux modes d'administration.
- Etant donné que le P25 n'a pas été dispersé avant exposition, les animaux ont été davantage exposés à de grands agglomérats plutôt qu'à des particules libres ou à des petits agrégats. Même si cela n'est pas protecteur considérant que les particules de plus petites tailles présentent une plus forte réactivité, cette exposition semble plus proche de la réalité de l'exposition chez l'Homme.

Au vu de ces données, et au regard des autres études disponibles, l'étude de Bermudez *et al.* (2004) reste l'étude la plus pertinente pour l'établissement de la VTR. Il est également à noter que les autres études de toxicité répétée par inhalation, même si elles sont réalisées avec d'autres formes de TiO_2-NP et avec des durées d'exposition inférieures (Ma-Hock *et al.* (2009); Landsiedel *et al.* (2014); Yu *et al.* (2015); Oyabu *et al.* (2017)) confortent les résultats de Bermudez *et al.* (2004).

Méthode	Résultats	Commentaires	Référence
Etude subchronique par inhalation Femelles rats (CDF(F344)/CrlBR), souris (B3C3F1/CrlBR) et hamsters (Lak:LVG(SYR)BR) TiO_2 P25 (80% / 20% anatase/rutile, 21 nm) 0, 5 ; 2, 0 ; 10 mg/m^3 pendant 90 jours par inhalation ; exposition corps entier Période de récupération : 4, 13, 26 ou 52 semaines (49 pour les hamsters) Paramètres évalués : mortalité, observations cliniques, prolifération cellulaire, histopathologie des poumons	**<u>Souris :</u>** 4 morts reliées au traitement pendant la phase d'exposition ; diminution réversible du poids corporel dans tous les groupes testés. Augmentation de la charge pulmonaire avec des temps de demi-vie de rétention en fonction de la concentration de 48, 40 et 319 jours. Présence de particules libres dans les macrophages alvéolaires et les régions septales à toutes les concentrations. A la concentration de 10 mg/m^3 : augmentation du nombre de cellules et du nombre total de protéines (non réversible) et de la LDH (réversible) dans le liquide broncho-alvéolaire. Augmentation réversible de la réplication cellulaire. Agrégation de macrophages remplis de particules, avec translocation vers des zones interstitielles. Prolifération lymphoïdes périvasculaires. **Rats :** Diminution réversible du poids corporel dans tous les groupes testés. Augmentation de la charge pulmonaire avec des temps de demi-vie de rétention en fonction de la concentration de 63, 132 et 395 jours. Légère accumulation de macrophages contenant des particules dès 0,5 mg/m^3. Cette accumulation s'accompagne d'une hypertrophie et hyperplasie des cellules épithéliales alvéolaires de type II ainsi que	NOAEC souris = 2 mg/m^3 NOAEC rat = 0,5 mg/m^3 NOAEC hamster = 10 mg/m^3 Limites : uniquement des femelles testées, pas de caractérisation complète dans l'étude, pas de résultats détaillés pour l'histopathologie, pas de dispersion du P25 avant administration. Score Klimisch = 1	Bermudez *et al.*, 2004

	d'une augmentation réversible de la réplication cellulaire à 2 mg/m^3. A la concentration de 10 mg/m^3 : augmentation réversible du nombre de cellules et du nombre total de protéines et de la LDH dans le liquide broncho-alvéolaire. Lésions métaplasiques (bronchiolisation de l'épithélium alvéolaire) avec accumulation de macrophages remplis de particules. **Hamsters :** Perte de poids à la fin de la période d'exposition, partiellement réversible. Augmentation de la charge pulmonaire avec des temps de demi-vie de rétention en fonction de la concentration de 33, 37 et 39 jours. Augmentation des neutrophiles dans le liquide broncho-alvéolaire. Augmentation réversible de la réplication cellulaire à la plus forte concentration, avec présence de particules dans les macrophages.		

- Choix de la dose critique

A ce jour, la plupart des études réalisées avec du TiO_2-NP expriment les expositions en mg/m^3. De nombreuses discussions sont en cours sur la façon d'exprimer les concentrations pour les particules faiblement solubles et en particulier celles sous forme nanométriques. En effet, les concentrations peuvent également être exprimées en aire de surface, en nombre de particules ou en volume. Certaines études suggèrent que la réponse biologique dépend davantage de l'aire de surface que de la masse (Oberdorster, 2002, NIOSH (2011)). L'expression de la concentration en masse reste cependant toujours pertinente et a le mérite d'être communément utilisée (Sager and Castranova (2009) and Noel *et al.* (2017), NIOSH (2011)). Ainsi, en l'absence de consensus, l'expression de la concentration en mg/m^3 est retenue pour la dérivation de la VTR.

D'après l'étude de Bermudez *et al.* (2004), les effets rapportés chez le rat à la concentration de 0,5 mg/m^3 sont une diminution réversible du poids corporel, la présence de particules dans les macrophages alvéolaires et une faible accumulation de macrophages dans les poumons. A la concentration de 2 mg/m^3, des hypertrophies et hyperplasies des cellules alvéolaires de type II et une augmentation réversible de la réplication des cellules alvéolaires et bronchiolaires sont également observés, ainsi qu'une accumulation des macrophages alvéolaires. Les effets deviennent plus sévères à la concentration de 10 mg/m^3 avec des changements métaplasiques dans la région centro-acinaire.

Sur la base de l'effet d'augmentation de la prolifération cellulaire, dans un premier temps, une modélisation BMD a été réalisée, considérant l'existence d'une relation dose réponse. Cependant, cette approche a été écartée aux motifs suivants : faible nombre d'animaux analysés par dose pour le paramètre considéré (n=5), et forte variabilité interindividuelle. Certains critères d'acceptation d'une BMD n'étaient en effet pas remplis (US EPA, 2012) :

- Le ratio BMD/BMDL est d'environ 10, ce qui démontre une forte incertitude ;
- La BMDL est 10 fois plus faible que la plus faible dose testée;
- La valeur de la BMD se situe entre le groupe contrôle et la plus faible dose.

Une BMD ne pouvant pas être établie, un couple NOAEL/LOAEC est proposé.

Sur la base des effets précédemment décrits, la LOAEC retenue est donc de 2 mg/m^3 et la NOAEC de 0,5 mg/m^3.

- Choix de l'hypothèse de construction

Les substances cancérogènes sont traditionnellement divisées en deux catégories selon le mode d'action: génotoxique ou non génotoxique.

Comme indiqué ci-dessus, le TiO_2-NP est un génotoxique faible, dont l'effet n'apparaît qu'à des doses élevées et avec une relation dose-réponse identifiée dans de nombreuses études expérimentales. Les données disponibles indiquent qu'une génotoxicité secondaire, consécutive à un stress oxydatif, serait le principal mécanisme d'action. Les effets cancérogènes apparaissent également à des concentrations élevées, associées à une altération de la clairance pulmonaire et à une réponse inflammatoire.

La qualité des études est importante pour évaluer la génotoxicité d'une substance et choisir entre la construction d'une VTR à seuil ou sans seuil. Pour le TiO_2-NP, la majorité des résultats positifs sont obtenus à partir de tests des comètes. Bon nombre des tests des comètes disponibles ont été réalisés *in vitro*. Ce test des comètes *in vitro* n'est pas un protocole faisant l'objet d'une ligne directrice de l'OCDE, qui sont considérées comme des protocoles standards pour évaluer la mutagénicité des substances chimiques. De plus, ces tests mesurent les lésions précoces de l'ADN qui peuvent être réparées par la suite (Charles *et al.* (2018)).
Pour chercher à évaluer la génotoxicité d'une substance chimique, Brusick *et al.* (2016), dans une approche fondée sur le poids de la preuve, ont attribué un faible poids de preuve à ce type de tests. L'OCDE, citée dans le document méthodologique de l'Anses sur l'élaboration de la VTR (Anses 2017), précise que « *lors de l'évaluation du potentiel mutagène d'un produit chimique à l'essai, il faudrait accorder plus de poids à la mesure des changements permanents de l'ADN (c'est-à-dire les mutations) qu'aux événements réversibles* » (OCDE, 2015). Par conséquent, conformément à la méthodologie Anses, les réponses positives obtenues avec les tests « indicateurs » (mesure des cassures de l'ADN, échanges de chromatides sœurs, etc.) sont certainement associées à l'exposition mais doivent être considérées comme insuffisantes pour déterminer un effet mutagène.

En conclusion : considérant la faible génotoxicité du TiO_2-NP associé à un mécanisme d'action génotoxique décrit dans les études comme majoritairement secondaire et du poids "faible" des tests positifs disponibles pour parvenir à cette conclusion, la construction d'une VTR à seuil est considérée comme le choix le plus pertinent pour le TiO_2-NP.

- Ajustements allométrique et temporel

Le calcul de la concentration équivalente humaine (CEH) pour le TiO_2-NP, a été basé principalement sur la méthodologie utilisée par la « *Maximale Arbeitsplatz-Konzentration* » (MAK) pour le calcul de la valeur limite de la fraction de poussière respirable des poussières granulaires biopersistantes (MAK, 2012).
Cette méthodologie est fondée sur l'hypothèse que la sensibilité du rat et de l'Homme au TiO_2-NP ne diffère pas, pour une même dose de particules par unité de surface pulmonaire. Elle suit les étapes suivantes :

1. Evaluation de la **fraction de dépôt** dans le poumon.

La fraction de dépôt pulmonaire est le ratio du nombre de particules déposées dans les poumons sur le nombre de particules entrant dans le tractus respiratoire.

Pour estimer cette fraction, le modèle MPPD (*Multiple Path Particle Dosimetry*) (version 3.04, 2016) a été utilisé. Ce modèle a été développé par le *Chemical Industry Institute of Toxicology* (CIIT), NC (Caroline du Nord), USA, et l'Institut néerlandais de santé publique et de l'environnement (*Rijksinstituut voor Volksgezondheid en Milieu* (RIVM)). Les valeurs physiologiques (volumes courant, fréquence respiratoire…) utilisées dans les calculs sont celles rentrées par défaut dans le modèle MPPD (cf. ci-dessous). Les demi-vie d'élimination sont issues des publications de Brown *et al.* (2005) pour le rat et Kreyling and Scheuch (2000) pour l'Homme.

Rat : Fraction de dépôt: 0,056 (sans unité)
Homme : Fraction de dépôt : 0,1485 (sans unité)

2. Calcul du **volume de dépôt**, en m^3/jour :

Volume de dépôt = fraction de dépôt × volume courant × fréquence respiratoire × temps d'exposition

Rat : volume de dépôt = 0,056 x (2,1/1 000 000) x 102 x 60 x 6 x 5/7 = 0,003084 m^3/jour
2.1 ml = volume courant du rat
102/min = fréquence respiratoire du rat
60 min x 6 x 5/7 = temps d'exposition de l'étude, exprimée en jours
Homme : volume de dépôt = 0,1485 x (625/1 000 000) x 12 x 60 x 24 = 1,6038 m^3/jour
625 ml = volume courant de l'Homme
12/min = fréquence respiratoire de l'Homme
60 min x 24 = temps d'exposition, exprimé en jours

3. Calcul de la **constante d'élimination**, en jours :

Constante d'élimination = -ln(0,5)/Demi-vie d'élimination

Rat : Constante d'élimination = -(ln0,5)/60 = 0,0116/jour
Homme : Constante d'élimination = -(ln0,5)/400 = 0,00173/jour

4. Calcul de la **charge pulmonaire à l'état d'équilibre**, en m^3 :

Charge pulmonaire à l'état d'équilibre = volume de dépôt /constante d'élimination

A noter que la charge pulmonaire à l'état d'équilibre exprimée en mg/poumon est obtenue en multipliant cette valeur par la concentration de poussière dans l'air en mg/m^3, c'est-à-dire la NOAEC.

Rat : Charge pulmonaire à l'état d'équilibre = 0,003084/0,0116 = 0,2659 m^3
Charge pulmonaire à l'état d'équilibre = 0,2659 x 0,5 = 0,1329 mg/poumon
Homme : Charge pulmonaire à l'état d'équilibre = 1,6038/0,00173 = 927,0520 m^3
Charge pulmonaire à l'état d'équilibre = 927,0520 x 0,5 = 463,5260 mg/poumon

5. Enfin, la charge pulmonaire ramenée à la surface des poumons est calculée pour le rat et l'Homme, et le rapport de ces valeurs est utilisé pour le calcul de la **concentration équivalente humaine** en multipliant par la NOAEC :

$NOAEC_{CEH}$ = NOAEC x (charge pulmonaire à l'état d'équilibre/surface spécifique pulmonaire)$_{rat}$ /(charge pulmonaire à l'état d'équilibre /surface spécifique pulmonaire)$_{humain}$

$NOAEC_{CEH}$ = 0,5 x (0,2659/0,297)/(927,0520/57,22) = 0,5 x (0,8953/16,2015) = 0,5 x 0,055
$NOAEC_{CEH}$ = 0,028 mg/m^3

- Choix des facteurs d'incertitude

Le calcul de la VTR à partir de la $NOAEC_{CEH}$ a été effectué à l'aide des facteurs d'incertitude suivants (Anses, 2017) :

- Variabilité inter-espèce (UF_A): une valeur de 2,5 a été retenue pour prendre en compte la variabilité toxicodynamique et les incertitudes résiduelles.

- Variabilité inter-individuelle (UF_H): en l'absence de données permettant de réduire le facteur par défaut, une valeur de 10 a été retenue.

- Transposition subchronique – chronique (UF_S) : considérant que l'étude de Bermudez *et al.*, (2004) est une étude de toxicité subchronique, une valeur de 3 a été retenue par défaut.

- Utilisation d'une BMDL, LOAEC ou NOAEC (UF_L) : une valeur de 1 a été retenue, le point de départ étant une NOAEC.

- Incertitudes dues aux lacunes de la base de données (UF_D) : une valeur de 3 a été retenue. En effet, aucune étude disponible n'a investigué tous les paramètres, tels que recommandés par les lignes directrices de l'OCDE. De plus, les études disponibles avec le P25 présentaient diverses limites méthodologiques (administration intra-trachéale, une seule concentration testée, etc.). Dans ces conditions, il ne peut être exclu que des effets néfastes puissent apparaitre à des concentrations inférieures à celle produisant des effets inflammatoires.

Le facteur d'incertitude global est donc de 225.

- Proposition de VTR chronique

Une VTR chronique par inhalation de 0,12 µg/m^3 a été dérivée.

Cette valeur est directement applicable au P25 qui est la forme de TiO_2 testée dans l'étude de Bermudez *et al.* (2004).

La pertinence de cette valeur pour les autres formes de TiO_2-NP n'a pu être évaluée considérant l'existence de plus de 350 formes différentes de TiO_2, ayant des propriétés physicochimiques variées. En effet, sur la base de la littérature disponible, les propriétés intrinsèques d'un nanomatériau semblent influencer sa cinétique et sa réactivité.

Concernant la taille du TiO_2, il est attendu une plus forte réactivité des nanoparticules en comparaison des particules sous forme micrométrique du fait d'une augmentation de la réponse pulmonaire consécutive à une altération de la clairance, d'une plus longue biopersistance et d'une pénétration plus en profondeur dans les régions interstitielles des alvéoles. Ainsi, de nombreuses publications rapportent une inflammation pulmonaire plus sévère avec des formes plus petites de TiO_2-NP (Drew *et al.* (2017), Halappanavar *et al.* (2015), Han *et al.* (2012), Hashizume *et al.* (2016), Kobayashi *et al.* (2009), Rahman *et al.* (2017), Noel *et al.* (2013)). Cependant, *a contrario*, d'autres auteurs n'ont pas identifié de lien direct entre inflammation et taille des particules de TiO_2 (Li *et al.* (2007), Rossi *et al.* (2009), Roursgaard *et al.* (2011)).

L'importance de la forme cristalline sur la toxicité a été confirmée par de nombreux auteurs (Okada *et al.* (2016), Warheit *et al.* (2007), Rushton *et al.* (2010), Rahman *et al.* (2017) and Numano *et al.* (2014), Roursgaard *et al.* (2011), Park *et al.* (2014)), même si l'ensemble des données disponibles à ce jour ne permet pas d'identifier la forme cristalline la plus toxique.

La présence d'un revêtement de surface peut également influer sur la cinétique, la production d'espèces réactives et les interactions du TiO_2-NP avec les macromolécules mais aussi potentiellement relarguer des substances toxiques issues de ce revêtement. Bien que cette question ait été peu étudiée, il ressort de la littérature que les formes revêtues avec de l'alumine, avec des groupes amino- chargés positivement, avec des substances hydrophiles ou avec de la silice, pourraient induire une plus forte inflammation pulmonaire en comparaison avec les formes non revêtues en surface (Hashizume *et al.* (2016), Halappanavar *et al.* (2015), Rahman *et al.* (2017), Rossi *et al.* (2009)).

Enfin, l'influence des différentes formes de TiO_2-NP, telles que les nanosphères, les nanotubes, les nano-fibres etc…, sur la toxicité pulmonaire a été étudiée dans la littérature. Il est généralement montré que les formes fibreuses sont plus toxiques que les formes sphériques (Hamilton *et al.* (2009), Porter *et al.* (2013), Silva *et al.* (2013)). Cependant, ces études ont été réalisées avec du TiO_2-NP fabriqué en laboratoire, et la présence des formes non sphériques sur le marché européen reste à ce jour à définir.

Il ne peut pas être établi à ce stade que les données disponibles sur le P25 soient représentatives de toutes les formes de TiO_2-NP. Il ne peut pas également être exclu, en l'état actuel des connaissances, que le P25 soit moins toxique que d'autres formes de TiO_2-NP.

- Niveau de confiance

Un niveau de confiance global a été attribué à cette VTR chronique par voie respiratoire en se basant sur les critères suivants :

- Niveau de confiance dans la nature et la qualité des données :

Faible : les données toxicologiques sont insuffisantes pour évaluer toutes les propriétés toxicologiques de ce composé. De plus, la plupart des études n'ont pas été jugées pertinentes pour la dérivation de la VTR (aucune étude humaine pertinente, voie intra-trachéale, administration unique, forte concentration testée, matériel non caractérisé de façon suffisante, etc…dans des études expérimentales)

- Niveau de confiance dans le choix de l'effet critique et le mode d'action :

Moyen : c'est un effet retrouvé dans de nombreuses études. Cependant, le poumon est le seul organe investigué dans la plupart des études. Les études s'intéressant à d'autres organes ont été réalisées à une seule concentration, élevée et n'ont en général pas évalué la toxicité pulmonaire.

- Niveau de confiance dans le choix de l'étude clé :

Moyen : il s'agit d'une étude bien détaillée. Cependant, cette étude n'a pas été réalisée selon les lignes directrices de l'OCDE. Les autres limites méthodologiques sont décrites dans la section « choix de l'étude critique » du présent avis.

- Niveau de confiance dans le choix de la dose critique :

Moyen : une relation dose-réponse a été observée. Une BMD n'a pas pu être établie mais une NOAEC a été identifiée.

Le niveau de confiance global est donc **: Moyen**.

4. CONCLUSIONS ET RECOMMANDATIONS DE L'AGENCE

L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail endosse les conclusions et recommandations du CES « Valeurs Sanitaires de Référence » qui portent sur la proposition d'une valeur toxicologique de référence chronique par voie respiratoire pour le TiO_2-NP.

Tableau 2 : VTR chronique par voie respiratoire pour le TiO_2 P25

Effet critique (étude clé)	Concentration critique	UF	VTR
Hypertrophies et hyperplasies des cellules alvéolaires de type II, augmentation réversible de la réplication des cellules alvéolaires et bronchiolaires, accumulation des macrophages alvéolaires	NOAEC = 0,5 mg/m³ Ajustement temporel Ajustement allométrique 0,028 mg/m³	225 UF_A = 2,5 UF_H = 10 UF_S = 3 UF_L = 1 UF_D = 3	0,12 µg/m³
Bermudez *et al.* (2004)			Niveau de confiance moyen

L'Anses rappelle que l'expertise a porté exclusivement sur la toxicité par inhalation de la forme nanométrique du TiO_2. Elle n'avait pas pour objectif d'analyser la toxicité par ingestion du TiO_2 sous forme anatase, contenu dans l'additif E171, qui fait l'objet par ailleurs d'une autre expertise par l'Agence.

La forme TiO_2-P25 a été retenue par les experts pour la construction de la VTR chronique par voie respiratoire, s'agissant de celle utilisée dans l'étude clé (Bermudez *et al.*, 2004). Cette étude, qui date de 2004, reste à ce stade l'étude de toxicité répétée disponible la plus robuste. Les résultats de cette étude ont été confirmés depuis sa publication par des études plus récentes. Toutes ces études ont cependant pour limite de n'avoir exploré que les effets pulmonaires. Cette expertise a mis en évidence qu'aucune étude relative aux effets du TiO_2-NP n'a réalisé une évaluation complète (sans organe cible *a priori*) de sa toxicité.

L'Anses souligne que le domaine d'application de cette VTR chronique par voie respiratoire est celui de la forme TiO_2-P25 (anatase/rutile 80/20; 21 nm). En effet, au regard des données disponibles, il ne peut pas être établi que les effets toxiques observés avec le TiO_2-P25 soient représentatifs de toutes les formes de TiO_2-NP, considérant l'existence de plus de 350 formes répertoriées. De même, il ne peut pas être affirmé, en l'état actuel des connaissances, que le TiO_2-P25 soit la forme la plus toxique de de TiO_2-NP par voie respiratoire.
En conséquence, l'analyse de l'extension du champ d'application de cette VTR aux autres formes de TiO_2-NP nécessite la réalisation d'une expertise ad hoc.

Considérant la base de données disponibles sur le TiO_2-NP, des études complémentaires seraient donc utiles au vu des incertitudes existantes, notamment pour évaluer l'influence des différents paramètres physicochimiques sur la toxicité, ou encore pour évaluer la toxicité du TiO_2-NP après une exposition chronique par inhalation et en examinant tous les paramètres physiologiques, et non plus uniquement la toxicité pulmonaire.

Faisant suite à la présente expertise, une réflexion a été engagée par l'Anses sur la possibilité d'élaborer une Valeur limite d'exposition professionnelle (VLEP) applicable au TiO_2-NP.

Pour mémoire, l'Anses instruit par ailleurs auprès de l'ECHA une évaluation des dangers et des risques du TiO_2 pour la santé humaine et l'environnement dans le cadre du règlement REACh.

Dr Roger Genet

MOTS-CLÉS

Valeur toxicologique de référence, VTR, dioxyde de titane, TiO_2, nanoparticule, inhalation, chronique
Toxicological reference value, TRV, titanium dioxide, nanoparticle, inhalation, chronic

Toxicological Reference Value (TRV)

Establishment of Chronic Reference Value by inhalation for Titanium Dioxide under nanoform

Request No. 2017-SA-0162 "TiO_2 TRV"

Collective Expert Appraisal
REPORT

Expert Committee on "Health Reference Values"

December 2018

French Agency for Food, Environmental and Occupational Health & Safety,
14 rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex
Telephone: +33 (0)1 49 77 13 50 - Fax: + 33 (0)1 49 77 26 26 - www.anses.fr

Key words

Titanium dioxide, TiO2, nanomaterial, Toxicological reference value, TRV.

