ANNUAL REVIEWS

Annual Review of Pharmacology and Toxicology

Mechanism of Action of TiO_2: Recommendations to Reduce Uncertainties Related to Carcinogenic Potential

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Annu. Rev. Pharmacol. Toxicol. 2021. 61:203–23

First published as a Review in Advance on April 13, 2020

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

https://doi.org/10.1146/annurev-pharmtox-101419-100049

Keywords

TiO_2, carcinogenicity, mechanism of action, adverse outcome pathway, inhalation, oral exposure

Abstract

The Risk Assessment Committee of the European Chemicals Agency issued an opinion on classifying titanium dioxide (TiO_2) as a suspected human carcinogen upon inhalation. Recent animal studies indicate that TiO_2 may be carcinogenic through the oral route. There is considerable uncertainty on the carcinogenicity of TiO_2, which may be decreased if its mechanism of action becomes clearer. Here we consider adverse outcome pathways and present the available information on each of the key events (KEs). Inhalation exposure to TiO_2 can induce lung tumors in rats via a mechanism that is also applicable to other poorly soluble, low-toxicity particles. To reduce uncertainties regarding human relevance, we recommend gathering information on earlier KEs such as oxidative stress in humans. For oral exposure, insufficient information is available to conclude whether TiO_2 can induce intestinal tumors. An oral carcinogenicity study with well-characterized (food-grade) TiO_2 is needed, including an assessment of toxicokinetics and early KEs.

1. INTRODUCTION

Titanium dioxide (TiO_2) particles are widely used in pigments and paints due to their high refractive index, which gives them a bright and natural white color (1). The desired light-scattering effect of TiO_2 particles occurs in the particle size range of 200–300 nm (2). A smaller particle size (nanosized, <100 nm) is targeted when properties such as transparency and maximum ultraviolet (UV) scavenging potential are desired in sunscreens or for (photo)catalyst functions in air- and water-purification systems (3). TiO_2 occurs in nature in three mineral crystal structures: anatase, rutile, and brookite (4, 5). Generally, the anatase crystal structure is the most effective form of TiO_2 for its UV scavenging and catalyst potential (6, 7). For use as food pigments, both anatase and rutile structures are allowed in the European Union, but anatase is most commonly used (8).

Due to the range of applications of TiO_2, people are exposed to (both nano- and microsized) TiO_2 via different exposure routes. Based on dermal exposure studies (9–11), the Scientific Committee for Consumer Safety has issued an opinion stating that there is strong evidence suggesting a lack of penetration of TiO_2 nanoparticles into viable epidermis or dermis cells (12). Inhalation and ingestion of TiO_2 pose a higher risk and can occur, for example, during TiO_2 production, packing, milling, or site cleaning in occupational settings (13); via TiO_2-containing foods, medicines, and consumer products such as toothpaste (14); and when using products such as paint sprays, rim sprays, or sunscreen sprays.

In September 2017, the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) issued an opinion that TiO_2 meets the Classification, Labeling, and Packaging criteria to be classified as a suspected human carcinogen (category 2) upon inhalation (15) and that this classification should cover all sizes, shapes, and crystal structures of TiO_2. This opinion is based on the induction of lung tumors in rats after chronic inhalation exposure to TiO_2 (16, 17). However, there is debate on whether this is due to the chemical or the physical (i.e., particle) nature of the TiO_2 particles. The biological mechanism leading to lung carcinogenicity induced by particles is only partly understood. Furthermore, the representativeness of rat lungs for those of humans is also criticized. For the oral exposure route, some recent studies report the induction of epithelial hyperplasia in the colon of rats and mice after subacute to subchronic exposure to food-grade TiO_2 (E171) (18–20). However, other oral (sub)chronic studies do not find this (21–23).

Clearly, there are many concerns but also uncertainties on whether TiO_2 can potentially lead to or promote tumor formation in humans. This is one of the reasons for controversy in how to deal with TiO_2 in policy-making, as is apparent by the repeatedly postponed decision of the European Commission on the classification and labeling of TiO_2 and the difference in policy on the use of the food additive E171 between France and other member states of the European Union.

Given the widespread exposure to TiO_2, the uncertainties regarding its carcinogenic potential need to be decreased, which may be achieved by collecting information on the biological mechanism of action (MOA) of TiO_2. Here we make use of adverse outcome pathways (AOPs), a conceptual framework that uses existing knowledge of events at the molecular level [initiating event (IE)] and links it to an adverse outcome (AO) via key events (KEs). Based on scientific peer-reviewed literature, we evaluate the ability of TiO_2 to induce each KE in the AOPs for inhalation and oral exposure leading to tumor formation. This analysis helps to increase our understanding of whether TiO_2 may indeed be carcinogenic, how representative rat data are for humans for effects of TiO_2, and what information gaps need to be filled to reduce uncertainties related to the carcinogenic potential of TiO_2. We conclude with research needs and recommendations for decision-makers in regulatory settings on TiO_2.

2. RESULTS AND DISCUSSION

2.1. Overview of Literature Search

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Studies that have addressed the carcinogenic potential of TiO_2 after inhalation or oral exposure were identified via Embase and PubMed searches (for details on the search strategy, see **Supplemental Appendix 1**). In addition, reports on TiO_2 were collected from various organizations, including the International Agency for Research on Cancer (IARC) (**https://www.iarc.fr/**), the Scientific Committee on Consumer Safety (24), the ECHA (**https://echa.europa.eu/**), the European Centre for Ecotoxicology and Toxicology of Chemicals (**http://www.ecetoc.org/publications/**), and the Swedish Chemicals Agency (**https://www.kemi.se/en**). For all included research papers, the following information was gathered: type of study, animal species and strain, animal sex, exposure route, exposure duration and postexposure period, commercial name of the studied material, crystal structure, primary particle size, agglomerate particle size, particle shape, particle surface area, surface charge, surface coating, purity and impurities, administered dose, internal dose, and observed effects.

In total, 9 reports (8, 12, 15, 25–29), 16 scientific reviews (1, 2, 5, 7, 13, 30–40), and 85 research papers were included. The research papers included 9 epidemiology studies, 1 human case report, and 64 animal studies (animal studies are specified in **Supplemental Tables 1** and **2**). A total of 25 inhalation, 18 intratracheal instillation, and 2 intrapulmonary spraying studies were reported, most of which were conducted in rats (35 studies). Effects of oral exposure have been studied using gavage (10 studies), intragastric administration (4 studies), and diet or drinking water (4 studies) as methods for exposure. All studies have been included in the search for a MOA of TiO_2 related to genotoxicity and carcinogenicity, although many studies lack information on crystal structure, (agglomerate/aggregate) particle size, or TiO_2 purity. The main complication of the lack of information on particle characteristics is that it is impossible, from the available literature, to correlate specific physicochemical characteristics of TiO_2 to a mechanism and observed effects.

2.2. Adverse Outcome Pathways for TiO_2

TiO_2 is poorly soluble under normal physiological circumstances as well as in aqueous suspensions, based on the dissolution rates in artificial lung fluids and the slow pulmonary clearance in humans, with a halftime of greater than 100 days upon short-term exposures (25, 41, 42). It is also considered a low acute toxicity substance compared to, for example, highly soluble zinc and copper oxide. Based on these features as well as data reported on its toxicity, TiO_2 is generally considered to be one of the poorly soluble, low-toxicity (PSLT) particles (25, 41, 42) or the granular biodurable particles without known significant specific toxicity (43). The MOA of PSLT particles after inhalation exposure has been described previously (5, 25–27, 31).