Dioxyde de Titane, TiO2, nanomatériau, Valeur toxicologique de référence, VTR.

Presentation of participants

PREAMBLE: The expert members of the Expert Committees and designated rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organisation.

RAPPORTEURS

Mr Stéphane BINET – Pharmacist toxicologist at the scientific head of INRS – Expertise: general and occupational toxicology

Ms Irina GUSEVA-CANU - Professor of epidemiology, Université de Lausanne, deputy head of health research and teaching, IST – Expertise: Epidemiology, toxicocogy

Mr Claude EMOND – Assistant clinical professor, University of Montréal, Canada – Expertise: Toxicology, Physiologically based pharmacokinetic (PBPK) modelling, toxicokinetics, nanotoxicology, endocrine disruptors

Ms Valérie FESSARD - Head of "Contaminants toxicology unit", Anses – Expertise: genotoxicity

Mr Emmanuel FLAHAUT - Research Director, CNRS – Expertise: physico-chemistry of nanomaterials

Mr Henri SCHROEDER – Research fellow, URAFPA (Research unit – Animal and functionality of animal products), INRA USC 340, Faculty of Sciences and Technologies, University of Lorraine – Pharmacist-biologist – Expertise: Neurotoxicity, animal behaviour, cerebral development, perinatal exposure

Mr Bernard SALLES - Professor of toxicology, unit head – Université de Toulouse and INRS – Expertise: genotoxicity

Mr Olivier SORG – Head of research group, University of Geneva, Switzerland – Doctor of science in biochemistry – Expertise: experimental toxicology, dermatotoxicology

Ms Lydie SPARFEL - Professor, Université de Rennes 1 / IRSET "Research Institute for Environmental and Occupational Health" UMR INSERM 1085 – Pharmacist toxicologist – Expertise: immunotoxicity, toxicogenomic, oncology, cellular and molecular biology.

Ms Paule VASSEUR - Retired Professor of toxicology, toxicologist and ecotoxicologist researcher, Université de Lorraine – Expertise: Toxicology, Environment

EXPERT COMMITTEE "HEALTH REFERENCE VALUES"

- Dates: 4 May 2018, 13 September, 18 October, 29 November

Chair

Mr Fabrice MICHIELS – Occupational physician-toxicologist, Intercompany association for occupational health, Corrèze

Vice-Chair

Mr Raymond VINCENT – Retired (formerly project manager at the Direction of Prevention Applications (INRS)) – Expertise: chemistry, pollutants metrology, occupational health risks assessment.

Members

Mr Marc BARIL – Associate professor, University of Montréal, Canada – Expertise: Toxicologist/chemist, occupational exposure limits (OELs)

Mr Stéphane BINET – Pharmacist toxicologist at the scientific head of INRS – Expertise: general and occupational toxicology

Ms Michèle BISSON – Study director, INERIS – Expertise: Pharmacist-toxicologist, general toxicology

Ms Anne CHEVALIER – Retired epidemiologist, French Institute for Public Health Surveillance (InVS) - Expertise: Epidemiology

Ms Fatiha EL-GHISSASSI – Scientist, IARC Monographs Section (IMO) International Agency for Research on Cancer – Expertise: biochemistry. Cancerogenicity and genotoxicity

Ms Mounia EL-YAMANI – Unit head, InVS – Expertise: Biochemistry, toxicology

Mr Claude EMOND – Assistant clinical professor, University of Montréal, Canada – Expertise: Toxicology, Physiologically based pharmacokinetic (PBPK) modelling, toxicokinetics, nanotoxicology, endocrine disruptors

Mr Rex FITZGERALD – Regulatory toxicology expert at the Swiss Centre for Applied Human Toxicology – Expertise: Reprotoxicity, developmental neurotoxicity, human risk assessment

Mr Robert GARNIER – Toxicologist physician, Paris poison control center – Expertise: Medical toxicology, occupational medicine

Ms Perrine HOET – Professor, Université catholique de Louvain. Institute of Experimental and Clinical Research – Expertise: Medicine, occupational and environmental toxicology

Ms Yuriko IWATSUBO – Epidemiologist physician, SPF – Expertise: occupational risks epidemiology

Ms Cécile KAIRO – Health risks assessor, SPF – Expertise: Doctor of Pharmacy specialized in environmental medicine, general toxicology and risks assessment

Ms Laila LAKHAL – Engineer, INRA, Toxalim unit – Expertise: toxicology, metabolism, endocrine disruptors

Mr Frédéric LIRUSSI – Lecturer – Hospital doctor (PU-PH), Health Sciences & Dijon University Hospital UFR – Expertise: Clinical toxicology, analytical toxicology, innate immunity, reprotoxicity

Ms Anne MAITRE – Professor – Hospital doctor (PU-PH), Occupational and environmental toxicology laboratory, Grenoble CHU; Team leader of “Environment and Health Prediction in Populations” TIMC laboratory, Université Grenoble Alpes – Expertise: Medicine, toxicology, biomarkers of exposure, pollutants metrology, occupational hygiene

Ms Florence PILLIERE – Medical advisor, INRS – Expertise: occupational medicine, toxicology, biomarkers of exposure

Ms Anne PLATEL – Lecturer, Faculty of biological and pharmaceutical sciences of Lille – Genetic toxicology laboratory, Institut Pasteur de Lille – Expertise: toxicology, genotoxicity, QSAR

Mr Henri SCHROEDER – Professor Associate, CALBINOTOX, EA 7488, Faculty of Sciences and Technologies, University of Lorraine – Pharmacist, neurobiologist – Expertise: Neurotoxicity, animal behaviour, cerebral development, perinatal exposure

Mr Olivier SORG – Head of research group, University of Geneva, Switzerland – Doctor of science in biochemistry, experimental toxicology, dermatotoxicology

Mr Jérôme THIREAU – Research assistant, CNRS – Doctor of science, animal physiology, cell biology, cardiotoxicity

Mr Claude VIAU – Retired full professor and currently adjunct professor Department of Environmental and Occupational Health, School of Public Health, University of Montreal – Expertise: Toxicology, Biological Exposure Indices, pollutant metrology,

ANSES PARTICIPATION

Scientific coordination

Ms Pauline GUILLOU – Scientific Project Leader

Scientific contribution

Ms Pauline GUILLOU – Scientific Project Leader

Ms Sandrine CHARLES – Scientific Project Manager

Ms Hélène SERRA – Scientific Project Leader

Mr Christophe ROUSSELLE – Head of the Chemical Risk Assessment Unit

Administrative assistance

Ms Séverine BOIX-PETRE

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Acronyms and abbreviations

Ach	Acethylcholine
ANSES	*Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail* [French Agency for Food, Environmental and Occupational Health & Safety]
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AST	Aspartate Transaminase
BALF	Bronchoalveolar Fluid
BER	Base Excision Repair
BET	Brunauer–Emmett–Teller surface area analysis
BMD	Benchmark Dose
BUN	Blood Urea Nitrogen
BSA	Bovine Serum Albumin
BW	Body Weight
CAMKIV	Calcium/calmodulin-dependent protein kinase type IV
CES	ANSES Expert Committee
CIIT	Chemical Industry Institute of Toxicology
CINC-1	Cytokine-Induced Neutrophil Chemoattractant 1
CLP	*Classification Labelling and Packaging*
CMR	Carcinogenic, Mutagenic, Reprotoxic
COX	Cyclo-oxigenase
CRC	Chemical Rubber Company
DAF	Dosimetric Adjustment Factor
DGCCRF	*Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes* [Directorate General for Competition Policy, Consumer Affairs and Fraud Control]
DFOSB	Truncated form of FosB missing the C-terminal 101 amino acids
DGS	*Direction Générale de la Santé* [Directorate General for Health]
DHPN	N-bis(2-hydroxypropyl)nitrosamine
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DNEL/DMEL	Derivative No Effect Level / Derivative Minimum Effect Level
DSP	Daily Sperm Production
ECHA	European Chemicals Agency
ESTR	Expanded Simple Tandem Repeat
FGF-18	Fibroblast Growth Factor-18
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GD	Gestational Day
GLP	Good Laboratory Practice
GSD	Geometric Standard Deviation
HCSP	High Council for Public Health

HDL-C	High-Density Lipoproteins Cholesterol
HEC	Human Equivalent Concentration
HIF-1α	Hypoxia-Inducible factor 1-alpha
HMPC	Hydroxypropylmethylcellulose
HO-1	Heme oxygenase 1
IARC	International Agency for Research on Cancer
IFN-γ	Interferon gamma
IL	Interleukine
INERIS	Institut national de l'environnement industriel et des risques [French National Institute For Industrial Environment And Risks]
LDH	Lactate Dehydrogenase
LY/LYP	Lymphocytes/Percentage of Lymphocytes
LOAEC	Lowest-Observed-Adverse-Effect Level
M-CSF	Macrophage Colony-Stimulating Factor
MAK	Maximale Arbeitsplatz-Konzentration
MCP	Monocyte Chimoattractant Protein
MCV	Mean Corpuscular Volume
MDA	Maleic Dialdehyde
MDC	Macrophage-Derived Chemokine
MIP-2	Macrophage Inflammatory Protein 2
MMAD	Mass Median Aerodynamic Diameter
MMP-9	Matrix Metalloproteinase 9
MPPD	Multiple Path Particle Dosimetry
NER	Nucleotide Excision Repair
NIOSH	National Institute for Occupational Safety and Health
NMDA	*N*-methyl-D-aspartate receptor
NO	Nitric Oxid
NOAEC	No-Observed-Adverse-Effect Level
NP	Nanoparticle
NK	Natural Killer
OECD	Organisation for Economic Co-operation and Development
OEL	Occupational Exposure Limit
OVA	Ovalbumin
PBS	Phosphate-Buffered Saline
PBS-HEC	Phosphate-Buffered Saline - Hydroxyethyl cellulose
PCNA	Proliferating Cell Nuclear Antigen
PMN	Polymorphonuclear Neutrophils
PND	Postnatal Day
QSAR	Quantitative structure-activity relationship
RAC	Risk Assessment Committee
REACh	Regulation (EC) No 1907/206 of 18/12/06 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACh)

RDI	Relative Deposition Index
RDW	Red Blood Cell Distribution Width
RIVM	*Rijksinstituut voor Volksgezondheid en Milieu* [Netherlands National Institute for Public Health and the Environment]
RMOA	Risk Management Option Analysis
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAA	Serum Amyloid A
SMR	Standardized Mortality Ratio
SNP	Sodium Nitroprusside
TG	Triglycerides
TEM	Transmission Electron Microscopy
TiO_2	Titanium dioxide
UF	Uncertainty Factors
VEGF-α	Vascular Endothelial Growth Factor-alpha
VT	Tidal Volume
WBC	White Blood Cell

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1 Background, purpose and procedure for carrying out the expert appraisal

1.1 Background

A toxicological reference value, or TRV, is a toxicological index qualifying or quantifying a risk to human health. It establishes the link between exposure to a toxic substance and the occurrence of an adverse health effect. TRV are specific of a duration of exposure (acute, subchronic or chronic) and a route of exposure (oral or inhalation). The construction of TRV differs according to the knowledge or assumptions made about the mechanisms of action of the substances. Currently, the default assumption is to consider a monotonous relationship between exposure, or dose, and effect, or response. Based on current knowledge and by default, it is generally considered that, for non-carcinogenic effects, toxicity is only expressed when a dose threshold is exceeded (Anses, 2017a).

In practice, the construction of a TRV involves the following steps:

- identify and analyse available toxicity data, based on epidemiological and/or experimental studies;
- identify the target organ(s) and critical effect;
- identify the establishment assumption, with or without a dose threshold, depending on the mode of action of the substance;
- choose a key study of good quality that allows establishing a dose-response relationship;
- define a critical dose in humans or animals from this study, and in the case of a critical dose obtained in animals, adjust this dose to humans;
- for a threshold TRV, apply uncertainty factors to this critical dose in order to derive a TRV applicable to the entire population concerned;
- for a TRV without threshold, perform a linear extrapolation at the origin to determine an excess of unit risk.

The development of TRVs follows a highly structured and stringent approach involving collective evaluations by groups of specialists.

1.2 Purpose of the request

On the 28 June 2017, Anses received a formal request from INERIS to provide an advice on the proposal of INERIS benchmark values set for titanium dioxide under nanoform (N° DRC-16-157027-10246A) dated 04 November 2016.

In 2017 July 4, Anses was also mandated by the Directorate General for Health (DGS), Directorate General for Risk Prevention (DGPR), and Directorate General for Labour (DGT) to establish a chronic TRV by inhalation for TiO_2 under nanoform. In parallel, they also requested the opinion of the French High Council for Public Health (Haut Conseil de Santé Publique – HCSP) on management measures designed to protect workers in sites that manufacture and handle titanium dioxide nanoparticles (TiO_2-NPs) with dimensions inferior to 100 nm, and populations in the vicinity of such sites. HCSP released its opinion on 29th April 2018.

Those requests are the results of the analysis of the R-Nano database indicating that many industrial sites in France handled TiO_2 under nanoform, which can be responsible of worker exposure but also exposure of general population *via* environmental emission.

1.3 Procedure: means implemented and organisation

Ten external and Committees experts have been named rapporteurs.

Anses entrusted examination of this request to the Expert Committee (CES) on "Health Reference Values".

The methodological and scientific aspects of the rapporteurs' expert appraisal work were regularly submitted to the CES. The report issued by the rapporteurs takes into account the comments and additional information provided by the members of the CES.

This work was therefore conducted by a group of experts with complementary skills.

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

1.4 Prevention of risks of conflicts of interest

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals.

The experts' declarations of interests are made public via the Anses website (www.anses.fr).

2 General Information

2.1 Substance identity

Table 1 : Substance identity

Name	Titanium Dioxide
CAS number	13463-67-7 (All forms) 1317-70-0 (Anatase) 1317-80-2 (Rutile) 12188-41-9 (Brookite)
EC number	236-675-5
Synonymes	Dioxotitanium
Molecular formula	TiO_2
Structural formula	

TiO_2 exists under micro and nanosize. The present report refers specifically to TiO_2 under nanoform (namely TiO_2-NP in this report). According to European Commission (EC, 2011), a nanomaterial means:

"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %."

2.2 Physico-chemical properties

Table 2 : Physico-chemical properties (CRC Handbook of Chemistry and Physics, Lide 2000, Weast 1991)

State of the substance at 20°C and 101,3 kPa	Solid, crystalline, white, odourless inorganic substance.
Molecular weight (g/mol)	79.87
Boiling point	ca. 3000 °C
Melting/freezing point	Anatase: 1560 °C, Rutile: 1843 °C, Brookite: 1825 °C
Relative density (g/cm^3, 20 °C)	Anatase = 3.9, Rutile = 4.26, Brookite = 4.17
Water solubility	Not soluble
Solubility in organic solvent	Not soluble
Partition coefficient n-	Not soluble

octanol/water	

2.3 Discussions on different forms of TiO_2

Intrinsic physico-chemical properties of a nanomaterial, such as particle crystallinity, size, surface area and surface modification, are presumed to influence its reactivity and behaviour.

Regarding **crystallinity**, three main naturally titanium dioxide polymorphs exist: rutile, anatase and brookite, the most commonly studied and used being rutile and anatase (Carp, Huisman, and Reller 2004, NIOSH 2011, IARC 2010).

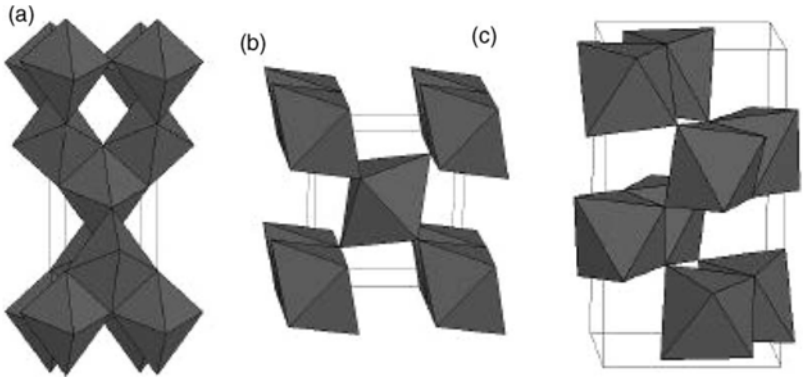


Figure 1 : Crystal structures of anatase (a), rutile (b), and brookite (c) from Carp, Huisman, and Reller (2004)

The importance of crystal form in assessing the pulmonary toxicity of TiO_2-NP was confirmed by different authors but with contradictory conclusions.

A distinct inflammatory potential was noted by Aragao-Santiago et al. (2016) between anatase TiO_2-NP and rutile TiO_2-NP**,** in which the latter did not induce inflammatory response. Okada et al. (2016) found that mixed-crystal phase and amorphous TiO_2-NP engender the most severe fibrosis compared to anatase and rutile forms. Warheit et al. (2007) and Rushton et al. (2010) also observed a more pronounced inflammation with mixed-crystal phase. 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. A higher inflammatory response for rutile form compared to anatase and amorphous TiO_2-NP was also reported by Roursgaard et al. (2011); but in addition, they identified amorphous polymorph TiO_2-NP as the most potent in regard to acute tissue damage, based on the level of total protein in BALF. Park et al. (2014) studied differences in pulmonary toxicity of anatase and brookite TiO_2-NP nanorods prepared at laboratory. They found that the brookite form caused more severe and frequent lesions in the lung than the anatase form, along with higher cytokine levels in BALF.

Regarding **particle size**, nanoparticles are expected to be more reactive than bulk materials with an increase in the pulmonary response due to a delayed clearance, longer biopersistence and

deeper penetration into interstitial regions of alveoli. In particular, Drew et al. (2017) found that metrics related to 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 BALF, lung histopathology) after an intratracheal exposure to various forms of titanium dioxide (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-NP could possibly be due to their less efficient penetration into cells (the smaller size of particles would facilitate their possible and rapid translocation[1]). Contrasting with these findings, other authors did not evidence a direct association between particle size and inflammatory potential of TiO_2-NP (Li et al. 2007, Rossi et al. 2009, Roursgaard et al. 2011)

Moreover, the behaviour of nanoparticles in the medium, the production of reactive oxygen and nitrogen species or the interaction with macromolecules, can also be influenced by the **presence of a coating** which may itself also release toxic material (Charles et al. 2018). Even if coated forms of TiO_2-NP are not commonly 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-NP 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 the important role of coating with an exacerbation of the pulmonary response when animals were exposed to TiO_2-NP covered with an hydrophilic coating, compared with no or hydrophobic coating. Among different forms of TiO_2-NP tested, Rossi et al. (2009) found that only Si-coated rutile TiO_2-NP elicited clear pulmonary inflammation compared to uncoated TiO_2-NP.

Finally, the influence of different **shapes of TiO_2-NP** such as nanospheres, nanobelts, nanorods, nanodots, needles, tubes, fiber-like, on lung toxicity have been studied in the literature. For example, Hamilton et al. (2009) demonstrated that alteration of TiO_2-NP into a fibre structure greater than 15 µm creates a highly toxic particle which initiates an inflammatory response by alveolar macrophages. Similar conclusions were reached by Porter et al. (2013) or Silva et al. (2013) who reported more severe pulmonary responses with nanobelts compared to nanospheres. In contrast, Warheit et al. (2006) did not find significant differences in the pulmonary responses between anatase nanodots and anatase nanorods, despite a six-fold difference in the surface area. It is worth to note that these publications refer to self-synthesized TiO_2-NP which actual relevance of on the European market cannot be assessed.

[1] Translocation : movement away from the site of deposition either within or outside the lungs (Elder, Nordberg, and Kleinman 2015)

3 Summary of the toxicological data

The synthesis of toxicological data was prepared on the basis of a systematic literature review performed in December 2017. Key words were selected in order to obtain a toxicological profile of TiO_2-NP based on studies published from 2000.

Studies not related to toxicological experimentand studies not performed with TiO_2-NP alone have been excluded based on the title and/or abstract.

The review of the articles was then focused on *in vivo* studies by inhalation performed with TiO_2-NP, which had to be sufficiently characterised. For transparency and tracability, reading grids were filled for all full-text articles read. In order to assess the reliability of the studies potentially eligible for the establishment of the chronic TRV by inhalation, Klimisch criteria were applied using ToxRTool software.

Details of this bibliographic research are presented in Appendix 2.

3.1 Toxicokinetics

As an introduction, it has to be noted that kinetics of TiO_2-NP after inhalation, as an inorganic particle, depends only on the extent of lung deposition and clearance. This section will therefore mainly deal with respiratory tract kinetics.

Lung kinetics:

Lung is the portal of entry for inhalation exposure to TiO_2-NP, and many studies have focused on local pulmonary fate of TiO_2-NP.

Oyabu et al. (2017) assessed the biopersistence of TiO_2-NP (spindle-shaped; 12x55 nm) in the lung after instillation and inhalation for 4 weeks. With the two conditions of exposure, the biological half-life was approximately 2 months. The authors suggest the biopersistence to be a good indicator of TiO_2-NP hazard, as a good correlation was found between biopersistence and effects observed. Similar retention half-times in the lung (≥ 60 days) were reported for TiO_2-NP (P25; anatase/rutile; 21 nm) after inhalation for 13 weeks in rats (Bermudez et al. 2004) or after 3 instillations at a 4-day interval (Relier et al. 2017). This biological half-life value was obtained at concentrations inducing lung inflammation but without being in an overload situation.

Eydner et al. (2012) nose-only exposed rats to 10 mg/m^3 TiO_2 P25, 6h/day for 21 consecutive days, with the aim to assess the Relative Deposition Index (RDI). Particle deposition took place mainly in alveolar macrophages and to a lesser extent in type-I pneumocytes, and no particles were found in cell organelles such as mitochondria or nuclei.

Shinohara et al. (2014) reported a dose-dependent accumulation following acute intratracheal exposure in rat and proposed a simple model to describe the clearance of TiO_2 P25 in lung. They concluded that the translocation is a slower process than the lung clearance, and, in addition, lung clearance is more influenced by the dose, the higher the dose, the lower the elimination.

Zhang et al. (2015) compared pulmonary TiO_2-NP (P25) microdistribution in rats administered intratracheally with one or multiple dose at the same total dosage. The results suggested that multiple-dose administrations do not offer more advantages over single-dose administration in the study of pulmonary NP microdistribution: there are no prominent differences in the pattern of the pulmonary microdistribution of TiO_2. However, the multiple-dose administration reduced variations in the TiO_2 content in each lung lobe (Zhang et al. 2016).

Distribution to other organs:

It is generally recognized that an estimated 1% or less of TiO_2-NP deposited in the lungs translocates to systemic circulation and enters other organs. To investigate this postulate, a few recent studies investigated the translocation from lung and distribution of TiO_2-NP in the organism.

Based on the detection of nanoparticles in a granulocyte located inside a capillary, Eydner et al. (2012), suggested that distribution to other organs *via* the blood circulation is possible, although only to a minimal extent.

Kreyling and colleagues analysed the tissue distribution of anatase TiO_2-NP following a single intratracheal instillation exposure (Kreyling, Holzwarth, Haberl, Kozempel, Wenk, et al. 2017, Kreyling, Holzwarth, Schleh, et al. 2017). They observed a translocation of TiO_2-NP across the air-blood barrier into the circulation, leading to small but persistent TiO_2-NP accumulation in almost all studied organs and tissues. The largest fraction of translocated TiO_2-NP was found in soft tissue followed by skeleton while the highest concentrations per organ weight were found in kidneys, liver and spleen. This resulted in a similar distribution pattern compared to the gavage exposure (Kreyling et al. 2017b), but very different from intravenous exposure (Kreyling, Holzwarth, Haberl, Kozempel, Hirn, et al. 2017). Moreover, the authors confirmed that the TiO_2-NP cleared from the lungs after instillation can be absorbed in the gastrointestinal tract.

Another interesting study explored the distribution of TiO_2-NP (20 nm anatase) in rat tissues following a 6h nose only inhalation of 15 mg/m^3 TiO_2-NP (Pujalte, Serventi, et al. 2017). The authors confirmed translocation of particles to blood and distribution in others tissues (liver, kidneys or pancreas). They also found detectable amount of TiO_2-NP in brain.

Husain et al. (2015) demonstrated the presence of TiO_2-NP in liver and heart after acute instillation exposure to TiO_2 UV-Titan L181.

Gate et al. (2017) focused on the differences between young and elderly rats exposed 4 weeks by inhalation to TiO_2 P25. They confirmed translocation to liver and spleen of TiO_2-NP, but never found difference with control group for kidneys or brain. They observed that the amount recovered in spleen and liver was higher in old than in young adults. According to the same authors, the elimination from lung was slower in old rats.

Excretion:

Pujalte, Dieme, et al. (2017) showed that TiO_2-NP is mainly excreted in feces following inhalation, compared to urine. The authors stated that these data combined with the observed time courses of TiO_2-NP in lung, blood and lymph nodes are compatible with a mucociliary clearance from the respiratory tract and ingestion of particles, as concluded by Kreyling et al. (2017c).

3.2 Acute toxicity

3.2.1 Pulmonary effects

The following acute toxicity studies focused on pulmonary effects. All studies reported pulmonary inflammation to various extent depending on the protocol used.

Grassian and colleagues investigated the pulmonary effects of two different TiO_2-NP (5 nm anatase and 21 nm anatase/rutile) with a comparable protocol of exposure (about 0.7 and 7 mg/m^3 by inhalation for 4 hours). Similar effects were reported with both forms at high concentration, including inflammation in the BALF without histopathological changes in the lung (Grassian, Adamcakova-Dodd, et al. 2007, Grassian, O'Shaughnessy P, et al. 2007).

Macrophage-engulfed pigment-like components were reported in the lung after 6 hour-exposure to rutile TiO_2 (10x50 nm) at 4 mg/m^3 by whole body or nose-only protocol, suggesting no difference between these two types of administrations (Oyabu et al. 2016).

Noel et al. (2012) also showed that an acute (6 hours) inhalation of 5 nm TiO_2 (anatase) with two distinct agglomeration states, smaller or larger than 100 nm, induced mild pulmonary effects at 7 mg/m^3.

In a study of Leppänen et al. (2011), mice were exposed by nose-only inhalation to anatase:brookite (3:1) 20 nm TiO_2 for 30 min to 0, 8, 20 and 30 mg/m^3. The main effect was an airflow reduction, which occurred at each studied concentration. Thereafter, the same authors investigated respiratory effects in mice following 30 min exposure to nano silica-coated rutile TiO_2 (10 x 40 nm) at 0, 5, 10, 20 and 30 mg/m^3. The exposure induced first phase of pulmonary irritation (rapid and shallow breathing), starting at 10 mg/m^3 exposure, but did not induce inflammation (Leppanen et al. 2015)

Numerous acute toxicity studies by intratracheal instillation are also available. Although not transposable quantitatively, as they are not representative of normal exposure (the upper respiratory tract is bypassed), they can bring additional information for hazard identification. Most of those studies showed similar pulmonary effects as studies by inhalation (Oberdörster et al. 2000, Renwick et al. 2004, Chen et al. 2006, Nemmar, Melghit, and Ali 2008, Nemmar et al. 2011, Liang et al. 2009, Sager and Castranova 2009, Cho et al. 2010, Roberts et al. 2011, Tang et al. 2011, Hurbankova et al. 2013, Husain et al. 2013, Husain et al. 2015, Lee et al. 2014, Choi et al.

2014, Oyabu et al. 2013, Yoshiura et al. 2015, Kobayashi et al. 2016, Wallin et al. 2017, Saber et al. 2013). In contrast, another study did not show any effect of anatase TiO_2-NP 7 nm at 0.08 mg/ml (0.2 mg/0.4 ml) (Horie et al. 2012).

Unfortunately, the doses used in instillation studies are difficult to compare with each other due to different metrics used by the authors, and even more so with inhalation studies.

3.2.2 Cardiovascular effects

A series of publications from the same team studied the effect of TiO_2 P25 (anatase/rutile; 21 nm) exposure on microvascular function (Nurkiewicz et al. 2008, Nurkiewicz et al. 2009, LeBlanc et al. 2009, LeBlanc et al. 2010, Knuckles et al. 2012, Stapleton, McBride, et al. 2015). Animals were exposed to different time-concentration combinations in the first study chronologically, and then to 6 mg/m^3 for 4 hours in the other ones.

Those studies showed that:

- Inhalation of P25 caused an impaired vasodilatation capacity in the systemic microcirculation;
- This reduced vasoreactivity was observed after co-exposure to ACh (activation of endothelial NO synthase and prostaglandin production), A23187 (interaction with endothelial cells to increase intracellular Ca^{2+} concentration and subsequently stimulation of nitric oxide production), or an active hyperemia (through the stimulation of muscular contraction), while smooth muscle responsiveness to NO remained unaltered (co-exposure to sodium nitroprusside (SNP), an NO “donor”, causes no differences between control and exposed group);
- Those changes are consistent with an endothelial dysfunction (microvascular NO bioavailability compromised after nanoparticle exposure);
- COX inhibition significantly decreased arteriolar-induced dilation in exposed animals ;
- This observation is consistent with a COX mediated compensation for the reduced NO bioavailability;
- Exposure to P25 increased ROS (reactive oxygen species) and RNS (reactive nitrogen species) production in the microvascular wall;
- The impairment of vasodilation was restored by incubation with ROS scavengers;

These studies shows that acute exposure to P25 induces vascular dysfunction via ROS generation, which leads to reduce NO bioavailability, and ultimately impairs vasodilation. These results suggest that COX pathways would mediate a compensation mechanism for the reduced NO bioavailability. Moreover, considering the observations described above, it can be estimated that the cardiovascular effects observed are concomitant with a weak pulmonary inflammatory effect.

The effects on cardiovascular function after acute exposure were also observed in other studies performed by instillation:

In the study of Savi et al. (2014), male rats were exposed to TiO_2-NP (25-35 nm; anatase/rutile) at a single dose of 2 mg/kg by instillation. The authors observed that TiO_2-NP enhanced the susceptibility to cardiac arrhythmias, via shortening of repolarization time, and increasing of cardiac excitability. The authors also demonstrated the presence of TiO_2-NP into cardiomyocytes via Transmission Electron Microscopy (TEM). In the study of Saber et al. (2013), female mice were exposed intra-tracheally once to 18, 54 and 162 µg of TiO_2-NP (UV Titan L181, rutile surface coated, 17 nm). Exposure to UV Titan L181 increased pulmonary Serum Amyloid A (SAA, a risk factor for cardiovascular disease in mice) mRNA expression in a time- and dose-dependent manner. The strongest response was seen at the early time points (400-fold increase in Saa3 mRNA expression in lung at day 1 at the highest dose). Saa3 expression remained significantly increased 28 days after exposure in all mice exposed to the highest dose. According to the authors, this result indicates a long-lasting induction of acute phase response.

3.3 Repeated dose toxicity

3.3.1 Animal data

Table 3 presents the repeated studies conducted by inhalation, with several or single concentrations. The studies conducted by instillation route are discussed in the text below the table.

Table 3 : Repeated studies by inhalation route

Method	Results	Remarks	Reference
Studies with several concentrations			
13-week study by whole-body exposure Females B3C3F1/CrlBR mice, CDF(F344)/CrlBR rats, Lak:LVG(SYR)BR hamsters 25/species/time point Uf-TiO_2 (P25, average primary particle size of 21 nm) 0.5, 2.0, or 10 mg/m^3 Corresponding to actual concentrations - mice: 0.54 ± 0.06, 2.2 ± 0.1 and 10.8 ± 1.0 mg/m3 - rats: 0.52 ± 0.03, 2.1 ± 0.1, and 10.5 ± 0.7 mg/m3 - hamsters: 0.53 ±0.03, 2.1 ± 0.1, and 10.7 ± 0.6 mg/m3 6 h/day, 5 days/week, for 13 weeks, whole body Additional recovery groups for post-exposure periods of 4, 13, 26, or 52 (49 for hamster) weeks in clean air. MMAD = 1.37 µm (1.29-1.44µm) Parameters: mortality, clinical observations, body weight, BALF, lung cell proliferation	**Mice:** Treatment-related mortalities during the exposure phase of the study (4). During post-exposure, 4 deaths not treatment related. Reversible depression of body weight gain in all groups. Increased TiO_2 burden in the lung and in lymph nodes at 10 mg/m^3): retention half-times in the lung: 48, 40, and 319 days for each concentration, respectively. At 0.5 and 2 mg/m^3: Particles free and within alveolar macrophages and in alveolar septal regions. At 10 mg/m^3: ↑ total number of cells and total proteins (still significant at 52 weeks) and LDH (not significant at 26 week post-exposure) in BALF. Reversible increase in terminal bronchiolar cell replication Aggregations of heavily particle laden macrophages in central lobar centriacinar sites which concentrated over time post-exposure and moved to interstitial areas. Perivascular lymphoid proliferation. **Rats:** In the post-exposure phase, 7 deaths not treatment related. Reversible depression of body weight gain in all groups. Increased TiO_2 burdens in lung (similar to mice) and in lymph nodes (mid and high doses): retention half-times in the lung: 63, 132, and 395 days, for each concentration, respectively. At 0.5 mg/m^3: Particles within alveolar macrophages and very minimal changes in the patterns of alveolar macrophage accumulation in the lungs. At 2.0 mg/m^3: Particle laden macrophage accumulation with minimal	NOAEC = 2.0 mg/m^3 in mice NOAEC = 0.5 mg/m^3 in rats NOAEC = 10 mg/m^3 in hamsters No sonication performed before administration. Only females tested. Only examination of lung response. No full characterization of the tested material but P25 is a well-characterized form of TiO_2. No detailed results on histopathology. Reliability = 1[2] **Key study**	Bermudez et al. (2004)

[2] Reliability was assessed via the software ToxRTool

and lung histopathology	hypertrophy and hyperplasia of type II alveolar epithelial cells. Significant increase in terminal bronchiolar cell and in alveolar cell replication (mid and high dose) reversible during recovery period. At 10 mg/m^3: ↑ total number of cells (normal by 26 weeks post-exposure), total protein (normal by 4 weeks post-exposure) and LDH (normal by 26 weeks post-exposure) in BALF. Metaplastic changes in the centriacinar region (bronchiolization of alveolar epithelium) associated with particle and particle-laden macrophage accumulation – not fully reversible at the 52-week final sacrifice. **Hamsters:** ↑ morbidity and mortality (35 animals during the postexposure phase of the study), not treatment related BW loss at the end of the exposure (9–15%) with a slow recovery. ↑ TiO_2 lung burdens lower than rats and mice; retention half-times in the lung: 33, 37, and 39 days, for each concentration, respectively. BALF: significant ↑ neutrophils at the end of exposure. Significant terminal bronchiolar cell replication at the high dose (normal at 4 week post-exposure). Alveolar and interstitial macrophages containing particles and occasional aggregation of particle-laden macrophages in the high dose group. No pathology.		
Repeated-dose toxicity study by nose-only inhalation Male Wistar rats 6 rats/time point/dose Uncoated TiO_2 with hydrophobic surface (14% rutile, 86% anatase). Average primary particle size : 25.1±8.2 nm (13-71 nm)	No significant effect on BW. Increase in lung weights immediately after exposure in the 50 mg/m^3 group. Concentration related increases in total cell counts (due to increased numbers of PMN), total protein content and enzyme activities. After exposure, increased PMN counts and increased γGT activity in animals exposed to 10 mg/m^3, whereas exposure to 50 mg/m^3 resulted in increased total protein content and activities of all 4 enzymes examined. Three days after exposure, minimal effects on total protein and on some enzyme activities also observed at 2 mg/m^3 ➔ most prominent changes in biochemical and cytological parameters 3 days after exposure. After recovery period of 16 days most of these parameters returned to control level. Immediately after exposure, MCP-1, MCP-3, M-CSF, MDC, MIP-2, myeloperoxidase and osteopontin increased in rats exposed	LOAEC = 2 mg/m^3 Probably TiO_2 P25 but not specifically named in the publication. Only males. Too high concentrations tested as no NOAEC was identified Reliability = 2 Supportive study	Ma-Hock et al. (2009)

2.0, 10 or 50 mg/m^3 Nose-only exposure 6h/day for 5 days followed by a recovery period of 3 or 16 days Parameters: lung burden analysis, BALF, cell mediators in BALF and serum, haematology and serum troponin I, histopathology (lung, nasal cavity and larynx), cell proliferation and apoptosis	to 10 mg/m^3 and these parameters together with clusterin and haptoglobin also significantly increased in rats exposed to 50 mg/m^3. Three days after exposure, clusterin and haptoglobin levels increased in rats exposed to 2 mg/m^3. With exception of osteopontin, levels of all other mediators increased in both the 10 and 50 mg/m^3 groups. No significant changes in haematological parameters observed in all exposure groups. No evidence for any heart muscle damage. Minimal and minimal to mild diffuse alveolar infiltration with histiocytes at 10 mg/m^3 and 50 mg/m^3. Hypertrophy/hyperplasia of bronchioles and bronchi at 50 mg/m^3. Increases in labelling indices in both large/medium bronchi and terminal bronchioles in all treatment groups after the end of the exposure period.		
Repeated-dose toxicity study by nose-only exposure Male Wistar rats (8/group) nano-TiO_2 (T-Lite SF, 15x50 nm) rutile with minimal anatase, coated with dimethicone/methicone copolymer Concentration: 0.5, 2 and 10 mg/m^3 mg/m3 Exposure: 6 h/day for 5 days, and 3 week post-exposure for the group exposed to 10 mg/m^3 Parameters: measurement of cells and marker of inflammation in the BALF, and lung histopathology	No effects observed at 0.5 mg/m^3 At 2 mg/m^3 and 10 mg/m^3: -concentration-dependent increase in PMN and monocytes in the BALF - increase in LDH and ALP release At 10 mg/m^3: numerous pigment-loaded alveolar macrophages within the alveoli and slight diffuse histiocytosis not fully reversible after 3 weeks of recovery.	LOAEC= 2 mg/m^3 NOAEC = 0.5 mg/m^3 Only males tested. Good characterization of TiO_2-NP and exposure Reliability = 1 Supportive study	Landsiedel et al. (2014)

Repeated-dose toxicity study by nose-only inhalation Five-week-old A/J Jms Slc mice 10 mice/time point/dose Average primary particle size : 19.3±5.4 nm 2.5, 5, 10 mg/m^3 whole body, 4 weeks (5d/w, 6h/d) Protocol according to OECD 412 (2009) guideline Parameters assessed: Biochemical analysis of serum, Whole blood analysis, Haematoxylin & eosin staining and immunofluorescence (IF) assay, Western blot analysis, lung histology	No adverse effects on growth or food intake detected Significantly higher levels of ALT, AST, blood urea nitrogen (BUN), and triglycerides (TG) in exposed groups than in control group. Levels of MCV, RDW, LYP, and LY significantly higher in the TiO_2-inhaled blood samples than in the control blood samples. At 2.5 mg/m^3, hyperplasia and haemorrhage observed. In the middle-dose group, hyperaemia, bronchial atelectasis, and brown particle-laden alveolar macrophages detected. In the high-dose group, bronchial atelectasis and multifocal lymphoid tissue hyperplasia were observed. Expressions of CD31 and PCNA increased in a dose-dependent manner. Densitometry analysis further supported the Western blot results, and showed a significant difference in the high-dose group. In the middle- and high-dose groups, phospho-p38, NF-kB, and VCAM-1 increased in a dose-dependent manner. Dose-dependent ER swelling and mitochondrial disruption in exposed murine lungs. Penetration of TiO_2-NP into the lung cells (TEM). Expression levels of the proteins Grp78/Bip, CHOP, inositol-requiring enzyme 1 alpha (IRE-1a), LC3, p62, and Beclin 1 increased in the TiO_2 inhalation mouse lungs in a dose dependent manner (Western blot assay). Autophagosomes observed in the lungs.	LOAEC = 2.5 mg/m^3 No information on crystallinity of nano-TiO_2 used. Too high concentrations tested as no NOAEC was identified This study has to be disregarded due to the lack of sufficient characterization of the tested material. Even if it is stated that the study was performed according to OECD 412 guideline, only blood, serum and protein analysis, IF and lung histology were evaluated which is not in line with the guideline. Reliability = 3 Disregarded study	Yu et al. (2015)
Repeated-dose toxicity study by whole-body inhalation Male Fisher rats (10/group/time point) TiO_2 (MT-150AW); spindle-shaped; 12x55 nm; average agglomerated particle size: 44.9 nm; purity = 99.5%; surface area = 111 m^2/g Concentration: 0.50 ± 0.26 and 1.84 ± 0.74 mg/m^3 Exposure: 4 weeks (6 h/day, 5 days/week); sacrifice after 3 days, 1 or 3 month post-exposure Measurement of TiO_2 amount in the whole lung and BALF; observation of cells in the	Biological half-time were 2.0 months for the low tested concentration and 1.8 months for the high tested concentration. Nanoparticles were phagocytized by macrophages, and each particle seemed to exist individually inside the macrophage. Cells with TiO_2 particles were almost normal. Some alveolar macrophages with a pigment-like material deposition were observed in the alveoli at 3 days after exposure.	NOAEC = 1.84 mg/m^3 The aim of this study was to determine whether biopersistence is a useful indicator for evaluating the toxicity of nanoparticles. Therefore, there is only limited information on the toxicity effects reported. Only males tested. No information on crystallinity in this publication but information available from Morimoto et al., 2016 (rutile) In this study, groups of rats exposed by instillation to 0.2 mg, 0.36 mg or 1 mg were also included, showing similar results.	Oyabu et al. (2017)

BALF, lung histopathology (only at 3 days after exposure for the highest concentration)		Reliability = 2 Supportive study	
Studies with one concentration			
Repeated-dose toxicity study by whole body inhalation male C57Bl/6 mice (6/group) TiO_2 anatase, 2-5 nm, BET = 219 +/-3 m^2/g 8.88 ±1.98 mg/m^3, 4h/day for 10 days, sacrifice after last dose and after week 1, 2, 3 post-exposure Parameters: BALF (enumeration of cells, LDH, cytokines) and lung histopathology	Cumulative inhaled TiO_2 dose was 154 µg per mouse. Number of alveolar macrophages elevated in the groups of animals necropsied at weeks 0, 1, and 2 postexposure but not in mice necropsied at week 3 post-exposure. No other respiratory effect.	Only males treated.	Grassian, O'Shaughnessy P, et al. (2007) Grassian et al. (2007a)

Repeated-dose toxicity study by nose-only inhalation Female Wistar rats TiO_2 P25 10 mg/m^3 6h/day 21 consecutive days, followed by recovery periods of 3, 28 or 90 days Parameters: lung histopathology, haematology, BALF, electron microscopy of lungs, quantification of lung septum components and relative deposition index	Lungs of treated animals showed randomly distributed, multifocal, white foci of 0.5–2 mm after 3 days of recovery. In a few lungs, multifocal, acute alveolar emphysema, accentuated in caudal and marginal lung parts observed. Lungs of rats treated showed moderate alveolar infiltration with particle-laden macrophages. A few particle-laden macrophages also seen intraluminally and subepithelially in bronchi and bronchioles. Particle agglomerates rarely found in bronchiolar epithelium, in type-I pneumocytes, and as free particles in alveoli. Minimal interstitial infiltration with mononuclear cells and minimal alveolar infiltration with neutrophilic granulocytes observed. Few animals also showed minimal bronchiolo-alveolar hyperplasia. Three days after the last treatment, no statistically significant changes evident in haematology. After 28 days of recovery, the white blood cell and lymphocyte counts significantly reduced. Alterations in haematology parameters similar after a 90-day recovery period, with reduced white blood cell counts, lymphocyte counts and number of segmented neutrophils. No significant changes in red blood cell count. Decreased activities of β-glucuronidase after 3 days of recovery		Eydner et al. (2012)
Repeated-dose toxicity study by nose-only inhalation male Crl:WI (Han) Wistar rats (6-7 per time point) TiO_2-NP, anatase being the major crystal phase; spherical; wide range of sizes with a few particles up to 100 nm with some agglomerates Concentration: 0 and 11.39 ± 0.31 mg/m^3 Exposure: 2 weeks (6 h/day, 5 days/week) followed by recovery periods of 1, 7 or 15 days. Parameters: biochemistry in BALF and serum, liver, spleen and lung weights, histopathology (lung, nasal cavity)	No significant clinical sign induced. No significant changes in bw. No significant change of liver and lung weights. Increased relative spleen weight. No effect on cytology and biochemical parameters. No observation of significant differences in levels of IL-4, IL-6, or IL-10 between control and treated groups on 1, 7, and 15 days post-exposure Numerous brown pigmented macrophages in alveoli until 15 days post-exposure. At 1 day post-exposure, olfactory epithelium degeneration/regeneration with inflammatory cell infiltration in the nasal septum and ethmoid turbinate of the treated rats. Basal cell proliferation in the ethmoid turbinate at 7 day post-exposure. Lesions not observed at 15 days post-exposure.	This study has to be disregarded due to the lack of sufficient characterization of the tested material.	Kwon et al. (2012)
Repeated-dose toxicity study by nose-only inhalation	Ti only detectable in the lung and mediastinal lymph nodes of the exposed animals. In H&E stained sections TiO_2 observed mainly in alveolar	Relevance of the results questionable due to too high concentration used.	van Ravenzwaay et al. (2009)