Inhaled PSLT particles are able to induce pulmonary toxicity and lung cancer in rats at relatively high doses (25, 26), although a very recent 2-year inhalation study with cerium dioxide in rats (performed at BASF, Germany) did not reveal any exposure-related tumor formation (R. Landsiedel, personal communication). For TiO_2-induced tumors in rats, it has been concluded that the tumors appear to be associated with persistent inflammation. Inflammation is considered to be the consequence of impaired pulmonary clearance, resulting in large lung burdens (generally referred to as lung overload). Impaired clearance of PSLT particles like TiO_2 occurs when the deposition of particles in the lung overwhelms pulmonary clearance mechanisms. This results in an accumulation of particles in the lung that is greater than expected from linear kinetics (i.e., normal physiological clearance). Impaired clearance is associated with a recruitment of alveolar macrophages (AMs) and polymorphonuclear cells into the alveolar region (44) and can be

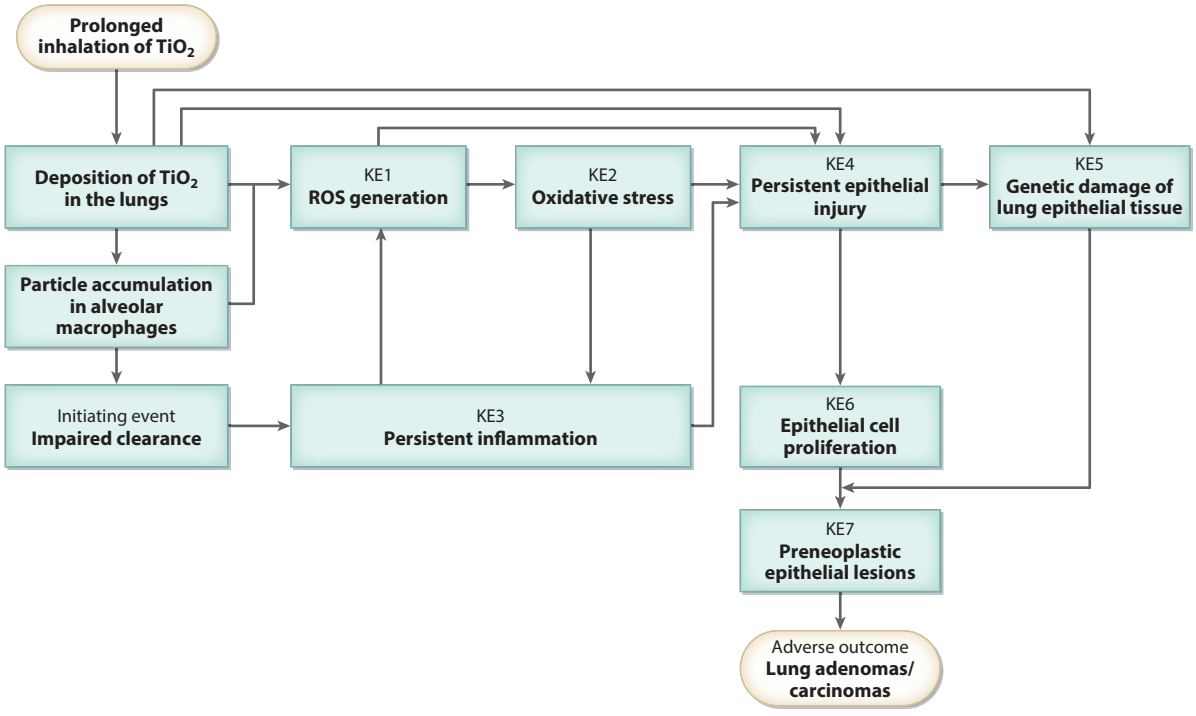

Figure 1

Postulated adverse outcome pathway of TiO_2 related to carcinogenicity after inhalation exposure (5, 17, 27–30). Abbreviations: KE, key event; ROS, reactive oxygen species; TiO_2, titanium dioxide.

demonstrated by assessing the clearance halftime of tracer particles such as fluorescent polystyrene particles (45) or radioactively labeled aerosols (17) after the TiO_2 inhalation exposure. Impaired particle clearance has occurred when a statistically significant retardation in lung particle clearance compared to controls can be demonstrated. This may subsequently lead to chronic inflammation, cell proliferation, and ultimately lung cancer in rats (26, 27). Based on the available knowledge of the MOA of PSLT particles and on the RAC's opinion on TiO_2 (15), the latest evaluation by a European scientific committee on the intrinsic hazardous properties of TiO_2 by inhalation, we suggest an AOP for TiO_2 leading to lung tumors after prolonged inhalation (**Figure 1**). A direct particle interaction of TiO_2 with DNA can also not be excluded as a possible mode of action (genotoxicity) for the induction of neoplastic lung lesions (24).