7 week-old male Crl:WI (Han) Wistar rats Anatase/rutile (70/30) TiO_2 particles were in the size range 20–30nm Concentration : Target : 100 mg/m^3 (measured : 88 mg/m^3); 6 h/day, 5 days/week followed by 14 days of recovery Parameters: histopathology of the respiratory tract, electron microscopy and BALF	macrophages. Number of alveolar macrophages moderately increased and few numbers of neutrophils within the alveolar space. Particles mainly located extracellularly in the lumen of the alveoli and bronchi. Moreover, particles detected in the cytoplasm of alveolar macrophages. TiO_2 found in the lung mostly agglomerates of about the same size as found in the atmosphere; no signs of disagglomeration of the inhaled agglomerates. Significant increases in total cell count and polymorphonuclear neutrophils also slightly increased lymphocytes and monocytes values in the lavage fluid. Levels of total protein and activities of lactate dehydrogenase, alkaline phosphatase, ɣ-glutamyltransferase and N-acetyl-glucosaminidase significantly increased. Increases of BALF parameters partly reversible within the recovery period. Exposure resulted in a >30% increase in lung weight. Diffuse histiocytosis and mild neutrophilic inflammation observed. In the mediastinal lymph nodes, lymphoreticulocelluar hyperplasia observed. After 14-day recovery period inflammatory response declined and only focal infiltrates of alveolar macrophages observed, still containing particles in their cytoplasm. Lung weight returned to control levels.		
Repeated-dose toxicity study by whole-body inhalation Male Kunming mice 15/animals/group Anatase TiO_2, 20 nm 6.34 +/- 0.22 mg/m^3 Every day for 3 weeks Parameters: distribution (brain, lung, liver, kidney, spleen), BALF and brain homogenate extract, differential blood count, prothrombin time and blood biochemical indexes, pathological examination of lungs,	TiO_2-NP mainly accumulated in the lungs. Concentration in liver blood and urine also increased. Significant increases of H_2O_2 and MDA concentrations in brain homogenate extracts observed. Decrease in WBC count and percentage of lymphocytes and increase in percentage of neutrophilic granulocytes, PLT and reticulocytes count. Significant increases in ALT and AST observed. No obvious pathological lesions in the lung, brain, liver or kidneys.	Results not well detailed and almost not discussed.	Yin et al. (2014)

brains, livers and kidneys			
Repeated-dose toxicity study by whole-body inhalation Female C57BL/6BomTac mice 17 animals: 9 controls and 8 exposed UV-titan L181 42 mg/m^3 Exposure: 1h/d for 11 days Parameters: BALF, Gene Expression Analysis, RT-PCR, Immunoassay	BALF: increase in the percentage of neutrophils; Transcriptomic analysis of the lungs: gene inductions for inflammation (cytokines and receptors), oxidative stress, chemotactism, complement; modulation of a few miRNAs; Transcriptomic analysis of the liver: no significant changes	Relevance of the results questionable due to too high concentration used.	Halappanavar et al. (2011)
Repeated-dose toxicity study by whole-body inhalation Outbred Crl:OF1 male mice 4-6/animals/group anatase + brookite (3:1)TiO_2, 20 nm 30 mg/m^3 1 h/day, 4 days/week for 4 weeks Parameters: respiratory rate, time of inspiration, time of expiration, time of pause after expiration, time of braking after inspiration, tidal volume, and airflow at midpoint of expiration	Airflow limitation stronger along the exposure period, Sensory irritation fairly minor, and observed also in the control group with the same intensity. Pulmonary irritation observed both in the exposure and control groups. The highest “time of pause” values increased along with the exposure days in the exposure group, whereas in the control group, such trend was not observed.	Relevance of the results questionable due to high concentration used.	Leppänen et al. (2011)
Repeated-dose toxicity study by whole-body inhalation	Pulmonary irritation, stronger during the first days of the exposures (days 1–4), and after, the effect was not as intense. Airflow limitation in the conducting airways.	Relevance of the results questionable due to high concentration used.	Leppanen et al. (2015)

female BALB/c/Sca mice 8/animals/group Rutile Si-coated TiO_2, 10x40 nm 30 mg/m^3 1 h/day, 4 days/week for 4 weeks on days 1–4, 8–10, 12, 15–18 and 22–25 Parameters: BALF, pathological examination of lungs, immunohistochemical staining	Inflammation in the airways: infiltration of inflammatory cells in peribronchial and perivascular areas.		

3.3.1.1 Pulmonary effects

Four reliable studies by inhalation with several concentrations are available on TiO_2-NP, and are described and discussed below. These studies mainly focus on pulmonary effect without considering other effects. The studies by van Ravenzwaay et al. (2009), Kwon et al. (2012), and Yin et al. (2015) reported in the table are not described in the text as they have been disregarded.

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

Particle size analysis and chamber concentrations of P25 aerosol are given table 4. It can be noted that the aerosol generated was made up of particle aggregates.

Table 4 : Summary of exposure conditions in Bermudez et al. (2004)

Species	**Chamber concentrations (mg/m^3)**	**Mass median aerodynamic diameter (µm)**
Hamster	0.54 ± 0.06 2.2 ± 0.1 10.8 ± 1.0	1.29 ± 0.30
Mouse	0.52 ± 0.03 2.1 ± 0.1 10.5 ± 0.7	1.45 ± 0.49
Rat	0.53 ± 0.03 2.1 ± 0.1 10.7 ± 0.6	1.44 ± 0.57

Treatment-related deaths were noted in 4 mice during the exposure phase. During the post-exposure phase, unscheduled mortalities, distributed over the different treatment groups, were reported in all species. Hamsters presented the greatest morbidity/mortality (35 animals), presumably due to severe chronic renal disease.

Following the end of the exposure period, a decrease in body weight was noted in all groups and all species. A more marked body weight loss was noted in hamsters (9-15%). Recovery occurred over the next three to four weeks in mice and rats but was slower in hamsters, with recovery within approximately 6 weeks.

Clear species differences in pulmonary clearance and lesions were observed, rats being the most sensitive.

Rats and mice exhibited equivalent TiO_2 lung burdens whereas lung burdens in hamsters were approximately 2 to 5 fold lower after 13 weeks of exposure. At the end of the recovery period, rats of the high-dose group retained approximately 57% of the initial burden compared to approximately 46% for mice and approximately 3% for hamsters. The calculated particle retention half-times for the three dose levels were 63, 132 and 395 days in rats, 48, 40 and 319 days in mice and 33, 37 and 39 days in hamsters. Therefore, under the conditions of this study, hamsters had better ability to clear TiO_2 nanoparticles than similarly exposed mice and rats.

Inflammation was noted in rats and mice at 10 mg/m^3, as evidenced by increases in macrophage and neutrophil numbers and in soluble indices of inflammation (LDH and protein) in BALF.

Significant terminal bronchiolar cell replication was observed at the end of the exposure period in mice and hamsters of the high-dose group and in rats of the mid- and high-dose groups. The indices returned to control levels at 4 weeks post-exposure. Alveolar cell replication was significantly increased at the end of the exposure in rats of the mid- and high-dose groups; and returned to control values by 4 weeks and 26 weeks in the mid- and the high–dose groups, respectively. In mice, a transient increase in alveolar cell replication was only noted at 13 and 26 weeks post-exposure. Hamster mitotic indices remained equivalent to controls throughout the study.

The histopathological evaluation showed that the pulmonary lesions were the most severe in rats compared to mice and hamsters. Only appearance of particles within alveolar macrophages and very minimal changes in the patterns of alveolar macrophage accumulation in the lung were noted in the rats exposed to the low concentration. At the mid- and high concentrations, epithelial and fibroproliferative lesions, which were progressive even following cessation of particle exposure and diminution of pulmonary inflammation, were reported. These effects consisted of alveolar hypertrophy and hyperplasia of type II epithelial cells surrounding aggregations of particle-laden macrophages of minimal to mild severity, which became more severe at the highest concentration of 10 mg/m^3. Alveolar metaplasia (bronchiolization) and septal fibrosis were also noted in rats of the high dose group by 52 weeks post-exposure. In contrast, no epithelial, metaplastic or fibroproliferative changes were observed in mice and hamsters. In mice, the findings were limited to the presence of particles free and within alveolar macrophages and in alveolar septal regions at the low and middle concentrations. Minor epithelial changes primarily consisted of aggregations of heavily particle-laden macrophages concentrated in central lobar centriacinar sites, with perivascular lymphoid proliferation. Over the post-exposure period, there was evidence of concentration of these cell aggregates and movement to interstitial areas, primarily around blood vessels and peribronchiolar interstitium. No pathologies associated with treatment exposure were noted in hamsters, except particle-laden alveolar and interstitial macrophages, and occasional aggregation of these particle-laden macrophages in the high concentration-exposed group.

The NOAEC for rats was established at 0.5 mg/m^3, based on inflammation evidenced in the BALF and pulmonary lesions (minimal hypertrophy and hyperplasia of type II alveolar epithelial cells) at 2

mg/m^3. The NOAEC for mice was set at 2 mg/m^3 based on inflammation evidenced in the BALF. The NOAEC for hamster was set at the highest tested concentration of 10 mg/m^3.

Some limitations associated with the testing protocol can be noted. First, the study was only performed on females and the analysis only focused on lung response, with limited level of details for the histopathological findings. Therefore, it cannot be completely ruled out that other findings occured at non-inflammatory concentrations in other organs. In addition, even if P25 is a well-known form of TiO_2 (80%/20% anatase/rutile, 21 nm), the nanoparticles were not fully characterised in the study. Finally, P25 was not dispersed (by sonication for example) before exposure in order to generate the largest amount of free and/or aggregate particles.

Male Wistar rats were treated by head-nose inhalation with concentrations of 2, 10 or 50 mg/m^3 of TiO_2-NP (uncoated TiO_2 with hydrophobic surface, 14% rutile and 86% anatase forms, average primary particle size: 25.1±8.2 nm) for 5 days (Ma-Hock et al. 2009). Groups of 6 animals for each time point were used in the study. Following the exposure period, animals were held for recovery periods of 3 or 16 days. Changes in BALF parameters (increased of total cells and neutrophils) were observed, more pronounced 3 days after the end of the exposure than immediately after and decreasing 16 days post exposure. There were also minimal and minimal to mild diffuse alveolar infiltration with histiocytes at 10 mg/m^3 and 50 mg/m^3, and hypertrophy/hyperplasia of bronchioles and bronchi at 50 mg/m^3. In addition, the authors observed an increase in labelling indices in both large/medium bronchi and terminal bronchioli in all treatment groups after the end of the exposure period. Based on this last observation, they establish the LOAEC at the minimal concentration of 2 mg/m^3. The results and conclusions of the current study are very consistent with those of Bermudez et al. (2004), although the duration of exposure in this study is shorter.

Male Wistar rats (8 animals/group) were exposed for 6h/day on 5 consecutive days by head-nose exposure to 0.5, 2 and 10 mg/m^3 nano-TiO_2 (T-Lite SF, 15x50 nm, rutile with minimal anatase, coated with dimethicone/methicone copolymer). An additional group exposed to 10 mg/m^3 was held for a recovery period of 3 weeks (Landsiedel et al. 2014). Exposure to T-Lite SF induced a concentration-dependent increase in PMN and monocytes in the BALF at 2 mg/m^3 and 10 mg/m^3. The inflammatory response was associated with an increase in LDH and ALP release at the same concentrations. The BALF parameters remained elevated at the end of the recovery period for the group exposed to 10 mg/m^3. In addition, numerous pigment-loaded alveolar macrophages were observed within the alveoli along with slight diffuse histiocytosis at this concentration, not fully reversible after 3 weeks of recovery. The authors report a NOAEC of 0.5 mg/m^3 based on the pulmonary inflammation evidenced in the BALF parameters. The concentration-dependent increase in pulmonary inflammation observed is this study is consistent with the findings of Bermudez et al. (2004).

Male Fischer rats were exposed whole body to 0.50 ± 0.26 and 1.84 ± 0.74 mg/m^3 of TiO_2 (MT-150AW; rutile; spindle-shaped; 12 x 55 nm; purity = 99.5%) for 4 weeks, 6h/day, 5 days/week (Oyabu et al. 2017). Following the exposure period, animals were held for recovery periods of 3

days, 1 or 3 months. The study primarily focused on biopersistence, but some information on lung histopathology was also reported. After exposure to MT-150AW for 4 weeks, the biological half-time was estimated to be approximately 2 months for both tested concentrations. Histopathologically, only alveolar macrophages containing nanoparticles were noted at 3 days after exposure to 1.84 mg/m^3. For comparison, other groups of animals were exposed by instillation to 0.2, 0.36 or 1 mg of TiO_2. Similar results in term of biological half-life and histopathological findings were reported. Based on these results, the authors conclude to a comparatively good correlation between biological half-life and total cell counts, PMN, LDH, cytokine-induced neutrophil chemoattractant 1 (CINC-1), and HO-1 in the BALF.

Additional studies were performed by inhalation, but with only one concentration, which does not allow to establish a dose-response relationship.

Male C57Bl/6 mice exposed to 5 nm anatase TiO_2 for 10 days at 8.88 +/- 1.98 mg/m^3 showed modest but significant inflammatory response (number of alveolar macrophages elevated) among animals necropsied at week 0, 1, or 2 after the last exposure. Mice fully recovered from the inflammatory response at week 3 post-exposure (Grassian, O'Shaughnessy P, et al. 2007)

Female Wistar rats were nose-only exposed to 10 mg/m^3 P25 TiO_2, 6h/day for 21 consecutive days. Following the exposure period, animals were held for recovery periods of 3, 28 or 90 days. Toxicological investigations, limited to the description of lung toxicity, were not the primarily aim of this study, which was to assess the RDI of TiO_2-NP as described in section 3.1. The authors reported, in a few lungs, multifocal, acute alveolar emphysema, accentuated in caudal and marginal lung parts. Minimal interstitial infiltration with mononuclear cells and minimal alveolar infiltration with neutrophilic granulocytes were also observed. A few animals showed minimal bronchiolo-alveolar hyperplasia. The leucopenia observed after 28 and 90 days recovery (reduced white blood cell and lymphocytes count) could be explained by the infiltration of the lungs with these cells (Eydner et al. 2012). This leucopenia was also observed after a daily whole body exposure for 3 weeks to 6 mg/m^3 of anatase TiO_2 (20 nm) (Yin et al. 2014)

Persistent inflammation was also reported in female mice when TiO_2-NP (UV Titan L181; rutile surface coated, 17 nm, Chemical composition: Na_2O (0.6%), SiO_2 (12.01%), Al_2O_3 (4.58%), ZrO_2 (1.17%), TiO_2 (70.81%), polyalcohol adding to the remaining wt %) was administered whole-body 10 days during gestation at 42 mg/m^3, 1 hour per day (Jackson et al. (2013) - see section 3.4.4 for details). At the same dose and with similar exposure scenario (11 days, 1h/day), Halappanavar et al. (2011) exposed mice by whole body inhalation and reported slight changes in pulmonary inflammation biomarkers (induction of genes associated with inflammation and increased in neutrophils proportion in BALF). However, the relevance of the results from these studies is questionable considering the relatively high concentration used.

In the study of Leppänen et al. (2011), mice were exposed by whole body inhalation to anatase:brookite (3:1) 20 nm TiO_2, 30 min, 4 days/weeks for 4 weeks to 0 and 30 mg/m^3. In this study, the authors observed airflow limitation, sensory irritation and pulmonary irritation. However, sensory and pulmonary irritations were both reported in the exposure and control groups.

In the study of Leppanen et al. (2015), mice were exposed by whole body inhalation to silica-coated rutile TiO_2 (10x40 nm) 30 min, 4 days/weeks for 4 weeks to 0 and 30 mg/m3. An inflammation was observed in the murine airways as evidenced by the infiltration of inflammatory cells in peribronchial and perivascular areas. This inflammatory response was not likely due to radical formation since silica-coated nano TiO_2 did not significantly produce °OH radicals under UV radiation.

All the studies described above are in line and confirm the results from Bermudez et al. (2004), showing inflammatory effects at doses above 0.5 mg/m^3.

In line with acute toxicity, repeated dose toxicity studies are also available by intratracheal instillation, with doses varying from 1.25 to 10 mg/kg. These studies cannot be used in risk assessments because they are not representative of normal inhalation (the upper respiratory tract is bypassed) and they do not provide external exposure concentrations. However, even if they are not transposable quantitatively, they can provide additional useful information for hazard identification. Overall, those studies showed similar effects on the pulmonary tract as studies by inhalation (Sun, Tan, Ze, et al. 2012, Sun, Tan, Zhou, et al. 2012, Li et al. 2013, Hong et al. 2017)

3.3.1.2 Cardiovascular effects

Five studies identified from the literature evaluated cardiovascular effects of TiO_2-NP after repeated exposure.

Pregnant Sprague-Dawley female were exposed to 10 mg/m^3 P25 TiO_2 5h/d during approximately 8 days. The exposure induced a significant uterine microvascular dysfunction, with an alteration of reactivity after pharmacologic stimulation (ACh, NO donor...) or shear stress (Stapleton et al. (2013) – see section 3.4.4 for details).

Saber et al. (2013) analysed mRNA response of SAA, after an inhalation exposure to UV-Titan L181 at 42 mg/m^3 10 days during pregnancy (gestation days 8-18) in mice. The authors showed an increased in pulmonary Saa3 mRNA 5 days and 26–27 days after exposure compared to controls exposed to filtered air, but those results have to be taken with reservation because of the high dose used.

In the study performed by Yu et al. (2014), atherosclerosis occurred in mice after a 9-month instillation treatment (1.25; 2.5 and 5 mg/kg) with anatase 5 nm, with effects seen at the low dose, increasing dose-dependently. The authors hypothesized that inhaled particles exert cardiovascular effects indirectly through the passage of inflammatory mediators from the lung to the systemic circulation, as the increased atherogenesis was concomitant with pulmonary inflammation and oxidative stress.

ApoE male knockout mice were exposed to 5-10 nm anatase TiO_2 twice a week during 6 weeks by instillation to 10, 50 or 100 µg/week. In accordance with studies described above, endothelial dysfunction, evidenced by dose dependant decrease in NO concentration and eNOS activity, and progression of atherosclerosis were observed. Lipid metabolism was also impacted with increased

serum total cholesterol and decreased high density lipoprotein cholesterol (HDL-C) (Chen et al. 2013).

In contrast to the above-mentioned studies (acute and repeated exposures) reporting effects of TiO_2-NP on cardiovascular system with impaired vasodilation, there was no alteration of vasodilatory function in aorta segment in mice exposed by instillation of UV-Titan L181, and even an increased NO production in *ex vivo* experiment on human cells. This study also shows a modest increased atherosclerotic plaque progression (Mikkelsen et al. 2011).

3.3.1.3 Immunotoxicity

Several studies have evaluated immune effects of TiO_2: five of them used intratracheal instillation at doses up to 32 mg/kg for 4 or 5 weeks; six of them used an allergen sensitization and challenge to ovalbumin (OVA) with various routes of exposure (aerosolized, inhalation, intranasal exposure, nose-only application) at doses up to 15.7 mg/m^3 or 200 µg/mouse for single or repeated exposures (6 hours, 3 days, 4 weeks).

Rossi et al. (2010) observed that exposure to 10 ± 2 mg/m^3 of TiO_2-NP (silica coated, needle-like; 10 x 40 nm) for 4 weeks (3 times a week for 2 hours) induced Th1 type inflammation in healthy mice, with an increase in neutrophil attracting chemokine CXCL5 mRNA expression and in neutrophil influx in the lungs. Fu et al. (2014) observed a slight congestion and brown particulate accumulation in spleen along with increased T and B cells and an enhanced NK cell activity after instillation of P25 twice a week for 4 consecutive weeks in rats. In a similar protocol, Chang et al. (2015) observed that rat immunologically competent cells CD3+, CD4+, and CD8+ were significantly lower after exposure to P25 than in control group. Also, the ratio of CD4+ to CD8+ was significantly increased showing a disturbance of cellular immune function. However, no significant changes in IFN-γ and IL-4 were observed.

During OVA sensitization and challenge studies, exposure to TiO_2-NP can display an adjuvant activity on allergic airway inflammation depending on the timing of exposure. Indeed, this timing may account for differences in effects induced by TiO_2 nanoparticles by either promoting inflammation when TiO_2 exposure occurs prior to challenge (De Haar et al. 2006, Kim et al. 2017) or suppressing such effect when exposure is subsequent to the challenge (Scarino et al. 2012, Rossi et al. 2010). All these studies have used only one dose, except the study by Scarino et al. where rats were exposed by nose-only inhalation to 9.4 or 15.7 mg/m^3 of anatase 5 nm for 6 hours. Interestingly, two studies (Kim et al. 2017, Rossi et al. 2010) have evaluated a functional parameter i.e. airway hyperresponsiveness, in addition to biochemical markers of inflammation. In the study by Rossi et al. (2010), TiO_2-NPs (silica coated, needle-like; 10 x 40 nm) administered at 10 mg/m^3, 3 times a week for 2 hours for 4 weeks downregulated Th2 type inflammation (i.e. infiltration of eosinophils and lymphocytes in the lungs and expression of Th2 cytokines) and reduced the OVA-induced air hyperresponsiveness to the control levels of non-sensitized mice. In another study with sensitization and challenge with TDI, the authors showed that acute instillation of TiO_2-NPs (anatase; 15 nm) at 0.8 mg/kg significantly increased the inflammatory response in

TDI-sensitised animals (significantly higher neutrophils, macrophages infiltration) (Hussain et al. 2011). In rats primed with endotoxin, anatase (20 nm) TiO_2-NPs caused a significant amplification of the inflammatory response induced by endotoxin or the particles alone after acute instillation (Oberdörster et al. 2000).

Liu et al. (2010) observed a reduced chemotactic ability and decreased expression of both Fc receptors and MHC-class II molecules on the alveolar macrophage cell surface after acute instillation exposure to anatase 5 nm. The mechanism responsible for this effect appears to involve increased nitric oxide and tumour necrosis factor-α.

Gustafsson et al. (2011) demonstrated that intra-tracheal exposure to P25 at 5 mg/kg in rats induced a transient influx in eosinophils and a more sustained neutrophilic response, followed by a recruitment of dendritic cells and lymphocytes expressing NK receptors. In line with the study of Chang et al. (2015) described above, a late-phase influx of lymphocytes to the rat lungs was dominated by CD4+ T-cells with smaller fractions of CD8+ T-cells and B-cells.

Scuri et al. (2010) exposed newborn, young, and adult rats for 3 days to 12 mg/m^3 of P25. Although no differences were observed in BALF analysis and in lung histopathology, they showed that exposure of newborn and weanling rats to P25 influenced the expression of lung neurotrophins (NGF and BDNF), which play a critical role in the pathophysiology of childhood asthma.

3.3.1.4 Neurotoxicity

Eleven studies related to the neurotoxicity of TiO_2-NP have been evaluated: eight of them were from the same laboratory in China and investigated in mice:

1) The brain toxicity of the daily intranasal administration (500 µg in 10 µL Milli-Q water/mouse/day) for 30 days of 4 preparations of TiO_2-NP that differed by size and surface coating (Zhang et al. 2011);

2) Differences in brain toxicity of TiO_2-NP according to the crystalline form of the preparation (anatase vs rutile), 500 µg in 10 µL Milli-Q water/mouse/day for 30 days (Wang, Liu, et al. 2008, Wang, Chen, et al. 2008);

3) The effect on brain and behaviour of the daily intranasal administration of TiO_2-NPs for 90 days at 3 doses (2.5, 5 and 10 mg/kg/day) (Ze et al. 2013, Ze, Sheng, Zhao, Ze, et al. 2014, Ze, Sheng, Zhao, Hong, et al. 2014, Ze, Hu, et al. 2014);

4) The neurotoxicity of the chronic intranasal administration (9 months) at doses of 1.25, 2.5 and 5 mg/kg/day (Ze et al. 2016).