The rationale behind the suggested AOP is that, upon chronic inhalation exposure to TiO_2 at relatively high concentrations, the particles can accumulate in the lungs. In case the accumulation of particles overwhelms the clearance capacity of the lungs, impaired clearance is induced (the IE). This event results in a continuous recruitment of neutrophils and persistent inflammation (KE3). Due to the persistent inflammation and/or cellular interaction with the surface of the TiO_2 particles, reactive oxygen species (ROS) are generated (KE1), which can induce oxidative stress (KE2) in case the antioxidant capacity of the lungs is exceeded. The generation of ROS and induction of oxidative stress promote the inflammation response (KE3), creating a vicious circle of inflammation upon chronic exposure. Both inflammation (KE3) and oxidative stress (KE2) can become persistent upon chronic exposure to TiO_2 and can induce persistent epithelial injury (KE4). This leads to regenerative cell proliferation (KE6), hyperplasia (KE7), and ultimately lung tumors (the

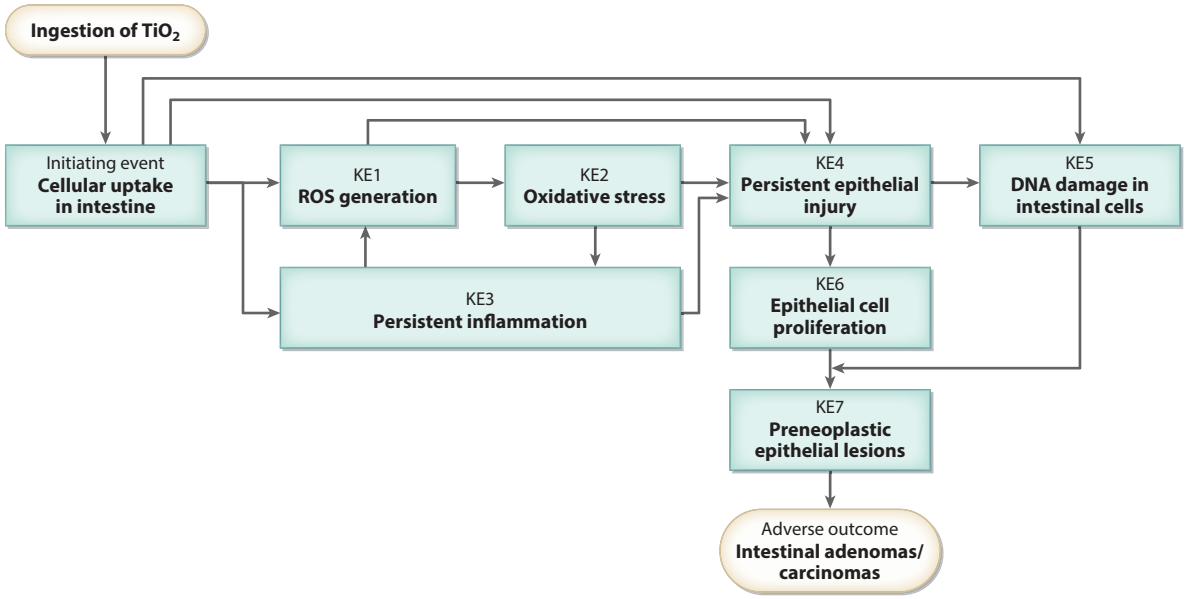

Figure 2

Postulated adverse outcome pathway of TiO_2 related to carcinogenicity after oral exposure (22, 31–33). Abbreviations: KE, key event; ROS, reactive oxygen species; TiO_2, titanium dioxide.

AO). It should be emphasized that the induction of KE6 and KE7 does not automatically lead to the AO; it is likely that there is a threshold. The persistent oxidative stress (KE2) and epithelial injury (KE4) could lead to DNA damage of the epithelial cells (KE5), which is fixed with ongoing cellular proliferation and potentially results in epithelial lung tumors (e.g., adenomas and carcinomas). Besides indirect DNA damage, the TiO_2 particles might be able to induce direct DNA damage to the epithelial cells (KE5), which could also result in lung tumors.

The MOA of possible TiO_2 carcinogenicity after oral ingestion is not completely understood. Similar to the inhalation route, it is suggested that TiO_2 might induce or promote colon tumors via inflammation and ROS production (19, 20). In case of excess ROS production, oxidative stress might be induced, leading to tissue damage. The suggested AOP for TiO_2 leading to intestinal tumors is very similar to that for lung tumors, as shown in **Figure 2**.

The rationale behind the suggested AOP is that oral ingestion of TiO_2 can lead to the cellular uptake of the particles in the intestine (the IE). In the cells, TiO_2 can generate ROS (KE1), which can induce oxidative stress (KE2) in case the antioxidant capacity is exceeded. The generation of ROS and induction of oxidative stress promote the inflammation response (KE3). TiO_2 particles could also directly induce inflammation (KE3) via lysosomal membrane permeabilization (46). Both inflammation (KE3) and oxidative stress (KE2) can induce epithelial injury (KE4). Persistent inflammation and oxidative stress due to chronic exposure to TiO_2 can thus induce persistent epithelial injury (KE4). This leads to regenerative cell proliferation (KE6), hyperplasia (KE7), and ultimately intestinal tumors (the AO). The persistent oxidative stress (KE2) and epithelial injury (KE4) can lead to DNA damage of the epithelial cells (KE5), and proliferation will make the mutation permanent (if unrepaired); thus, subsequently, intestinal tumors can be induced. Besides indirect DNA damage, the TiO_2 particles might be able to induce direct DNA damage to the epithelial cells of the gut (KE5).

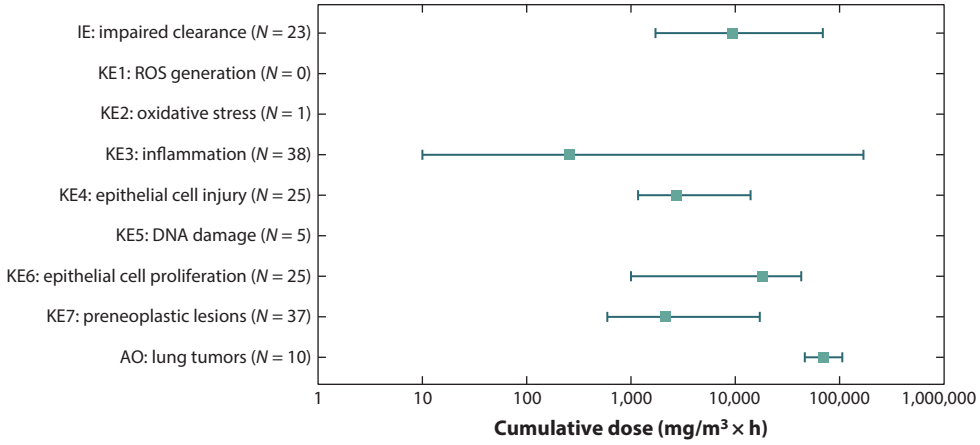

Figure 3

Quantitative analysis of occurrence of KEs after inhalation exposure to TiO_2 in rats and mice. The *x* axis represents the cumulative dose, calculated by multiplying the exposure concentration by the total number of hours of exposure. The *y* axis represents all KEs in the adverse outcome pathway, including the number of data points available for analysis. The graph shows the ED50 values, including 95% confidence intervals, which can be interpreted as the doses at which the average animal changes from nonresponding to responding. Abbreviations: AO, adverse outcome; IE, initiating event; KE, key event; ROS, reactive oxygen species; TiO_2, titanium dioxide.