The results obtained from Zhang et al. (2011) showed that the most deleterious effects on the brain (histological lesions of hippocampus and cortex tissue, and disturbances of extracellular dopamine, serotonin and noradrenergic levels measured in the same regions) were observed with rutile Si-coated TiO_2-NP (diameter = 10 or 50 nm) intranasally instilled (500 µg in 10 µL Milli-Q

water/mouse/day) for 30 days compared to rutile uncoated TiO_2-NP (1 µm or 10 nm in diameter according to the preparation studied) administered using the same protocol.

The two studies by Wang and colleagues aimed to compare the neurotoxicity of a once every two days intranasal administration of 10 µL of two suspensions of non-coated TiO_2-NP for 30 days in female mice, one preparation containing rutile TiO_2 (diameter = 80 nm) and the other one anatase TiO_2 (diameter = 155 nm) (Wang, Liu, et al. 2008, Wang, Chen, et al. 2008). The results indicated the same histological and neurochemical alterations with both forms after 30 days of exposure, which were more pronounced with the anatase form compared to the rutile one. Results also showed a higher susceptibility of hippocampus to TiO_2-NP compared to the other brain regions studied that was correlated with the greater accumulation of TiO_2 observed in this part of the brain.

Studies from Ze and colleagues assessed the neurotoxicity in hippocampus of a repeated intranasal instillation of TiO_2-NP for 90 days at doses of 2.5, 5.0 or 10 mg/kg/day in mice. TiO_2-NP (anatase 6 nm, surface area 175 m^2/g form) was suspended in HMPC 0.5% for administration (Ze et al. 2013, Ze, Sheng, Zhao, Ze, et al. 2014, Ze, Sheng, Zhao, Hong, et al. 2014, Ze, Hu, et al. 2014). The findings of the four studies showed a translocation and accumulation of TiO_2-NP in brain with an overall proliferation of glial cells, tissue necrosis and signs of cellular degeneration observed in the hippocampus considered as a brain region of interest. Changes in hippocampal cell ultrastructure were observed in both TiO_2-NP exposed groups and were indicative of cell apoptosis (Ze, Hu, et al. 2014) possibly related to oxidative stress (Ze et al. 2013, Ze, Hu, et al. 2014) through activation of the p38-Nrf-2 signalling pathway (Ze et al. 2013), neuroinflammation and alterations of cytokine expression (Ze, Sheng, Zhao, Hong, et al. 2014). Down- or up-regulations of brain gene expression in genes associated with oxidative stress, immune response, apoptosis, memory and learning, brain development, signal transduction, metabolic process, DNA repair, response to stimulus, and cellular process were observed with the dose of 10 mg/kg TiO_2-NP in the same region (Ze, Hu, et al. 2014). Finally, subchronic TiO_2-NP exposure induced significant long-term potentiation reduction and down-regulation of glutamate NMDA receptor subunits (NR2A and NR2B) expression associated with the simultaneous inhibition of CaMKIV, cyclic-AMP responsive element binding proteins (CREB-1, CREB-2), and FosB/DFosB in mouse hippocampal tissues, with a spatial memory recognition impairment (Ze, Sheng, Zhao, Ze, et al. 2014). Taken together, all these results suggest dose-dependent neurotoxicity of TiO_2-NP in anatase form, with hippocampus as a brain region of higher susceptibility, leading to impairments of synaptic plasticity and learning performances possibly related to neuroinflammation and oxidative stress.

Three other studies using intratracheal instillation (Horvath et al. 2017) or inhalation (Disdier et al. 2017, Yin et al. 2014) for TiO_2-NP exposure were also considered. In the study from Horvath et al. (2017), adult rats were dosed intratracheally (1 mL/kg b.w., 5days/week) daily for 28 days with a suspension of TiO_2-NP (diameter = 10 nm) in PBS-HEC 1% at doses of 1, 3 or 10 mg/kg/day, and were studied for various electrophysiological activities including spontaneous cortical activity, sensory evoked potentials and tail nerve action potential. Results showed the slow-down of the sensory evoked potentials and tail nerve action potential which were moderately correlated with

brain Ti level and oxidative stress parameters. In the study of Disdier et al. (2017), young and aged rats (12-13 weeks and 19 months of age, respectively) were exposed to TiO_2-NP (75% anatase and 25% rutile, Aeroxide P25, diameter = 21 nm) nose-only 6 hours/day, 5 days/week for 4 weeks. Results showed increasing blood-brain barrier permeability in aged rats associated with neuroinflammation and decreased synaptophysin, a marker of neuronal activity. Yin et al. (2014) observed significant increases of H_2O_2 and MDA concentrations in mice brain homogenate extracts after whole body inhalation exposure to 6 mg/m^3 of 20 nm anatase TiO_2-NP for 3 weeks, suggesting that the brain was injured after inhalation of TiO_2-NP. No lesions were observed in this study.

3.3.1.5 Liver toxicity

Few studies have investigated liver toxicity induced by TiO_2-NPs. Halappanavar et al. (2011), in a study described above (cf. 3.4.1.1) observed no changes in the liver in a transcriptomic analysis.

A 4-week instillation study performed with TiO_2-NP (80% anatase/20% rutile, 21 nm) at concentrations of 0.5, 4 and 32 mg/kg in male Sprague-Dawley rats showed statistically significant increases in the AST level and oedema and loose cytoplasm on liver cells (Chang et al. 2015).

3.3.1.6 Kidney toxicity

Huang et al. (2015) studied effects of P25 TiO_2-NP exposure by instillation for 4 weeks at concentrations of 0.1, 0.25 or 0.5 mg/week on kidneys of ICR mice.

TiO_2 contents in the kidneys of P25-treated mice were significantly increased as compared with controls. Incidences of histological changes such as tubular dilation, necrosis, and loss of the brush border were statistically significant at 0.5 mg/week and dose dependent. Alterations in oxidative stress markers (HO-1, nitrotyrosine, HIF-1α...) and renal function markers (BUN and creatinine) were also observed.

3.3.1.7 Human data

Nine human studies have been identified on toxicological effects following inhalation exposure of TiO_2 in workers. Among them, three studies dealt with Chinese workers (Zhen et al. 2012, Zhao et al. 2018, Ichihara et al. 2016) and 5 investigated a sample of workers in the Czech Republic (Pelclova et al. 2017, Pelclova, Zdimal, Kacer, Vlckova, et al. 2016, Pelclova, Zdimal, Kacer, Fenclova, et al. 2016, Pelclova, Zdimal, Fenclova, et al. 2016, Pelclova et al. 2015). All these studies have been considered inadequate, due to selection bias, classification bias for exposure to TiO_2 and confounding factors.

The study by Zhen et al. (2012) focused on short-term cardiopulmonary effects after exposure to inhalable TiO_2 in an (unspecified) finished-product production workshop. Zhao et al. (2018), who

belonged to the same team as Zhen et al., re-analysed short-term cardiopulmonary effects in a sample of workers in a nano-TiO_2 manufacturing plant using a cross-sectional design. Ichihara et al. (2016) undertook a pilot study, with a cross-sectional design, in a plant handling TiO_2 in Shanghai, China. The number of exposed workers included in these studies was between 7 and 83. Finally, the five publications by Pelclova and colleagues were part of the same research project, sub-divided into several studies depending on the type of effect studied and the analysis time. All of the studies had a cross-sectional design, but some were repeated, with differences in exposure measurement and biological sampling protocols. The authors distinguished between several sub-groups of workers considered as exposed, all from the same production plant for paints and pigments containing TiO_2 and other compounds (iron oxide). The sizes of the sub-groups varied depending on the publication, except for researchers (n=4) and exposed office workers (n=22). The authors studied several types of effects including respiratory function with markers of oxidative stress and lipid peroxidation.

On the whole, none of the nine studies considered enable a causal relationship to be established between exposure to nano- or micro-metric (both corresponding to the respirable fraction) size ranges of TiO_2 and the occurrence of biological or health effects in workers. These studies suggest possible effects on respiratory and cardiovascular function whose mechanisms may include oxidative stress reactions, inflammation, and the regulation of the parasympathetic nervous system. However, none of them include dose-response analyses based on the concentrations of TiO_2 measured at the workstations or individually. The lack of a validated method for assessing individual exposure is a common limitation of all of the studies with on the one hand, the inability to compare nanometric TiO_2 concentrations with the background noise formed by other indoor air particles, and on the other hand, the inability to assess the internal dose or effective dose (TiO_2-NP deposited in the airways).

3.3.2 Mutagenicity

Publications related to mutagenicity of TiO_2-NP have been summarized in several reviews (Chen, Yan, and Li 2014, Magdolenova et al. 2012, Charles et al. 2018, ANSES 2016).

In vitro:

Many *in vitro* genotoxicity studies are available for TiO_2-NP. A review of *in vitro* data published between 2010 and 2016 was performed by Charles et al. (2018). A summary of the studies with the higher level of confidence is presented in the table below.

Most of the published results refer to the anatase form as well as mixture of anatase and rutile (generally P25). Very few studies assessed the genotoxicity of coated TiO_2-NP or rutile forms.

Table 5: Summary of genotoxicity studies on TiO_2-NP (from Charles et al. (2018))

Form of the TiO_2-NPs tested	MN assay	Comet assay	Chromosomal aberrations assay	Total
Anatase	14/25 (56%)	46/77 (58%)	1/3 (33%)	61/105 (58%)
Rutile	3/3 (100%)	12/24 (50%)	0/0 (0%)	15/27 (55%)
Mixture anatase/rutile	7/15 (47%)	25/37 (68%)	0/2 (0%)	32/54 (59%)
Coated-rutile	2/3 (67%)	8/13 (61%)	0/0 (0%)	10/16 (62%)
Rutile-brookite-anatase	0/1 (0%)	0/0 (0%)	0/0 (0%)	0/1 (0%)
Coated-anatase	1/2 (50%)	1/4 (25%)	0/0 (0%)	2/6 (33%)
Anatase-brookite	0/1 (0%)	4/6 (67%)	0/0 (0%)	4/7 (57%)
Coated-anatase-brookite	1/2 (50%)	15/16 (94%)	0/0 (0%)	16/18 (89%)
Total	18/52 (35%)	111/177 (63%)	1/5 (20%)	140/234 (60%)

[a]Since specific protocol parameters (e.g. cells, media, exposure-duration, standard or modified protocol, etc.) and forms of TiO_2-NPs varied among the 88 assays, the number of total testing conditions ended up to 234.

According to Charles et al. (2018), both negative and positive results are reported in the *in vitro* mutagenic assays. Most of the positive results were found at high doses in micronucleus and Comet assays, with a dose-response relationship. Inconsistencies observed in the results of the studies may be the result of differences in test materials (e.g. size, crystallinity, coating). However, it currently 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, cell/organ examined and parameter assessed. Moreover, numerous interferences with TiO_2 can occur due to fluorescence and absorbance interaction. Other interactions with the proteins or enzymes used during the assay are likely to occur; unfortunately, these interferences were not properly tested in most of the publications. All these elements did not permit an easy comparison of the studies.

In vivo:

A review of the available *in vivo* studies has been performed by ANSES (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. Several limitations can be noted from almost all studies including the lack of positive control, the absence of evidence of uptake, insufficient characterization of the tested material etc*".

Table 6: Summary of positive responses in function of crystalline phase of TiO_2-NP according to the authors – all routes of exposure (extracted from ANSES (2016))

Assays	Micronucleus assay	Comet assay	Mutation assay	DNA oxidative lesions	DNA adducts	H2Ax phosphorylation assay	Total
Nanoforms							
Anatase	2/9	5/22	0/7	5/6	0/0	1/1	13/45
Rutile	0/2	0/0	0/0	1/3	0/0	0/0	1/5

Anatase/rutile	3/7	8/20	2/2	1/2	0/1	1/1	15/33
Anatase coated	0/1	1/5	0/0	0/0	0/0	0/0	1/6
Rutile coated	0/6	5/22	0/1	0/0	0/0	0/0	5/29
Anatase/rutile coated	0/0	0/0	0/0	0/1	0/0	0/0	0/1
Brookite/anatase	0/1	0/1	0/0	0/0	0/0	0/0	0/2
Unspecified	1/1	1/1	0/0	1/2	0/0	0/0	3/4
Total	6/27	20/71	2/10	8/14	0/1	2/2	38/125

Some studies include several experiments with different NM and some NM can show negative and positive results within a study, depending on the organ examined. Each result was counted in all the relevant sections. An experiment is defined by a tested material and a specific protocol (ex. organ examined, duration…).

Based on the ANSES (2016) review, an update of the literature search for TiO_2-NP has been performed up to December 2017, focusing on *in vivo* studies carried out by the respiratory route (see annex 2). The following new *in vivo* studies by respiratory route have been identified in the literature:

Table 7: Summary of the *in vivo* mutagenicity studies performed with TiO_2-NP by the respiratory route, identified during the update of the literature search

Method	Results	Remarks	Reference
INHALATION ROUTE			
BALB/CcJ female mice TiO_2 anatase, 10 nm, BET: 173 m^2/g, geometric mean diameter in dispersion: 504 nm (mostly aggregates/agglomerate) - $1.1x10^5$ particles/cm^3 271 mg/m^3 for 1 hour, inhalation head-only (as dry powder) Estimated deposited dose in airways: 90.5 µg Comet assay on BAL and lung 24h after inhalation	Comet: Positive in the lung Airway irritation, lung inflammation	No positive control presented Only one high concentration.	Larsen et al. (2016)
INSTILLATION ROUTE			
Male Sprague-Dawley rats (12/group) P25 in PBS (phosphate-buffered saline): primary particles (25 +/- 15 nm) and 100 nm agglomerates 3 endotracheal instillation at a 4-days interval. Sacrifice after 2h and 35 days.	**Comet assay:** Positive (lung, blood and liver after 2h and/or 35 days). No increase of hedgehog. **ɣ-H2AX assay:** Positive in lung (immediately after exposure at highest dose) and Negative in liver and blood	No negative or positive control presented Instillation route, worst case exposure (bolus administration)	Relier et al. (2017)

0.17, 0.83, and 3.33 mg/ml, respectively, per instillation --> total: 0.5, 2.5, and 10 mg/kg or 87, 437, and 1700 cm^2/lung. Comet assay on lung cells, blood cells and liver cells ɣ-H2AX assay on lung, blood and liver cells Pig A in blood cells 35 days after exposure.	**Pig A:** Negative Acute lung inflammation (only 2h after administration) but no oxidative stress. Kinetics part of the study: measured lung burdens after 3 months (20%, 63%, and 83% of initial lung burden for low, mid, and high doses, respectively) --> half-retention time > 60 days for the 2 highest doses.		
Female mice (8/dose/time point) NRCWE-001: TiO_2 unmodified rutile with endogenous negative surface charge; 10 nm; BET = 99 m^2/g NRCWE-002: TiO_2 rutile with positive surface charge by coating with 3-aminopropyltriethoxysilane (purity 99%); 10 nm; BET = 84 m^2/g Agglomerates smaller than 100 nm in water 18, 54 or 162 µg/mouse by instillation, mice were killed after 1, 3 or 28 days. Comet assay on BAL cells, lung and liver tissues.	**NRCWE-001:** Positive in the lung (at 1 and 28 days) and in the BAL (at 18 and 54 µg on day 3) but Negative in the liver **NRCWE-002:** Positive in the lung (at 1 and 28 days) and in the liver and the BAL (only on day 1; not consistent finding) Inflammation, time and dose-dependent which persisted at the highest dose 28 days after exposure.	No significant effect of the charge on the result of the comet assay Instillation route, worst case exposure (bolus administration)	Wallin et al. (2017)
gpt delta transgenic mice (4-8/groups; both sexes) TiO_2; 28 nm ; 10-60 nm by TEM; BET = 45 m^2/g; 90% anatase/10% rutile In physiological saline = 280 nm (DLS). 50 µg administrated by instillation once. Lung collected 4 months after exposure. Determination of gp mutant frequency (genomic DNA from lung) 8 oxodG measured in the lung by ELISA ɣ-H2AX assay	**gp mutant frequency**: negative **8 oxodG**: negative **ɣ-H2AX assay**: negative (slight but not significant increase) None or a mild inflammatory response, no obvious fibrotic response in the lungs at 4 months after TiO_2 exposure	Negative control included but no positive control. Instillation route, worst case exposure (bolus administration) Only one high concentration.	Wan et al. (2017)

Similarly to *in vitro* data, results from *in vivo* studies are inconsistent and positive results are provided mainly by Comet tests at high doses. It was again not possible to identify the reasoning behind the inconsistencies (e.g. different protocols, different forms of TiO_2-NP). It should also be noted that 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…).

Hypothesized genotoxic mode of action of TiO_2-NP:

Primary genotoxicity could be the result of direct interaction with DNA or indirect mechanisms with molecules interacting with the genetic material. Secondary genotoxicity could result from ROS generated by the catalytic potential of the particles, activation by UV light or during particle-elicited inflammation.

Theoretically, TiO_2-NPs may **interact with DNA**, since particles were detected (by TEM and/or Raman imaging) inside the nucleus in a few *in vitro* and *in vivo* studies. However, the possible mechanism of penetration into the nucleus is unclear.

DNA damage can also arise through **indirect mechanisms** where the NPs do not physically interact with the DNA molecule. In particular, decreased nucleotide excision repair (NER) and base excision repair (BER) activities reported in A549 cells exposed to TiO_2-NPs (Jugan et al. 2012, Armand et al. 2016) suggest an effect on repair mechanism. In addition, some publications reported disturbances of mitosis, and abnormal multipolar spindle formation, chromosomal alignment, and segregation during anaphase and telophase, as well as disturbance of the cell cycle checkpoint function (Magdolenova et al. 2012). It is difficult to judge the relevance of these results since there is no harmonized tool to investigate these types of mechanisms.

Secondary genotoxicity can be induced by ROS or reactive nitrogen species (RNS) generated at the surface of NPs or produced by inflammatory cells. Positive results reported both *in vitro* and *in vivo* were often associated with oxidative stress (evidenced by ROS production, glutathione content, lipid peroxidation, malondialdehyde level, antioxidant enzyme level or by the adding of specific DNA repair enzymes) and/or with inflammation (up-regulation of pro-inflammatory cytokines and increased cells such as neutrophils in the BALF)(Charles et al. 2018).

In conclusion, the production of ROS seems to be the major mode of action explaining these effects, as evidenced by oxidative stress/ inflammation reported in many positive studies. However, ROS production may not be the sole mechanism explaining the genotoxicity found with TiO_2-NPs. At present, other mechanisms of action cannot be formally disregarded but there is no validated tool to investigate other possible genotoxic modes of action.

Conclusion on genotoxicity of TiO_2-NP:

Data from published reviews on the genotoxic potency of TiO_2-NP are numerous resulting from a large panel of tests carried out both *in vitro* and *in vivo*, of variable quality. In addition, various forms of TiO_2-NP with different physico-chemical characteristics (shape, size, coating, surface reactivity, charge, crystallinity…) were tested in rodents by inhalation or intratracheal instillation and on several cell types and tissues under different conditions of exposure. Although an impressive data set is available, it is still difficult to distinguish the key physico-chemical parameter(s) of the nanoparticles that are related to the genotoxic effect.

Despite the inconsistency of the mutagenic response among all the available data, it is noted that TiO_2-NP can induce genotoxicity in rodent lungs but also in organs such as liver and in ex vivo cells or cell lines. Most of dose-related positive results were obtained in the Comet and micronucleus assays, and only at high concentrations. Such genotoxic effects are probably due to an increase in ROS/RNS secondary to an inflammatory process that is frequently involved in the toxicity of TiO_2-NP. On the other hand, it is still unclear whether other pathways than oxidative

stress could also play a role in the induction of DNA damage.

In summary, considering that:

- **The results from *in vitro* and *in vivo* mutagenic assays are rather inconsistent;**
- **The positive effects were primarily reported at high concentrations;**
- **The identification of the underlying reasons for the differences of responses reported is not possible;**
- **The data point to a secondary genotoxic mode of action involving ROS/RNS production;**
- **The carcinogenic effects appear only at high concentrations, associated with altered clearance and inflammatory responses (see also section 3.4.3)**

it can be concluded that TiO_2-NP is a weak genotoxic agent. These conclusions are in line with those from other organisms or reviews of TiO_2 genotoxicity (IARC 2010, NIOSH 2011, ANSES 2016, OECD 2018a, Charles et al. 2018).

3.3.3 Carcinogenicity

Animal data

Carcinogenic potential of TiO_2 (under micro and nanoforms) was reviewed by IARC (IARC, 2006 & 2010), by NIOSH (NIOSH 2011) and also recently by RAC (Risk Assessment Committee) of ECHA (ECHA 2017) in the framework of CLP Regulation (EC n°1272/2008), based on a classification proposal made by France (ANSES 2016, ECHA 2017).

Experimental studies on TiO_2-NP are summarized in table 8 below.