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As inflammation plays a central role in both inhalation and oral AOPs, we examined this KE in more detail compared to the other KEs. According to a recent paper by Villeneuve et al. (47), there are three hallmarks of inflammation that are independent of tissue and can be independently measured. These three hallmarks are tissue resident cell activation, increased proinflammatory mediators, and leukocyte recruitment/activation (**Supplemental Tables 3–8**). We reviewed available literature to evaluate each KE, including the three hallmarks of inflammation, in the proposed AOPs to investigate whether this mechanism could be operative for TiO_2. All collected information on TiO_2 has been summarized according to the modified Bradford Hill consideration for weight of evidence for each of the hypothesized AOPs (48). Data from in vivo inhalation and intratracheal instillation studies are summarized in dose-response temporality tables (**Supplemental Tables 3–6**) and in **Figure 3**; data from oral studies are summarized in dose-response temporality tables (**Supplemental Tables 7** and **8**) and in **Figure 4**. The major findings are discussed below.

2.3. Quantitative Analysis of Key Events

The correlation between KEs is both dose and time related. To obtain insight into these complex correlations, we reduced the complexity of the available data by combining the administrated doses and the exposure durations into a cumulative dose for each individual study. As most studies did not measure and report the delivered dose, we estimated the cumulative delivered dose by (*a*) multiplying the concentration in the test atmosphere by the total number of hours of exposure (expressed as $mg/m^3 \times h$) for inhalation studies, (*b*) multiplying the administration concentration by the number of administrations [expressed as mg/kg body weight (bw)] for intratracheal instillation aspiration studies, and (*c*) multiplying the administration concentration per day by the total number of days of exposure (expressed as mg/kg bw) for oral exposure studies. This cumulative delivered dose was not further adjusted for the deposition efficiency upon inhalation, as the aerodynamic diameter of the TiO_2 aerosols is within the same range for most of the inhalation studies;

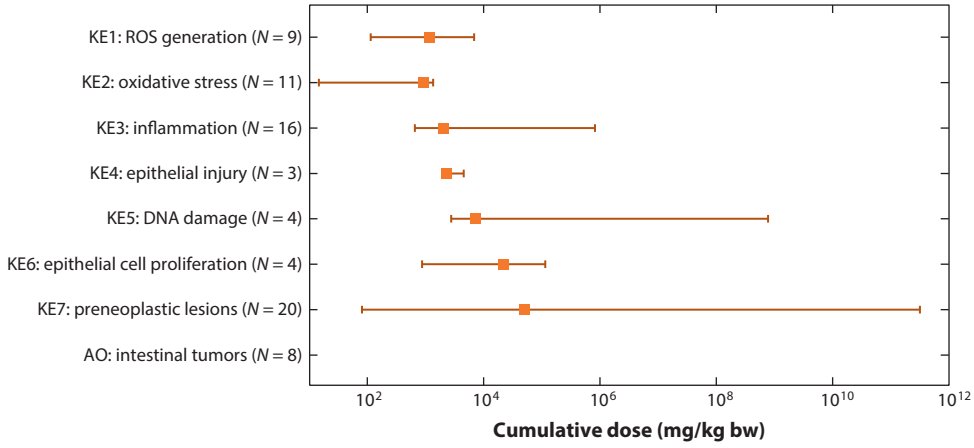

Figure 4

Quantitative analysis of occurrence of KEs after oral exposure to TiO_2 in rats and mice. The *x* axis represents the cumulative concentration, calculated by multiplying the administration concentration per day by the total number of days of exposure. The *y* axis represents all KEs in the adverse outcome pathway, including the number of available data points. The graph shows the ED50 values, including 95% confidence intervals, which can be interpreted as the doses at which the average animal changes from nonresponding to responding. Abbreviations: AO, adverse outcome; bw, body weight; KE, key event; ROS, reactive oxygen species; TiO_2, titanium dioxide.

however, the estimated cumulative dose is intended for comparison of studies with different exposure concentrations and durations and should be interpreted carefully.

We analyzed the cumulative doses at which each KE response changed from not observed to observed by applying binary logistic regression analysis using PROAST software version 67.0 (available at **https://www.rivm.nl/en/proast**) in R version 3.6.1. For each KE, an ED50 value and 95% confidence interval were calculated. The ED50 value can be interpreted as the dose at which the average animal changes from nonresponding to responding. We plotted the ED50 confidence intervals for all KEs in both AOPs (**Figures 3** and **4**).

2.4. Mechanism of Action After Inhalation

From the quantitative analysis (**Figure 3**), we can observe that most KEs are induced at similar cumulative doses, as indicated by the overlapping confidence intervals for the ED50 values. Only for the induction of lung tumors are clearly higher cumulative doses needed, compared to the other KEs. Nevertheless, we can observe a trend in increasing ED50 values from inflammation toward epithelial injury, epithelial cell proliferation, and ultimately lung tumors, indicating that the AOP leading to lung tumors might be operative for TiO_2. This is supported by the data from the individual studies showing that TiO_2 can induce impaired clearance (IE) in rats upon long-term exposure to high concentrations (16, 17, 45, 49–56). Impaired clearance has not been studied in mice. At lower exposure concentrations (<10 mg/m^3) and/or durations (after 5 days and 4 weeks exposure), impaired clearance was not observed (57–60). In addition, many studies show induction of inflammation (KE3) after inhalation exposure to high concentrations of TiO_2 (>10 mg/m^3) in both rats (16, 45, 51–55, 57, 58, 61–65) and mice (61, 66–69). Inflammation can also be observed after intratracheal instillation at cumulative doses greater than 1.8 mg/kg in rats (56, 70–78) and 0.05 mg/kg in mice (79–84). Epithelial injury (KE4) is also observed after exposure to TiO_2 in rats (45, 52, 56, 58, 61, 63, 64, 71–73, 75, 85, 86) and mice (61, 66, 79, 80, 83) at

cumulative doses above 1,000 mg/m^3 × h. Other studies, using lower cumulative concentrations, did not observe epithelial injury (57, 62, 67). The subsequent epithelial cell proliferation (KE6) is observed at cumulative concentrations (>1,000 mg/m^3 × h, corresponding to, e.g., >10 mg/m^3 for 4 weeks) in mice (61, 66) and rats (45, 49, 52, 56–58, 61, 62). Preneoplastic lesions, mainly type II alveolar cell hyperplasia, were reported upon long-term exposure to high cumulative concentrations of TiO_2 in rats (>500 mg/m^3 × h, corresponding to, e.g., >1 mg/m^3 for 90 days) (16, 17, 45, 51–61, 65). Finally, two inhalation studies and one intratracheal instillation study in rats showed the induction of lung tumors [benign squamous cell adenomas and carcinomas, bronchoalveolar adenomas and carcinomas, adenocarcinomas, cystic keratinizing epithelioma, nonkeratinizing epithelioma, and cystic keratinizing squamous carcinomas (later defined as nonneoplastic proliferative cysts)] (16, 17, 50, 51, 55, 87). Taken together, there is sufficient evidence that lung tumors are induced at high cumulative concentrations (>45,000 mg/m^3 × h or >10 mg/m^3 for 2 years) and that a mechanism via impaired clearance and persistent lung inflammation in rats is likely.

An interesting observation from **Figure 3** is that inflammation can be induced at lower cumulative doses compared to impaired clearance. An explanation for this observation is that inflammation can be induced via two pathways: via ROS generation and oxidative stress and via impaired clearance. In the case of TiO_2 exposures at lower cumulative doses, inflammation can be induced probably via ROS generation and oxidative stress; at higher cumulative doses, impaired clearance is induced, which contributes to the inflammation that becomes persistent and thus progresses toward the induction of epithelial regenerative cell proliferation, finally leading to the AO. Indeed, lung tumors are induced at cumulative concentrations that are above those inducing impaired clearance.