Table 8 : Summary of animal carcinogenicity data on TiO_2-NP by respiratory route (extracted from ANSES, 2016)

Method	Results	Remarks	Reference
Inhalation route			
Female Wistar rats [Crl:(WI)BR] and NMRI mice TiO_2, 15-40 nm, P25 (≈ 80% anatase and ≈ 20% rutile) Whole body exposure by inhalation: 18h/d, 5 days/week: 7.2 mg/m^3 for the first 4 months, then 14.8 mg/m^3 for 4 months followed by 9.4 mg/m^3 for 16 months for rats and 5.5 months for mice (cumulative particle exposure: 88.1 g/m^{3}xh for rats and 51.5 g/m^{3}xh for mice) Recovery period: 6 months for rats and 9.5 months for mice Not guideline, no GLP status	↑ benign keratinizing cystic squamous cell tumours, squamous-cell carcinomas, bronchioalveolar adenomas and adenocarcinomas in rats. Not carcinogenic in mice. ↑ mortality and ↓ body weight in both species. Impairment of clearance function, bronchioalveolar hyperplasia and interstitial fibrosis in rats.	Purity lacking. One concentration varying during the experiment, only females tested. High background tumour response in control mice. Non-neoplastic effects and lung clearance not reported in mice. R = 3	Heinrich et al. (1995)
Instillation route			
SPF Wistar female rats TiO_2 P25: 5x3mg, 5x6 mg or 10x6 mg by instillations TiO_2 P805: 15x0.5 mg or 30 x0.5 mg by instillation Animals sacrificed after 30 months. Not guideline, no GLP status	↑ benign tumours (adenomas and epitheliomas) and malignant tumours (adenocarcinomas and squamous cell carcinomas) at all tested doses.	TiO_2-NP P25, majority anatase, 25 nm TiO_2-NP P805 (P25 coated with trimethoxyoctyl-silane), 21 nm Purity lacking. Only females tested. Higher number of tumours with TiO_2-NP compared to fine TiO_2. R = 2	Pott and Roller (2005)
F344/DuCrl Crj male rats TiO_2-NP, 80 nm DHPN (initiation) for 2 weeks, then 0.5 mg/rat TiO_2 once in week 4 by instillation. Not guideline, no GLP status	No promotor potential by instillation. No lung lesion without pre-treatment with DHPN.	Many parameters did not match with standard protocol for carcinogenesis assessment; no valid positive control; only males tested; no clear information on crystallinity R = 3	Yokohira et al. (2009)

Hras 128 transgenic female rats TiO_2 non coated, rutile, 20 nm DHPN (initiation) for 2 weeks. Then, 250 µg/ml or 500 µg/ml TiO_2 once every 2 weeks from the end of the week 4 to week 16 by instillation. Not guideline, no GLP status	Promotor effect observed: ↑ multiplicity of DHPN-induced alveolar cell hyperplasia and adenomas in the lung at all doses, and the multiplicity of mammary adenocarcinomas at 500 µg/ml. Not carcinogenic without pre-treatment with DHPN.	Purity lacking. Little experience with this model. No positive control included. Only females tested. R = 3 Not reliable study.	Xu et al. (2010)

Only one carcinogenicity study by inhalation is available with adequately characterized TiO_2-NP (Heinrich et al. 1995). Female Wistar rats [Crl:(WI)BR] and NMRI mice were exposed whole body 18h/day, 5 days/week to aerosol of TiO_2 (P25, primary particle size 15-40 nm, ≈ 80% anatase and ≈ 20% rutile). The mean particle mass exposure concentration was 7.2 mg/m^3 for the first 4 months, followed by 14.8 mg/m^3 for 4 months and 9.4 mg/m^3 for 16 months for rats and 5.5 months for mice. Following the exposure period, the animals were kept under clean air conditions for an additional 6 months for rats and 9.5 months for mice. Rats developed lung tumours (benign keratinizing cystic squamous cell tumours, squamous-cell carcinomas, bronchioalveolar adenomas and adenocarcinomas) from 18 months of exposure. At the tested concentration, an increased mortality rate (60% versus 42% in the control group), a decreased body weight, an increase of lung wet weight, an alteration of alveolar lung clearance and non-neoplastic effects in the lung (bronchioalveolar hyperplasia and interstitial fibrosis) were also reported. No increased lung tumour rate was reported in mice. However, the high background tumour response in the control group might have limited the ability to detect any carcinogenic effects in this study.

Similar types of lung tumours were reported in rats intra-tracheally exposed to P25 (Pott and Roller 2005). Finally, two other intra-tracheal studies assessing the promotor potential of TiO_2-NP (rutile 20 nm or TiO_2-NP 80 nm) did not report any effect. However, the protocols used as not judged reliable and the studies have been disregarded (Xu et al. 2010, Yokohira et al. 2009).

An update of the literature search was performed until December 2017 focusing on studies carried out by the respiratory route (see annex 2). No reliable study was found in this update.

Human data

Seven epidemiological studies analyzed potential link between occupational exposure to TiO_2 and the occurrence of cancers, including 5 report historical industry-based cohorts (Chen and Fayerweather 1988, Fryzek et al. 2003, Boffetta et al. 2004, Ellis et al. 2010, Ellis et al. 2013) and 2 case-control studies (Boffetta et al. 2001, Ramanakumar et al. 2008). The characterization (size, crystallinity) of TiO_2 is not provided in the publications. In this context, it cannot be excluded that workers are exposed, at least partially, to TiO_2 under nanoform in these studies.

Despite the availability of five retrospective cohort studies, some of them share a large part of their population leading in fact to only three separate populations. All of these studies present bias for

worker selection and possible misclassification of exposure and health status. Even with these limitations, two publications on two different populations (one US and one European) reported statistically increased mortality by lung cancer (Ellis et al. 2013, Boffetta et al. 2004). Boffetta et al. (2004) reported a statistically significant increased standardized mortality ratio (SMR) for mortality from lung cancer (23% increased considering all studied countries with a 51% increased in Germany). Ellis et al. (2013) reported a significant increase of mortality by lung cancer (68% increased) when comparing to Dupont referent workers. In addition, increase mortality by lung cancer of borderline significance was also consistently found in all publications, except Chen & Fayerweather (1988). The absence of statistical relationship between duration / level of exposure to TiO_2 and an excess of lung cancer can be hidden due to the methodological deficiencies (bias selection, misclassification of exposure and health status, worker health effect, confounding factors, categorization by level of exposure etc).

Regarding case-control studies, they are judged of limited relevance to conclude on lung carcinogenicity of TiO_2 considering the mode of action expected to be primarily linked to its dust nature.

In conclusion, the epidemiological data are inadequate to prove the absence of a human hazard related to carcinogenicity.

Conclusion on carcinogenicity of TiO_2-NP:

Based on the induction of lung tumours reported after inhalation and instillation exposures (Pott and Roller 2005, Heinrich et al. 1995), TiO_2-NP (P25 as material tested) is considered as a lung carcinogen in rats at a concentration resulting in pulmonary inflammation and altered clearance. This conclusion is in line with those of IARC (2006 & 2010), which classified TiO_2 (without more specifications) as possibly carcinogenic to humans (Group 2B), of NIOSH (2011), which considered ultrafine TiO_2 as potential occupational carcinogen and of RAC (ECHA, 2017), which concluded that TiO_2 (without further physico-chemical description) should be classified as Category 2 carcinogen (suspected human carcinogen) according to CLP Regulation. Especially, the RAC conclusion is based on a following weight-of-evidence approach:

- *taking note that TiO_2 was not shown to be a multisite carcinogen,*
- *being aware that TiO_2 is a lung carcinogen especially in female rats,*
- *recognising that there are no robust carcinogenicity studies in species other than rats,*
- *recognising that the majority of rat lung tumours occurred late in life,*
- *recognising that rat lung tumours only developed under inhalation exposure conditions associated with marked particle loading of macrophages,*
- *presuming a practical threshold for lung tumour development (mutagenicity in lung cells is considered to depend on chronic inflammation and oxidative stress),*
- *taking note of experimental, mainly repeated dose toxicity data indicating a lower sensitivity of other small rodents, monkeys and humans compared to rats,*

- *being aware of TiO_2 epidemiology studies which do not consistently suggest an association between occupational exposure to TiO_2 and lung cancer mortality.*

3.3.4 Toxicity to reproduction (effects on fertility and developmental toxicity)

The studies presented in table below have been identified from the literature search performed up to 2017 focusing on studies carried out by the respiratory route (see annex 2).

No study performed with a standard protocol to assess fertility and development is available by respiratory route.

Table 9: Developmental toxicity studies identified in the literature for TiO_2-NP

Method	Results	Remarks	Reference
INHALATION ROUTE			
Pregnant female C57BL/6BomTac mice (22-23/group) UV-Titan L181 (rutile 70,8% modified with Zr Si, NaO, Al, coating polyalcohol); 21 nm 40 mg/m^3 (measured: 42.4 ±2.9 mg/m^3; 1.70 ± 0.2 x10^6 part./cm^3; peak size: 97 nm) whole-body for 1h/d; GD8-18 Parameters: maternal lung inflammation, gestational and litter parameters; offspring neurofunction and fertility (exposure C57BL offspring cross-mated to naïve CBA/J mice)	<u>Time-mated adult female mice:</u> Lung contained 38 mg Ti/kg on day 5 and 33 mg Ti/kg on day 26-27 after exposure. Low or no Ti in liver. Decreased absolute and relative lung weight. No effect on gestational and litter parameters. Lung inflammation (BAL) 5 day and 26-27 days following exposure termination. <u>Gestationally exposed offspring:</u> Moderate neurobehavioral alteration (spent significantly less time in the central zone of the field and visited the central zone less frequently, startled less). <u>Fertility part of the study</u>: Low or no Ti in liver and milk. No significant effect on fertility.	Only one high concentration tested	Hougaard et al. (2010)
C57BL/6 mice UV-Titan L181 (rutile 70,8% modified with Zr Si, NaO, Al, coated with polyalcohol); 20.6 nm; BET = 107.7 m^2/g 42.4 mg/m^3 for one hour/day; GD8-18 by inhalation, whole body. Female offspring were mated with unexposed CBA males. F2 descendants collected on PND2-7 or PND80 and ESTR germline mutation rates estimates from full pedigrees of F1 female mice	No evidence for increased ESTR mutation rates in F1 and F2 offspring. No effect on viability, no effect on sex-ratio.	Only one high concentration tested	Boisen et al. (2012)
Pregnant female C57BL/6 mice (n=12-13) UV-Titan L181 (rutile 70,8% modified with Zr Si, NaO, Al, coated with polyalcohol); 20.6 nm	Daily sperm production (DSP) not statistically affected in the F1 generation, although TiO_2 tended to reduce sperm counts (-12%). Time-to-first F2 litter increased with decreasing sperm production.	Only one high concentration chosen to correspond to half of the Danish OEL (8h).	Kyjovska et al. (2013)

Exposure to 42 mg/m^3 for 1h/d by inhalation whole body; GD8-18 F1 (C57BL/6J) offspring (n = 25) mated with unexposed CBA/J (cross-mating males/females) Assessment of male reproductive function in the two following generations (body and testicle weight, sperm content per g testicular parenchyma and daily sperm production (DSP))	Effect on sperm production in the F2 generation.	Need to optimize the method for measurement of DSP.	
Time-mated C57BL/6Bom-Tac female mice (22-23/group) UV Titan L181 Rutile surface coated, 17 nm, surface area: 70 m^2/g Chemical composition: Na_2O (0.6%), SiO_2 (12.01%), Al_2O_3 (4.58%), ZrO_2 (1.17%), TiO_2 (70.81%). UV-Titan is coated with polyalcohol adding to the remaining wt %. Geometric mean size during inhalation exposure: 97 nm 42 mg/m^3 for 1h/day whole body during GD8-18. Parameters: Comet assay in BAL +/- liver; hepatic gene expression; lung inflammatory response	Persistent inflammation in mothers and affected gene expression in the liver of offspring, with increased response in female offspring. The observed changes in gene expression in the newborn offspring 2 days after birth suggest that anti-inflammatory processes were activated in the female offspring related to retinoic acid signalling. Negative *in vivo* comet assay (BAL and liver in the non-pregnant females and dams; liver in the newborn at PND 2 or weaned offspring at PND 22).	Only one high concentration.	Jackson et al. (2013)
Pregnant female Sprague-Dawley Aeroxide (anatase/rutile ; 21 nm) Whole body exposure to 11.3 ±0,039 mg/m^3 for 5h/d from GD10 for an average of 8.2±0.85 days Microvascular tissue isolation (GD20) and arteriolar reactivity studies of the uterine premyometrial and fetal tail arteries	Significant maternal and fetal microvascular dysfunction. Isolated maternal uterine arteriolar reactivity consistent with a metabolically impaired profile and hostile gestational environment that impacted fetal weight. Isolated fetal microvessels demonstrated significant impairments to signals of vasodilation specific to mechanistic signalling and shear stress.	Only one concentration. Even if not clearly stated in the publication, tested material corresponds to P25	Stapleton et al. (2013)
Pregnant female Sprague-Dawley (6/group) Aeroxide (anatase/rutile ; 21nm) Exposure to 10.6 ±0.3 mg/m^3 for 5h/d GD6-12 (average of 6.8±0.5 days) by inhalation, whole body. Maternal or litter characteristics (maternal weight, implantation site, pup/litter, progeny weight at w 8, 12, 16 and 20) Microvascular reactivity, mitochondrial respiration and hydrogen peroxide production of the coronary and uterine circulations of the female offspring evaluated between 11 and 16 weeks of age	No significant differences within the maternal or litter characteristics. No significant differences in spontaneous active diameter, passive diameter or vascular tone with respect to coronary arterioles. No oxidative stress (hydrogen peroxide production). Endothelium-dependent dilation and active mechanotransduction in both coronary and uterine arterioles abolished. Significant reduction in maximal mitochondrial respiration (state 3 – maximal mitochondrial state) in the left ventricle and uterus.	Only one concentration. May be attributed to altered NO signalling (decreased NO bioavailability associated with oxidative NO scavenging). Even if not clearly stated in the publication, tested material corresponds to P25	Stapleton, Nichols, et al. (2015)
Pregnant female Sprague-Dawley	No effect on maternal weight,	Few animals	Engler-

(4/group) P25; count median aerodynamic diameter of 171 nm Exposure to 10.4 mg/m^3 for 5h/d; 4d/w; GD7-20 (for 7.8±0.5 days) by inhalation whole body Behaviour and cognitive functions of male pups at 5 months of age	implantation site number or pup number per litter. No effect on locomotor, balance, affective, anxiety-like or depressive-like behaviour in the male pups. Reference memory learning, retention, and perseveration not markedly altered. Prenatal TiO_2-NP exposure induced significant working or short-term memory impairments and initial motivation: alteration in cognitive behaviour.	tested. Only one concentration.	Chiurazzi et al. (2016)
Pregnant female Sprague-Dawley P25 (anatase/rutile ; 21nm) Whole body exposure to 10.35+/-0.13 mg/m^3; 5h/d treatments; from GD~6, with the last exposure given 24 h before birth, for a total of approximately 8 exposures (7.79+/-0.26 days) Physiological and bioenergetic effects on heart function and cardiomyocytes across three time points, fetal (GD20), neonatal (4–10 days), and young adult (6–12 wk).	Cardiac impairment of the progeny (systolic and diastolic abnormalities and cardiomyocyte contractile attenuation). Mitochondrial respiration dysfunction, with varying degrees of impairment across developmental stages.	Only one concentration.	Hathaway et al. (2017)
Pregnant female Sprague-Dawley P25 (anatase (80%) and rutile (20%)); primary particle size: 21 nm; specific surface area: 48.08 m^2/g; zeta potential: -56.6 mV Real-time TiO_2-NP mobility diameter: 129 nm, aerodynamic diameter: 143 nm Exposure by whole-body inhalation to 10 ± 0.5 mg/m^3, 4-6h/exposure, from GD5.78 ± 0.11 for 7-8 days (calculated, cumulative lung deposition = 217 ± 1 µg); isolation of 20 fetal hearts on GD20 Investigation of cardiovascular function.	No effect on progeny weight or total number of pups. Significant epigenetic and transcriptomic changes in the cardiac tissue (increased cardiac function); altered signalling liver and kidney pathways; increased inflammatory signalling and growth/survival	Only one concentration.	Stapleton et al. (2018)
INSTILLATION ROUTE			
C57BL/6 mice (5-6/group) TiO_2 anatase; about 6 nm (stable colloidal suspension of primary particles) – self-prepared in a Research laboratory Treatment at 1 mg/kg on PND4 or PND4, 7 and 10 Measurement of lung function (compliance and resistance), development (morphology), inflammation (histology; multiplex analysis of BALF for cytokines) on PND14	<u>Single dose</u>: inflammatory cell influx <u>3-doses</u>: increased inflammation and inhibition of lung development (increased mean linear intercept and decreased radial alveolar count) without effect on function Macrophages were noted to take up the TiO_2-NP, followed by polymorphonuclear infiltrate Inflammatory cytokines and matrix metalloproteinase-9 were increased in lung homogenates,	Only one concentration. Instillation route, worst case exposure (bolus administration)	Ambalavanan et al. (2013)

	and VEGF was reduced		
Pregnant female C57BL/6 mice TiO_2 anatase; 12.3 nm; BET: 96 m^2/g; zeta potential at pH7: 3.7 and hydrodynamic diameter: 1280 nm 3 instillations of a weekly dose of 100 µg on GD2.5, 9.5 and 16.5 Lung examination at GD17.5 (fetal stage); at PND 14.5 (pulmonary alveolarization) and at PND49.5 (lung maturity) Analysis of foetotoxicity on GD17.5 Measurement of cytokines on GD17.5 Chemical analysis of placenta and fetal lungs on GD17.5	Long-lasting impairment of lung development of the offspring. Increase of the alveolar space and a decrease of the number of alveoli on PND14.5 and 49.5. Decreased placental efficiency together with the presence of NPs in placenta, no increase of inflammatory mediators present in amniotic fluid, placenta or offspring lungs. Decreased pulmonary expression of vascular endothelial growth factor-a (VEGF-a) and matrix metalloproteinase 9 (MMP-9) at the fetal stage, and fibroblast growth factor-18 (FGF-18) at the alveolarization stage. No effect on uterine weight, fetal resorption rates and number of living fetuses. Decreased fetal weight. Increase of inflammatory cells in the lung of pregnant females.	Only one concentration. Instillation route, worst case exposure (bolus administration) Hypothesis: administration of NPs in pregnant mice is followed by an effect on the placenta with impact on the respiratory development of the offspring.	Paul et al. (2017)

Effects of *in utero* exposure of two forms of TiO_2-NP, UV-Titan L181 (Kyjovska et al. 2013, Jackson et al. 2013, Hougaard et al. 2010, Boisen et al. 2012) and P25 (Stapleton, Nichols, et al. 2015, Stapleton et al. 2013, Hathaway et al. 2017, Engler-Chiurazzi et al. 2016, Stapleton et al. 2018) were evaluated by inhalation by different groups .

Effects of **surface-coated TiO_2-NP, UV-Titan L181** (rutile (70.8 wt%) modified with unspecific amounts of zirconium (0.86-1.17 wt%), silicon (12 wt%), aluminium (4.58 wt%) and sodium oxide (0.6 wt%), and coated with polyalcohols (crystallite primary particle size 20.6 nm; surface area 107 m^2/g; geometric mean diameter 97 nm)) was studied in C56BL/6 mice and their offspring by the same group (Kyjovska et al. 2013, Hougaard et al. 2010, Boisen et al. 2012). In these studies, pregnant females were whole-body exposed 1h/day to either filtered clean air, or a target concentration of 40 mg/m^3 of UV-Titan L181 (42.4 ± 2.9 mg/m^3 measured over all the experiments) from gestational day 8 to 18.

Lung inflammation was noted in the BALF (with increased neutrophils) of the exposed dams, 5 and 26-27 days after exposure termination, relative to controls. In the offspring examined at age 11-15 weeks (males) or 12-16 weeks (females), cognitive functions were unaffected, while moderate neurobehavioral changes were noted (in open field test). In contrast, no significant effect on gestational and litter parameters or on fertility was reported (Hougaard et al. 2010).

No increased ESTR (expanded simple tandem repeat) mutations were measured in the F1 females exposed *in utero* to UV-Titan 181 and in the F2 offspring of prenatally exposed female mice, as compared to controls. There was also no effect on viability or sex ratio (Boisen et al. 2012).

Daily sperm production was not statistically significantly affected in F1 and F2 offspring. Only a trend in reduced sperm counts was recorded in the F1 generation with an increase in time-to-first F2 litter (Kyjovska et al. 2013).

No increase of DNA strand breaks was noted in BALF of time-mated mice or in the liver of both time-mated mice and their offspring. In contrast, exposure to UV-Titan 181 induced a persistent inflammation in mothers and affected gene expression in the liver of female offspring (Jackson et al. 2013).

It is worth to notice that the relevance of the results from the above mentioned studies is questionable regarding the high concentration used (42 mg/m^3).

Aeroxide TiO_2 P25 (anatase 80%, rutile 20%; primary particle diameter 21 nm, average aerodynamic diameter of agglomerates formed during aerolization: 149.1 ± 3.7 nm; surface area 48 m^2/g; zeta potential -56.6 mV) was studied on female Sprague Dawley rats and their offspring in different studies performed by the same group (Stapleton, Nichols, et al. 2015, Stapleton et al. 2013, Hathaway et al. 2017, Engler-Chiurazzi et al. 2016, Stapleton et al. 2018). In these studies, pregnant females were exposed whole-body 5h/day to either filtered clean air (control ; 0 mg/m^3) or a target concentration of 10 mg/m^3 after implantation (from gestation day 6, 7 or 10), with an average duration of about 7-8 days. The generated aerosols excluded agglomerates > 400 nm, and exposure started once the steady state aerosol concentration was achieved. Concentrations were monitored in real time, as well as the particle size distribution (using a scanning device).

Impact of the duration of exposure (≤ 7 days versus ≥ 7 days) was investigated in Stapleton et al. (2013). The authors showed significant decreases in the average litter size and weight, and in pup weight after 11 days of inhalation exposure, while an exposure of 7 days had no effects on maternal weight, implantation sites, litter size, sex of pups or female progeny weight gain. No significant differences within the maternal or litter characteristics (maternal weight, implantation site, number and weight of pups) were noted in the consecutive studies (Stapleton et al. 2013, Engler-Chiurazzi et al. 2016, Stapleton et al. 2018).

Microvascular characteristics were analysed by Stapleton, Nichols, et al. (2015), Stapleton et al. (2013). The authors reported fetal microvascular dysfunction after *in utero* P25 exposure, with an impaired ability of uterine arterioles to proper dilate and an impaired microvascular function. Endothelium-dependent dilation and active mechanotransduction in both coronary and uterine arterioles were significantly altered in the female progeny studied at 11-16 weeks of age. In addition, a significant reduction in maximal mitochondrial respiration in the left ventricle and uterus was noted. Hathaway et al. (2017) confirmed this decrease in basal and maximal respiration, and related this to the systolic and diastolic abnormalities and cardiomyocytes contractile attenuation observed in the progeny. Finally, Stapleton et al. (2018) reported a decreased cardiac dysfunctions (characterized by epigenetic and transcriptomic changes) in foetuses while showing a propensity toward hepatic and renal disease and increased inflammatory signalling.

Regarding neurotoxicity, the behaviour and cognitive functions of pups were evaluated at 5 months of age by Engler-Chiurazzi et al. (2016). They showed significant working memory impairments, especially under maximal mnemonic challenge, and possible deficits in initial motivation in male F1 adults. According to the authors, these results indicate that maternal exposure during gestation produces psychological deficits that persist into adulthood in male rats.

Two additional studies carried out by the instillation route have been identified. Both focused on effects of TiO_2-NP (anatase) on the development of the lungs.

Intranasal instillation of TiO_2-NP (anatase, 6 nm) were used in newborn C57BL/6 mice to study effects on lung development (Ambalavanan et al. 2013). A dose equivalent to 1 mg/kg body weight (5 µl of NP suspension) was instilled either at post-natal day (PND) 4 (single-dose experiment) or at PND 4, 7, 10 (multiple-dose experiment) and compared to mice exposed to vehicle. Administration of anatase caused inflammatory cell infiltrates and inhibited lung development in both single and multiple experiments. Inflammatory cells consisted of macrophages containing accumulation of TiO_2-NP surrounded by other inflammatory cells (polymorphonuclear and some mononuclear cells). No alteration of lung function or pulmonary vascular modeling was recorded, but gene expression and protein amounts of specific cytokines were increased in lung homogenates, as well as MMP-9 (known to be involved in lung injury and inhibition of development). There was also an overexpression of proinflammatory cytokines such as IL-1β, known to impair lung alveolarization. VEGF (vascular endothelial growth factor), important for normal lung development, was decreased, this decrease may contribute to impairment of alveolarization. The authors concluded that these effects possibly impact the risk of respiratory disorders in later life.

TiO_2-NP (anatase, 12 nm) was also shown to impair lung development of the offspring of C57BL/6 female mice (Paul et al. 2017). The pregnant mice were anesthetized and treated with 10 µl of nanoparticle suspension (10 mg/ml) by non-surgical intratracheal instillation at gestational day (GD) 2.5, 9.5, 16.5, while vehicle alone was injected for the saline group (control). The fetal resorption rate and the number of fetus/litter were not affected, but the fetus weight was decreased at GD 17.5 as well as placental efficiency (fetal weight/placental weight). Lung morphometric measurement (at PND 14.5 and 49.5) indicated a decrease in lung alveolar surface in offspring after anatase exposure during pregnancy. TiO_2-NP was significantly higher in the placenta of the treated group. Yet, no inflammatory response was detected in the amniotic fluid, placenta and lungs of fetuses from dams exposed to anatase. Therefore inflammation of dams' lungs did not appear to be the underlying mechanism contributing to lung impairment in the offspring. Decreased pulmonary expression of VEGF-α could be the mechanism leading to impairment of the lung. Other genes involved in lung development such as MMP-9 at the fetal stage and FGF-18 (fibroblast growth factor-18) at the alveolarization stage were shown to be downregulated. Contrary to Ambalavanan et al. (2013) who observed an increased MMP-9 expression in a context of a pro-inflammatory response, a decrease was recorded in the present study.

In all the studies described above, only a single concentration of TiO_2-NP was investigated and compared to the corresponding controls. Therefore, no dose-response relationship can be established. The results are useful to highlight mechanisms, but not to derive a TRV.

3.4 Population of concern

Only few studies provide information on potential populations which may be particularly sensitive to TiO_2-NP.

Roulet et al. (2012) induced emphysema in rats by instillation of elastase. Seven days after elastase or saline instillation (control), rats underwent intratracheal instillation with TiO_2-NP (100 µg/rat) or bovine serum albumin (BSA) (0.5 mg/ml). The authors showed that TiO_2-NPs did not aggravate elastase-induced pulmonary inflammation and emphysema. This result suggests that people with lung pathology may not be particularly at risk in case of TiO_2-NP exposure, but need to be confirmed by further studies.

Scuri et al. (2010), as stated in section 3.4.1.4, suggested a greatest sensitivity of young rats (newborn, 1-2 day old and weanling, 2 week old) compared with adults (12 week old), as evidenced by increased in lung neurotrophins, after 3-day inhalation to P25. In contrast, Gate et al. (2017) compared young adults (12–13-week old) and elderly rats (19-month old) in a biopersistence and translocation study (see 3.1) and showed that the amount of TiO_2 recovered in spleen and liver were higher in elderly rats. The study from Disdier et al. (2017) with rats of the same age, also underlines the age susceptibility for a potential neurotoxicity of inhaled TiO_2-NP, with older rat being more susceptible.

4 Chronic inhalation toxicological reference value proposal

For the time being, most of available studies described above present concentrations of TiO_2-NP in mg/m^3. There are currently ongoing discussions on the choice of the relevant dose metrics to use for poorly soluble particle, and specifically regarding nanometric forms. Other metrics are currently identified such as surface area, particle number, particle void volume... Indeed, several toxicology studies have suggested that the biological response following deposition of particles in the lung is dependent on particle area, rather than on mass concentration (Oberdorster (2002), NIOSH (2011)). Sager and Castranova (2009) and Noel et al. (2017) compared different exposure metrics after studying different size and agglomeration state (only in the study of Noel et al.) of TiO_2-NP. They confirmed that toxicity is related, at least in part, to surface area. However, regarding pulmonary effects, they also concluded that mass concentration, associated with agglomeration state could be appropriate, as it presents a good correlation with effects observed, and has the advantage of being commonly used and easier to determine. NIOSH (2011) reached similar conclusions.

Therefore, given the current lack of consensus on the dose metric to be used, the mass concentration is retained for the derivation of the TRV, as it is the most commonly used metric so far.

4.1 Critical effect

From the available repeated-dose toxicity studies, TiO_2-NP can induce toxicological effects in the lung (both non-neoplastic and neoplastic lesions), the cardiovascular system, the brain, the liver and the kidney. Developmental toxicity (neurotoxicity, impaired microvascular function) is also reported when TiO_2-NP is administered during pregnancy.

Considering all the repeated dose toxicity studies performed by inhalation, the most sensitive effect seems to be lung inflammation, which is observed at concentrations from 2 mg/m^3 in rats. More severe pulmonary effects including lung tumorigenesis occurred at higher concentration (≥ 10 mg/m^3). Effects on other organs are also reported at concentrations higher than 2 mg/m^3. For example, effects on the cardiovascular system were noted at 6 mg/m^3 but lower concentrations were not tested. Similarly, neurotoxicity and developmental effects were observed at a single tested concentration of 10 mg/m^3. Regarding toxicity on the liver and the kidney, the studies identified were all performed by instillation and cannot be adequately compared to inhalation conditions.

Based on the available data, lung inflammation is identified as the critical effect after TiO_2-NP exposure. However, most studies only focused on pulmonary response and the few assessing other potential target organs only tested a single high concentration. In this context, it cannot be completely ruled out that other adverse effects can occur at non-inflammatory concentrations.

Interspecies differences from experimental studies

Bermudez et al. (2004) compared the sensitivity of three rodent species to the lung toxicity of P25. The experimental findings suggest that the rat is particularly sensitive to lung toxicity of TiO_2-NP compared to other rodents. Indeed, clear species differences in pulmonary clearance and lung lesions were observed after inhalation exposure to P25 for 13 weeks in rats, mice and hamsters (Bermudez et al. 2004). In particular, pulmonary lesions were more severe and occurred at a lower concentration in rats, which was the only species developing progressive fibro proliferative lesions and alveolar epithelial metaplasia. The differences may be explained, at least partially, by biological diversity of detoxification systems, such as anti-oxidant defences, as described below.

Despite a lung burden similar to rats, inflammatory response occurred in mice at higher concentration, without developing metaplasia or fibrosis. This lower responsiveness could be explained by a lower sensitivity of this species to oxidative damage compared to rats. For instance, an increase of antioxidant (glutathione) levels in lung tissue was found during particle exposure in mice but not in rats (Oberdorster 1995).

In hamsters, the lack of lung adverse effect reported in this study can be related to a more efficient lung clearance system. Indeed, a markedly lower retention lung half-time was noted in hamsters compared to rats and mice. Furthermore, hamsters have antioxidant protection mechanisms different from rats and humans, suggesting that this species is not appropriate for testing particulate substances which may elicit inflammatory oxidative damage (ANSES 2016).

Extrapolation to humans

Regarding general particle mode of action, there are anatomical differences between the lungs of rodents and humans (e.g. lack of well-defined respiratory bronchioles in rats), resulting in different patterns of particle retention. In rats, the deposition of particles is rather uniform and principally observed in the alveolar lumen. In contrast, the deposition of particles in humans is more pronounced at bifurcations in the terminal airways (with observation of hot-spots) and in the interstitium. In addition, it has been shown that lung clearance of particles is slower in humans than in rats by approximately an order of magnitude, with about 60 days in rats (Brown, Wilson, and Grant 2005) and 400 days in humans (Kreyling WG and Scheuch G 2000).

Nevertheless, despite these differences, humans and rats display some consistency in response to dust exposure, including inflammatory reaction, lipoproteinosis, fibrosis and hyperplasia. These effects were not reported in mice and hamsters confirming that these species do not appear to be the most appropriate to predict the pulmonary toxicity of TiO_2-NP in humans (NIOSH, 2011).

In conclusion, considering

- the lack of specific mechanistic data to adequately compare humans to rats and their sensitivity to TiO_2-NP exposure;
- a slower lung clearance of particles in humans compared to rats;
- similar qualitative lung response to dust between humans and rats;

The findings reported in rats with TiO_2-NP (P25) are considered relevant for humans.

4.2 Choice of the establishment assumption

Carcinogenic chemicals have conventionally been divided into two categories according to the presumed mode of action: genotoxic or non-genotoxic.

As stated in the section above (3.3.2), based on the most recent studies, it can be concluded that TiO_2-NP is a weak genotoxin, with effect appearing only at high doses, with a dose-response identified in numbers of positive studies. Carcinogenic effects, as evidenced by lung tumours, appear only at high concentrations, associated with altered clearance and inflammatory response (cf. 3.3.3).

Genotoxicity data on TiO_2-NP point to secondary genotoxicity as the main mechanism of action. Indeed, several publications suggest correlation between inflammation and/or oxidative stress and genotoxicity. However, other mechanisms of action cannot be formally disregarded, but tests currently available do not allow demonstrating this. These conclusions are in line with those from other authorities or reviews of TiO_2 genotoxicity (IARC, 2010; NIOSH, 2011; Anses 2016; Charles & Jomini *et al.*, 2018; OECD 2018).

Weight of evidence is of importance in assessing genotoxicity of a chemical and choosing between the derivation of threshold or non-threshold toxicological reference value.

For TiO_2-NP, the majority of positive results are obtained from comet assays. First, many of the available comet assays are *in vitro* tests for which a harmonized protocol is not currently recommended as such by European legislation, in contrast to studies with validated OECD guidelines that are considered as standard protocols to assess mutagenicity of chemicals. In addition, one of the issues to be considered with comet assay is the biological relevance of results since this assay measures early DNA lesion that may subsequently be repaired (Charles & Jomini *et al.*, 2018). Applying a weight of evidence approach for genotoxicity, Brusick et al. (2016), reached a similar conclusion by allocating a low weight to “SSB/DSB (single strand break/double strand break) *in vitro* (including comet)” endpoint.

OECD, quoted in the Anses guidance on elaboration of TRV document (ANSES 2017), stated that “*When evaluating the mutagenic potential of a test chemical, more weight should be given to the measurement of permanent DNA changes (i.e. mutations) than to DNA damage events that are reversible*” (OECD 2017). Therefore, in accordance with Anses methodology, positive responses in "indicator" tests (i.e. the measurement of DNA breaks, sister chromatid exchanges, etc.) are

certainly associated with exposure but are to be considered insufficient to determine a mutagenic effect.

In summary, based on the hypothesized genotoxic mechanism of action of TiO_2-NP combined with the "low" weight of the assays available to reach this conclusion, the derivation of a threshold toxicological reference value is considered the most relevant approach.

4.3 Existing TRV analysis

At international level, only INERIS in 2016 (INERIS 2016) proposed an environmental reference value (by inhalation and oral routes), whose construction is detailed in the table below. INERIS underlines in its document that these values are toxicological benchmarks, which are indicative, provisional values that do not cover all the potential effects induced by the different forms of TiO_2-NP, and which could be reviewed according to the evolution of knowledge. Anses received a formal request from INERIS to provide advice on this proposal.

Table 10: Inhalation TRV proposed by INERIS

Value type	Critical effect (key study)	Critical concentration	UF[3]	TRV
Toxicological benchmark	Fibro-proliferative and progressive alteration of epithelium and alveolar bronchiolarization Bermudez et al. (2004)	NOAEC = 0,5 mg/m³ NOAEC adjusted = 0.089 mg/m³	900 UF_A = 3 UF_H = 10 UF_S = 3 UF_D = 10	0,1 µg/m³

Members of Anses CES recognised the quality of the work carried out, particularly considering the time available to carry out the assessment and the complexity of the subject. Nevertheless, the experts decided not to retain the values developed by INERIS for the following reasons:

- The CES considered it essential to update the bibliography in order to take into account the most recent publications on the subject. The CES notes that there is a rapid increase in knowledge on nanomaterials and in particular on TiO_2, and that a large number of scientific studies are published regularly on the subject. In addition, it has to be noted that the bibliography, which was finalised in 2016, is not exhaustive;

[3] UF: Uncertainty Factor. For details see section 4.4.4

- The choice and quality of the key studies and the starting points for the construction of the toxicological values were questioned by the CES;
- INERIS did not take into account in its report the existing epidemiological studies, and the CES considered that it is difficult to overlook this information;
- Many questions also arose regarding the characterisation of the forms of TiO_2 used in the studies, and its consideration in the construction of toxicological values. The CES also considered that the INERIS report does not provide sufficient detail on this point, which is fundamental for the construction of a reference value for TiO_2-NP.

4.4 Toxicological Reference Value establishment

4.4.1 Choice of the key study

Human studies available on TiO_2-NP, all considered as inadequate, do not allow the establishment of a TRV.

In animals, only few studies with repeated exposure are available by inhalation. Repeated-dose toxicity studies conducted by instillation were also found in the literature. As stated in OECD (2018), those studies cannot be used for risk assessment, mainly be because this exposure bypassed the upper respiratory tract and is therefore not representative of inhalation exposure.

Therefore, among inhalation studies, the one of Bermudez et al. (2004) is selected as the key study. This is indeed the most robust study available by inhalation, with the longest duration of exposure (13 weeks). The TiO_2-NP used (P25; 80% anatase/20% rutile; about 21 nm) is one of the OECD reference forms of TiO_2-NP and is fully characterized (OECD 2015). An adequate characterisation of the tested material is a critical point considering the wide variety of forms of TiO_2-NP (different products varying in composition, coating, sizing etc.) available on the European market. Since intrinsic physicochemical properties of a nanomaterial, such as particle crystallinity, size, surface area and surface modification, are presumed to influence its reactivity and behaviour, it is essential to have information on these parameters for the tested substance. Moreover, compared to most other studies available, the concentrations used (0.5, 2 and 10 mg/m^3) are adequate to observe a dose-response relationship and identify a no-observed effect concentration. Finally, three rodent species were included (mice, rats and hamsters). This feature is very interesting as it allows an assessment of the sensitivity of different species to TiO_2-NP rigorously under the same protocol.

However, this study has also some drawbacks:

- Only local/pulmonary toxicity was evaluated, with limited details on the results of lung histopathology. This is a critical point in the assessment of TiO_2-NP, as the majority of the repeated-dose toxicity studies available only focused on lung response. No repeated dose toxicity study with a full investigation of various organs, according to OECD guidelines, is available. This limitation of the database is important to keep in mind for the establishment of the TRV and especially in the setting of the uncertainty factors.

- Only females were exposed. However, considering the rest of the database, it is not considered as a major deficiency since a significant variability in the inflammatory lung response between sexes is not expected after TiO_2-NP inhalation as this is a local effect.
- This study was performed by whole-body inhalation. Nose-only is however the preferred mode of exposure recommended in the Test Guidelines as this mode of exposure minimises exposure or uptake by non-inhalation routes and thus allow evaluating the particle effect by inhalation only (OECD 2018b). However, in the case of TiO_2-NP, the critical effect identified from the available studies is lung inflammation. Thus, a significant impact of the mode of administration between nose-only and whole-body is not expected. This is confirmed by Oyabu et al. (2016) showing that the difference on pulmonary effects after whole-body and nose-only inhalation of TiO_2-NP is minimal or even non-existent.
- P25 was not dispersed (by sonication for example) before exposure. In this context, it is considered that animals are rather exposed to large agglomerate particles instead of free and/or small aggregate particles. However, this protocol can be considered close to real exposure scenario, compared to protocols with dispersion of particles.

Considering all these elements, the study of Bermudez et al. (2004) remains the most reliable study for the establishment of the TiO_2-NP TRV. It has to be noted that all other repeated-dose toxicity studies performed with several concentrations by inhalation, even if performed on other forms of TiO_2-NP (Oyabu et al. 2017, Ma-Hock et al. 2009, Landsiedel et al. 2014, Yu et al. 2015), support qualitatively and quantitatively the results obtained by Bermudez et al. (2004).

4.4.2 Choice of the critical dose

Histopathological observations in rats, identified as the most sensitive species, are used as a basis for the establishment of the point of departure. At the tested concentration of 0.5 mg/m3, the only effects reported were a reversible decrease in body weight, the presence of particles within alveolar macrophages and very minimal changes in the patterns of alveolar macrophage accumulation in the lungs. Lesions at 2 mg/m^3 were minimal to mild in severity and consisted primarily of particle laden macrophage accumulation and aggregation in subpleural regions and in centriacinar zones, associated with minimal hypertrophy and hyperplasia of type II alveolar epithelial cells. A significant but reversible increase in terminal bronchiolar and alveolar cell replication was also found at this concentration. At 10 mg/m^3, there were more severe epithelial proliferative changes, including metaplastic changes in the centriacinar region (bronchiolization of alveolar epithelium) associated with particle-laden macrophage accumulation and increase of inflammation markers in the BALF. The histopathological findings were progressive with increase of concentration, and time, also after cessation of exposure and decrease in inflammatory response.

Based on the increased of cellular proliferation, a Benchmark Dose (BMD) modelisation was performed. Although a dose response was observed, the results of the BMD modelling cannot be

accepted because of the low number of animals per dose (n=5) and the large inter-individual variability of the data set.

Indeed, several criteria of acceptance of the BMD are not fulfilled (US EPA 2012):

- The BMD/BMDL ratio is around 10 which means a too large uncertainty;
- The BMDL is 10 times lower than the minimum non-zero dose;
- The BMD stands between control and first dose;

In conclusion, a BMDL cannot be established based on these data.

Based on the abovementioned effects, the LOAEC is established at 2 mg/m^3 and the **NOAEC at 0.5 mg/m^3**.

4.4.3 Adjustments

To reduce the value of uncertainty on toxicokinetics inter-species variability, an allometric adjustment was performed. A Human Equivalent Concentration (HEC) is calculated.

The calculation of the HEC for TiO_2-NP, detailed below, has been mainly based on the methodology used by DFG for the derivation of the limit value for the respirable dust fraction of biopersistent granular dusts (MAK 2012).

This methodology is based on the assumption that the sensitivity to TiO_2-NP of rats and humans does not differ at the same dose per lung surface area.

The first step is the evaluation of the particle fraction deposited in the lung. Deposition fraction is the ratio of number of particles deposited on the lung to the number of particles entering respiratory tract. To estimate this fraction, the Multiple Path Particle Dosimetry (MPPD) model (v 3.04)[4] was used. This model was developed by the Chemical Industry Institute of Toxicology (CIIT), NC, USA, and the Dutch National Institute for Public Health and the Environment (RIVM). The MPPD model calculates the deposition and clearance of monodisperse and polydisperse aerosols in the respiratory tracts of laboratory animals and human adults and children (deposition only) for particles ranging in size from ultrafine (1 nm) to coarse (100 µm). Respiratory tract dosimetry models have been developed for several laboratory animal species including rat, mouse, rhesus monkey, pig, and rabbit.

The second step is the calculation of the deposition rate, in m^3/day:

$$\textit{Deposition volume} = \textit{deposition fraction} \times \textit{tidal volume} \times \textit{respiratory rate} \times \textit{exposure time}$$

The elimination constant can be calculated, expressed in days:

$$\textit{Elimination constant} = -\ln(0.5)/\textit{elimination half-time}$$

The steady state lung load is calculated, in m^3:

$$\textit{Steady state lung load} = \textit{deposition volume}/\textit{elimination constant}$$

[4] https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304

It has to be noted that the steady state load in mg per lung is obtained by multiplying this value by the dust concentration in mg/m^3, that is to say the NOAEC.

Finally, the lung load related to the lung surface area can be calculated for rats and humans, and the ratio of these values is used for the calculation of the HEC by multiplying by the NOAEC:

HEC = NOAEC x (steady state load/lung surface area)$_{rat}$ /(steady state load/lung surface area)$_{human}$

The calculation of the HEC is presented below, along with the graphe modelling of the calculations. The details and references of the parameters and data used for calculation, such as options selected in MPPD program are presented in annex 3.

Rat:

- Deposition fraction$_{rat}$: 0.056 (unitless)
- Deposition rate$_{rat}$ = 0.056 x (2.1/1000000) x 102 x 60 x 6 x 5/7 = 0.003084 m^3/day

  2.1 ml = tidal volume of the rat

  102/min = respiratory rate of the rat

  60 min x 6h x 5/7j = exposure time of the study, expressed in days
- Elimination constant$_{rat}$ = -(ln0.5)/60 = 0.0116/day

  60 days = elimination half-time of TiO_2-NP for the rat
- Steady state lung load$_{rat}$ = 0.003084/0.0116 = 0.2659 m^3
- Steady state lung load$_{rat}$ = 0.2659 x 0.5 = 0.1329 mg/lung

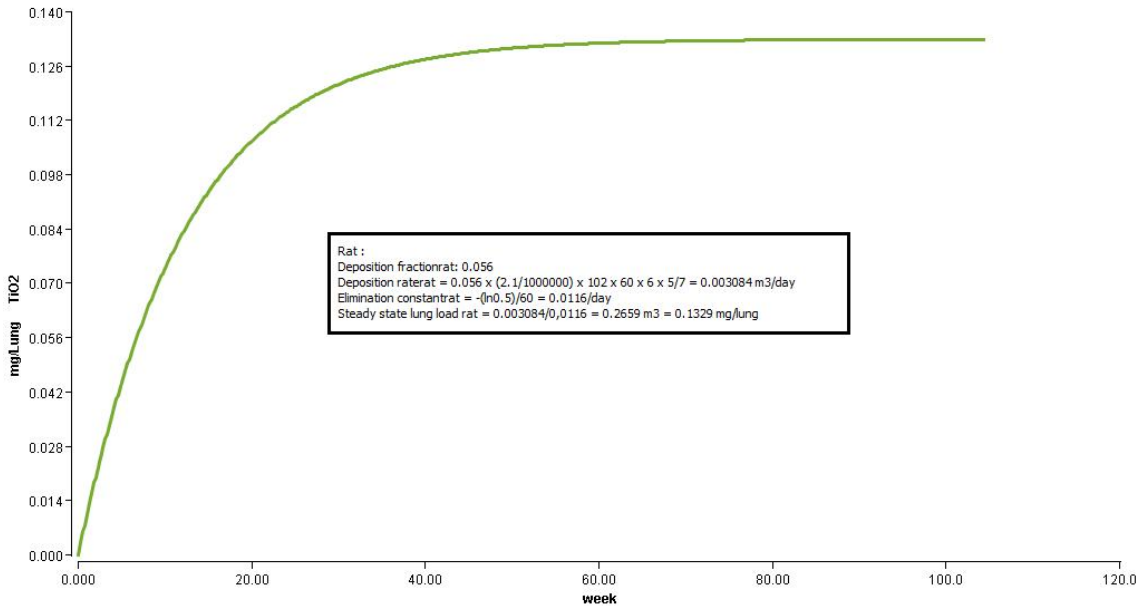


Figure 2: Modelling of steady state lung load in mg/lung in rats

Human:

- Deposition $fraction_{human}$: 0.1485 (unitless)
- Deposition $rate_{human}$ = 0.1485 x (625/1000000) x 12 x 60 x 24 = 1.6038 m^3/day

  625 ml = tidal volume of human

  12/min = respiratory rate of human

  60 min x 24h = exposure time, expressed in days
- Elimination $constant_{human}$ = -(ln0.5)/400 = 0.00173/day

  400 days = elimination half-time of TiO_2-NP for human
- Steady state lung $load_{human}$ = 1.6038/0.00173 = 927.0520 m^3
- Steady state lung $load_{human}$ = 927.0520 x 0.5 = 463.5260 mg/lung

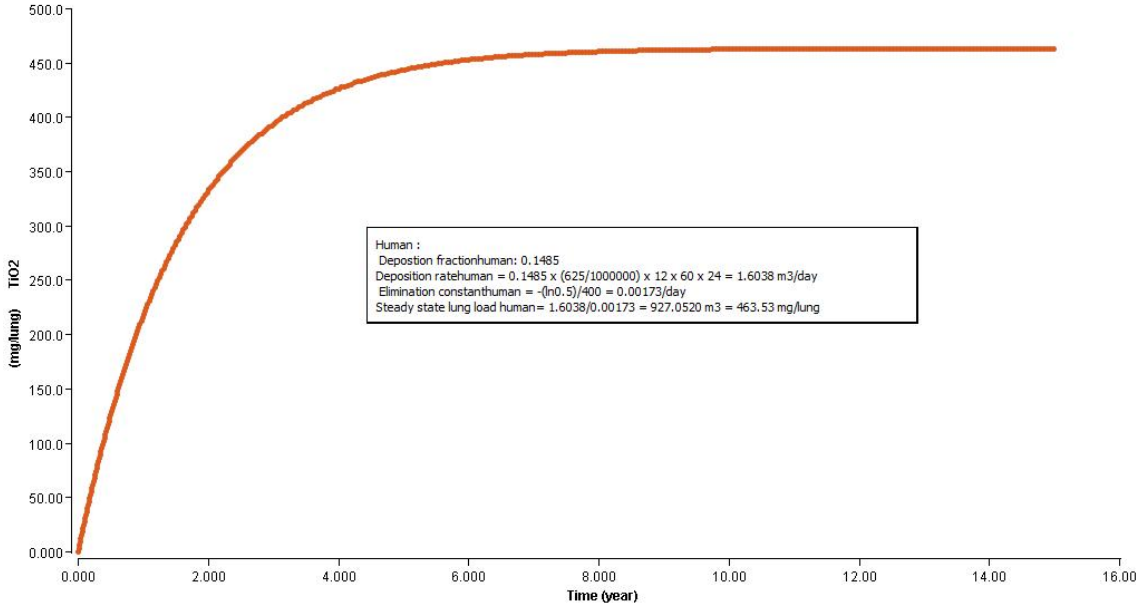


Figure 3: Modelling of steady state lung load in mg/lung in humans

Human Equivalent Concentration:

- **$NOAEC_{HEC}$** = NOAEC x (0.2659/0.297)/(927.0520/57.22) = 0.5 x (0.8953/16.2015) = 0.5 x 0.055
- **$NOAEC_{HEC}$** = 0.028 mg/m^3

4.4.4 Uncertainty factors

The chronic inhalation TRV was calculated from the $NOAEC_{HEC}$ using the following uncertainty factors (UF) (ANSES, 2017):

- ***Inter-species variability (UF_A) = 2.5***

The allometric adjustment performed by modelling enabled a human equivalent concentration to be calculated. To take toxicodynamic variability and residual uncertainties into account, an additional uncertainty factor was set at 2.5.

- ***Inter-individual variability (UF_H) = 10***

Because there were no scientific data available to reduce the default value, the value of 10 was used.

- ***Subchronic to chronic transposition (UF_S) = 3***

Because the key study was a subchronic study, with animals exposed for 13 weeks, the value of 3 was used for exposure duration extrapolation.

- ***Use of a BMDL, LOAEC or NOAEC (UF_L) = 1***

Because establishment of the TRV is based on a NOAEC, this factor does not apply.

- ***Inadequacy of the database (UF_D) = 3***

Most of the studies performed on TiO_2-P25 are not judged fully reliable for chronic risk assessment (e.g. intratracheal administration, single high concentration tested, no chronic study). In addition, because the majority of repeated-dose toxicity studies by inhalation investigated only one endpoint, it cannot be ruled out that other adverse effects could occur at sub-inflammatory concentrations. In this context, the value of 3 was selected.

A global uncertainty factor of 225 is therefore used for the derivation of the TRV.

4.4.5 Chronic inhalation toxicological reference value proposition

The TRV for TiO_2- P25 is calculated as it follows:

$$TRV = NOAEC_{HEC}/UF$$

$$TRV = 0.00012\ mg/m^3 = 0.12\ \mu g/m^3$$

This TRV is only applicable to TiO_2-NP as P25 (80% anatase/20% rutile; 21 nm) which is the substance tested in Bermudez et al. (2004) study.

In the present assessment, the relevancy of this TRV to all forms of TiO_2-NP cannot be evaluated considering the presence of more than 350 different TiO_2 products on the European market (varying in composition, coating, size etc., - parameters which are presumed to influence the reactivity and behavior of TiO_2-NP). Thus, it cannot be verified whether P25 is the most potent form and to what extent the data provided are representative for all forms produced, processed and placed on the market.

4.4.6 Confidence level

An overall confidence level was assigned to this chronic TRV by inhalation, based on the following criteria:

- Level of confidence in the nature and quality of the data:

Low: the toxicological database is not sufficient for assessing all the toxic properties of this compound. Moreover, most of the available studies are not considered fully reliable for risk assessment (e.g. intratracheal administration, single high concentration tested, material not adequately characterized)

- Level of confidence in the choice of the critical effect and the mode of action:

Moderate: it is a sufficiently robust effect for establishing a TRV. However, lung was the only investigated organ. Moreover, studies focusing on other endpoints (like cardiovascular effects, neurotoxicity) were performed with a single high concentration and did not generally assess pulmonary effects in parallel.

- Level of confidence in the choice of the key study:

Moderate: Bermudez et al. (2004) is a well-detailed study. However, the authors did not follow OECD guideline 413 and did not state whether the protocol complied with good laboratory practice. Other methodological limitations are detailed in section 4.4.1.

- Level of confidence in the choice of the critical dose:

Moderate: a dose-response relationship has been observed. A BMD could not be established, but a NOAEC was identified.

Thus, the overall level of confidence for this TRV is **moderate**.

5 Conclusions and recommendations of the Expert Committee

A chronic TRV by inhalation is proposed for TiO_2-P25, based on lung inflammation. A level of confidence was allocated to this TRV (Table 11).

Table 11: Chronic TRV by inhalation for TiO_2-P25

Critical effect (Key study)	Critical dose	UF	TRV
Lung inflammation Bermudez et al. 2004	NOAEC = 0.5 mg/m³ $NOAEC_{HEC}$ = 0.028 mg/m³	75 UF_A : 2,5 UF_H : 10 UF_S : 3 UF_L : 1 UF_D : 3	**TRV = 0.12 µg/m³** **level of confidence: Moderate**

Anses wants to insist that this value is only applicable to TiO_2-P25 (80% anatase/20% rutile; 21 nm), which is the substance tested in the study used for the establishment of this value (Bermudez et al. 2004), and might not apply to other forms of TiO_2-NP (different size, crystallinity, surface coating…) as it could not be representative of the toxicity of those other forms.

Date of validation of collective expert appraisal report by the expert committee: 29/11/2018

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ANNEXES

Annex 1: Formal Request Letter

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RÉPUBLIQUE FRANÇAISE

Ministère des Solidarités et de la santé	Ministère de la Transition écologique et solidaire	Ministère du Travail
Direction générale de la santé	Direction générale de la prévention des risques	Direction générale du travail

Monsieur le Directeur général

Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail

Paris, le - 4 JUIL. 2017

Objet : Définition d'une valeur toxicologique de référence et coordination d'actions concernant le dioxyde de titane sous forme nanométrique (TiO_2)

Monsieur le Directeur général,

L'analyse de la base de données R-Nano[1] indique que de nombreux sites en France manipulent le dioxyde de titane sous forme nanométrique. Ces manipulations peuvent être à l'origine d'exposition des travailleurs mais également d'émissions à l'extérieur des sites (rejets canalisés, rejets diffus, envols de poussière, ré-envol de matières déposées pendant les périodes précédentes) et d'exposition des populations.

De plus, le Centre international de recherche sur le cancer a classé le TiO_2 sous forme de particules respirables en cancérogène possible par inhalation. L'Anses a porté auprès de l'agence européenne des produits chimiques une demande similaire de classification dans le cadre du règlement européen (CE) n°1272/2008 « CLP », relatif à la classification, à l'étiquetage et à l'emballage des substances et des mélanges dangereux.

Le Haut Conseil de la santé publique (HCSP) a été saisi afin d'établir des recommandations sur les mesures de gestion à mettre en œuvre vis-à-vis des populations riveraines de sites manipulant du TiO_2 à l'échelle nanométrique ainsi que des travailleurs. Cette saisine fait suite à l'élaboration par l'Institut national de l'environnement industriel et des risques (INERIS), à la demande de la Direction générale de la prévention des risques (DGPR), d'une valeur repère pour l'exposition des populations riveraines de ces sites.

L'expertise de l'Anses est souhaitée afin de compléter ces travaux par la définition d'une valeur toxicologique de référence (VTR) pour le TiO_2 sous forme nanométrique. Cet avis fera le cas échéant l'objet d'un développement sur les critères pouvant justifier d'une VTR différente de celle de la substance conventionnelle non nanométrique.

Ces travaux pourront s'appuyer sur toutes les informations disponibles et en particulier les développements en cours dans le cadre des réglementations européennes, ainsi que les études de l'Institut national de recherche et de sécurité (INRS) et du réseau des préventeurs des Caisses d'assurance retraite et de la santé au travail (CARSAT).

[1] Décret n° 2012-232 du 17 février 2012 et https://www.r-nano.fr/

Un point spécifique sera également proposé par la Direction générale du travail (DGT) dans le cadre de la révision du programme de travail de l'Anses sur les valeurs limites d'exposition professionnelle (VLEP).

Ces travaux ont vocation à compléter à terme l'avis du Haut Conseil de la santé publique sur les mesures de gestion à mettre en place concernant l'exposition de la population et des travailleurs pour tous les sites manipulant du TiO_2 à l'échelle nanométrique.

Aux fins des différentes saisines, et des travaux par ailleurs engagés par les partenaires (HCSP, INRS et les CARSAT associées) sur le TiO_2, des données issues de la base R-nano pourront être communiquées aux partenaires, et ce, sous la responsabilité des directions générales. L'Anses assurera en tant que de besoin une coordination de ces différentes activités.

Nous vous remercions de bien vouloir accuser réception de la présente demande en nous précisant le ou les comités d'experts spécialisés qui seront associés au traitement de la saisine. Un avis relatif à la VTR est attendu sous 6 mois à compter de la date de la présente saisine.

Nous vous prions d'agréer, Monsieur le Directeur général, l'expression de nos salutations les meilleures.

Le Directeur général de la prévention des risques

Le Directeur général de la santé

Professeur Benoît VALLET

Le Directeur général du travail

Yves STRUILLOU

Annex 2: Bibliographic Search

Study question: Identification of toxicological studies performed with TiO_2-NP.

The ultimate goals of this systematic review are 1) the derivation of a chronic toxicological reference value by inhalation with TiO_2-NP; 2) Identification of toxicological concerns which needs to be clarified (by requesting new studies) during Substance Evaluation in the framework of Reach Regulation.

Description of the review method:

Publications were identified through two databases: PubMed and Scopus®. Secondary literature from IARC, OECD, NIOSH, ECHA, EFSA and SCCS was also taken into account.

The methodology of the review (eligibility criteria and key words) was defined between October and December 2017. The literature search was performed in January 2018. An update of the systematic review was performed in July 2018.

Key words and eligibility criteria for inclusion or exclusion:

The following key words were used for the records identification in the selected database (PubMed and Scopus®):

Identity:

"Titanium dioxide" OR Titania OR "TiO2" OR "TiO(2)" OR Rutile OR Anatase OR Brookite OR P25 OR "T-lite" OR "Titanium oxide*" OR "TiO2-NPs" OR Nanotitania OR Nanotitanium OR E171 OR NM101 OR NM102 OR NM103 OR NM104 OR NM105

"Titanium dioxide" OR titania OR "TiO2" OR "TiO(2)" OR rutile OR anatase OR brookite OR p25 OR "T-lite" OR "Titanium oxide*" OR "TiO2-NPs" OR nanotitania OR nanotitanium OR e171 OR nm101 OR nm102 OR nm103 OR nm104 OR nm105)) AND (ALL ("Ultra Fine" OR nanoscale OR nanomaterial OR "nanoparti*" OR nano OR nanocrystal OR nanosized OR "nanostructure*" OR synthetic OR nanobelt OR nanotube OR "nanofib*" OR "nanolayer*" OR modified OR coated OR "nanocomposite*" OR "functionali*" OR "nanopowder*" OR nanoamor OR nanotechnology OR "nanoadditive*" OR uncoated OR aggregate OR substituted OR agglomerate OR nm100 OR "Food-grade"

Exposure:

"Inhalat*" OR "respira*" OR airway OR nasal OR intranasal OR "intra tracheal" OR instillation OR lung OR chronic OR "pre natal" OR "post natal" OR subchronic OR "repeat*" OR "day*" OR "week*" OR olfactive OR "month*" OR "year*" OR "long term" OR subacute OR "short term" OR "nose only" OR acute OR oral OR gavage OR "drinking water" OR feed OR food OR diet OR "per oral"

"in vivo" OR animal OR "cohort*" OR "case control" OR epidemiology OR "review*" OR "chapter*" OR "poster*" OR "experiment*" OR occupational OR longitudinal OR "in vitro" OR cell OR "in silico" OR safety OR evaluation OR corona OR biokinetic OR "ex vivo"

Population:

Child*" OR "worker*" OR "adult*" OR occupational OR "rat*" OR mouse OR "rabbit*" OR "human*" OR "monkey*" OR "dog*" OR hen OR "guinea pig*" OR "animal*" OR "sensitive population" OR painter OR man OR woman OR men OR women OR "manufacturer*" OR asthmatic OR pregnant OR infant OR toddler OR male OR female OR mammalian OR mice OR elderly OR aging OR gestation

Outcome:

Toxicity OR toxicology OR "inflammat*" OR "neurotox*" OR "tumor*" OR neoplastic OR promotion OR cancer OR "oxidative stress" OR "reactive oxygen species" OR "reactive nitrogen species" OR ros OR rns OR fertility OR developmental OR effect OR "genotox*" OR "mutagen*" OR genetic OR aberration OR mutation OR "DNA damage" OR overload OR transformation OR diffusion OR translocation OR clastogenicity OR "micronucle*" OR comet OR "carcinogen*" OR hormone OR thyroid OR reproduction OR tumour OR non-neoplastic OR immunity OR metabolism OR heart OR brain OR lung OR kidney OR "blood barrier" OR "Blood-Brain-Barrier" OR "placental barrier" OR retention OR disease OR "adverse effect*" OR concern OR elimination OR kinetics OR absorption OR "reprotox*" OR safety OR noael OR loael OR loel OR noel OR mitochondria OR nucleus OR threshold OR bmd OR behavior OR reactivity OR benchmark OR hazard OR risk OR spleen OR irritation OR hematoencephalic OR assessment OR placenta OR development OR immune OR epigenetic OR promoter OR chromosome AND stability OR alveolar AND barrier OR injury OR lipid OR crossing OR excretion OR body AND burden OR distribution OR macrophage OR epigenome OR intestinal OR gut OR permeability OR renal

Only records published from 2000 were considered because it is generally considered that before this date, the tests often had missing information on the physicochemical characteristics of the testing nanomaterial and/or did not take into account nano-specificity. In addition, publications not written in English or French were excluded. A total of **1888** records were thus identified.

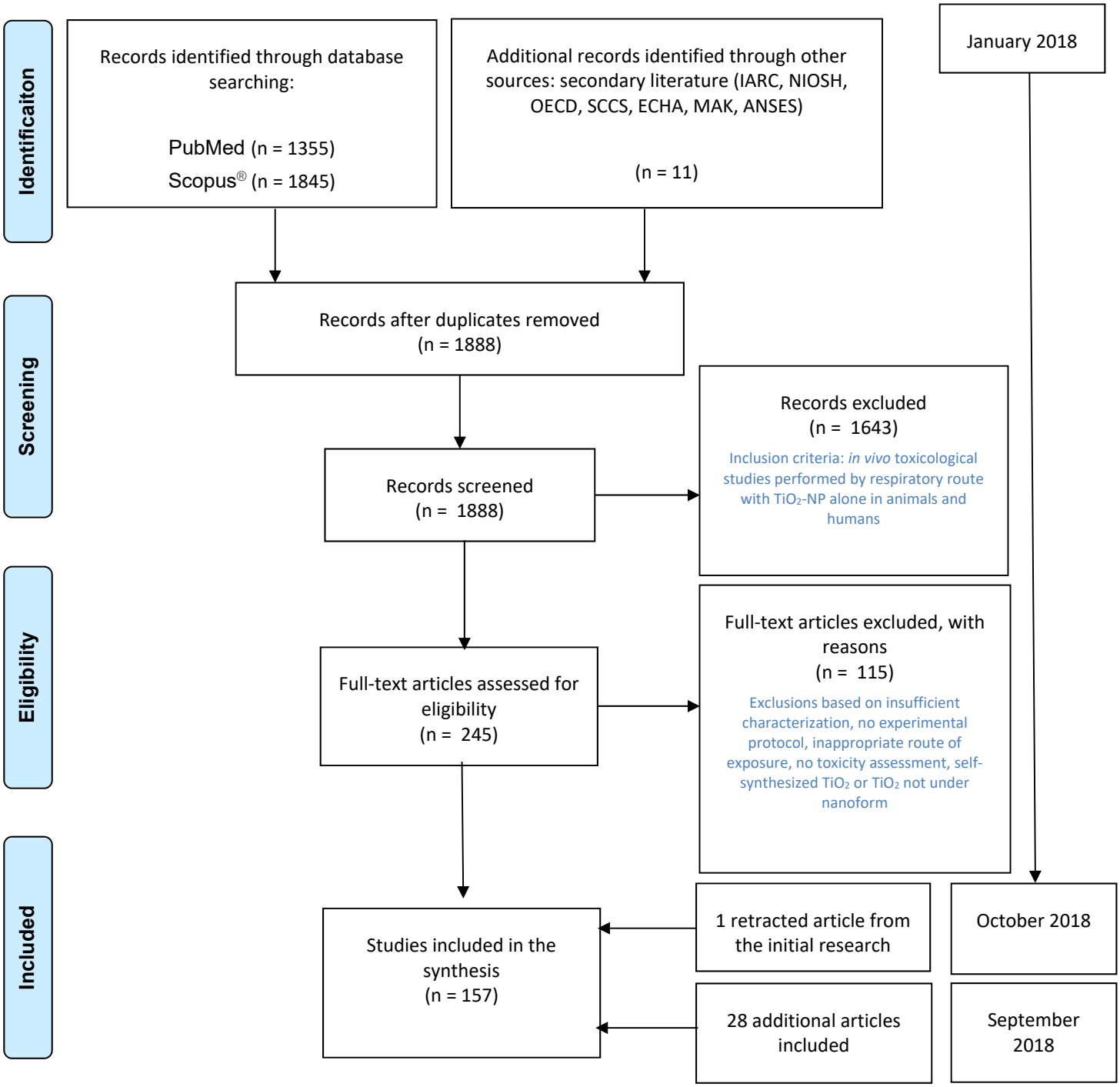
The following exclusion criteria were then applied leading to the exclusion of **1643** records based on title and abstract:

- Studies not performed with TiO_2-NP alone;
- Not toxicological studies (ex. ecotoxicological studies, studies on implants, water disinfection, biotechnology, nanomedicine, analytical method, etc.).

In addition, it was decided to only focus the assessment on *in vivo* toxicity of TiO_2-NP by respiratory route. Therefore, only full-text articles complying with this criterion were selected, leading to a total of **245** articles. In addition, in September 2018, **28** further full-text articles were included. Finally, **one** article sorted from the initial bibliographic research was retracted in October 2018.

Methodology quality assessment of included studies:

Among these 266 articles, 157 articles were judged relevant and included in the synthesis.


Identificaiton
Screening
Eligibility
Included
Records identified through database searching:
PubMed (n = 1355)
Scopus® (n = 1845)
Additional records identified through other sources: secondary literature (IARC, NIOSH, OECD, SCCS, ECHA, MAK, ANSES)
(n = 11)
January 2018
Records after duplicates removed (n = 1888)
Records screened (n = 1888)
Records excluded (n = 1643)
Inclusion criteria: in vivo toxicological studies performed by respiratory route with TiO2-NP alone in animals and humans
Full-text articles assessed for eligibility (n = 245)
Full-text articles excluded, with reasons (n = 115)
Exclusions based on insufficient characterization, no experimental protocol, inappropriate route of exposure, no toxicity assessment, self-synthesized TiO2 or TiO2 not under nanoform
Studies included in the synthesis (n = 157)
1 retracted article from the initial research
October 2018
28 additional articles included
September 2018

Annex 3: Details of parameters used for calculation of TRV

Information on the use of the MPPD model version 3.04

Parameter	Rat	Human	Reference
Airway Morphometry			
Model	Asymm. Multiple-Path long Evans	Yeh/Schum 5-lobe	/
FRC (ml)	4.0	3300	MPPD default value
URT Volume (ml)	0.42	50	MPPD default value
Inhalant properties – Aerosol			
Density (g/cm3)	4.26	4.26	P25 data, Sigma-Aldrich
Aspect ratio	1	1	/
Diameter (µm)	1.44 (MMAD)	1.44 (MMAD)	Bermudez et al. (2004)
GSD	2.6	2.6	
Inhability adjustment uncheck			Size < 3 µm
Equiv. Diam. Model uncheck			
Exposure condition – Constant exposure			
Aerosol concentration (mg/m3)	0.5	0.5	NOAEC Bermudez et al. 2004
Breathing Frequency (/min)	102	12	MPPD default value
Tidal Volume (ml)	2.1	625	MPPD default value
Inspiration Fraction	0.5	0.5	Default value
Pause Fraction	0	0	Default value
Breeathing Scenario	Whole-Body inhalation	Oronasal – normal augmenter	
Deposition/Clearance - Deposition only			

Details and justification of the parameters and data used for calculation of the TRV

- Elimination half-life:
    - Rat: 60 days (Brown et al. 2005), confirmed by the results of the Bermudez et al., (2004) study where an elimination half-life of 63 days was calculated for the concentration of 0.5 mg/m^3 for rats.
    - Human: 400 days (Kreyling and Scheuch 2000)
- Lung surface area
    - Rat: 57.22 m² (U.S. EPA, 2009)
    - Human: 0.297 m² (U.S. EPA, 2009)

Agence nationale de sécurité sanitaire
de l'alimentation, de l'environnement et du travail
14 rue Pierre et Marie Curie
F94701 Maisons-Alfort cedex
www.anses.fr
@Anses_fr

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