Another observation from **Figure 3** is that there are insufficient data on the generation of ROS and the induction of oxidative stress to allow analysis (73, 74, 82). The same holds for the induction of DNA damage: Only two studies are available that measured DNA damage after inhalation of TiO_2 (50, 86), and they showed no DNA-damaging potential of TiO_2. The ten intratracheal instillation studies showed inconsistent results. Especially when multiple genotoxicity assays were used in the same study, different outcomes were reported. No DNA damage in lung cells was detected using the Comet assay, γ-H2AX assay, 8-OHdG, and measuring *hprt* mutation frequency in rats (70, 72, 73, 77) or using the Comet assay and 8-OHdG in mice (81, 88). However, other studies did report DNA damage in the lungs measured via Comet assay, γ-H2AX, and 8-OHdG in rats (73, 76–78) and via Comet assay in mice (80, 82). ROS generation, the induction of oxidative stress, and the DNA-damaging potential of TiO_2 and the possible impact of particle form on that should be investigated in more detail. Clarity on its genotoxicity is important, especially since there is currently some indication for a mechanism of indirect DNA damage via oxidative stress, and a direct DNA-damaging effect of TiO_2 cannot be excluded.

Supplemental Material >

Regarding epithelial cell proliferation, there seems to be a difference between inhalation and instillation studies. Cell proliferation takes time and therefore cannot be measured directly after an acute exposure. This might be one of the reasons that no effect on proliferation was observed after intratracheal instillation studies that mainly used a single exposure and had a short follow-up time (56, 72, 76) (**Supplemental Tables 5** and **6**). The same holds for the induction of preneoplastic lesions; hyperplasia is a lesion that progresses over time and cannot be observed directly after an acute exposure. No induction of hyperplasia was observed after acute and short-term intratracheal instillation of TiO_2 (56, 71, 74–77, 79, 84) nor after short-term inhalation exposure (<2 weeks) (57, 62–64).

2.4.1. Species differences. Regarding the inhalation studies, there are indications for a species difference between rats and mice. The studies in rats do report the induction of hyperplasia

(alveolar type II hyperplasia, bronchoalveolar hyperplasia, and alveolar lesions) (KE7) upon long-term exposure to high cumulative concentrations of TiO_2 (>500 mg/m^3 × h, corresponding to, e.g., >1 mg/m^3 for 90 days) (16, 17, 45, 51–61, 65), whereas for mice, the induction of hyperplasia is less clear, with three studies finding no hyperplasia (17, 61, 67) and a single study showing induction of hyperplasia (66). Also, for other species, the carcinogenic potential of TiO_2 is less clear in the absence of robust carcinogenicity studies, as most investigators used the rat as a test species. No induction of lung tumors was observed in either mice or hamsters exposed to TiO_2 by inhalation or intratracheal instillation, respectively. Exposure in these studies, however, was too short (mouse) or the life span was markedly decreased (hamster), thereby impairing the detection of late lung tumors (15, 26).

2.4.2. Relevance to humans. Recent publications argue that humans are less susceptible to impaired clearance compared to rats. In rats, particles mainly remain in the alveolar spaces, whereas in humans, there is a greater tendency for particles to deposit in the interstitium (25, 89–92) due to anatomical and histological differences (93–95). Furthermore, the number of macrophages per surface area in the lung and the volume of lung lining fluid per surface area in the lung are higher in humans compared to rats. The clearance capacity of the human lung is assumed to be about sevenfold higher than rats, based on the number and volume of AMs and the volume of lung lining fluid (96).

When focusing on AMs that play an essential role in impaired clearance, there are substantial differences between rats and humans. The cell size differs, with AMs from humans being significantly larger than those from rats (25). Furthermore, human interstitial macrophages are less inflammogenic compared to AMs (30). Rat AMs have the ability to form nitric oxide, which rapidly interacts with any superoxide anion to form the relatively long-lived strong cytotoxic oxidant peroxynitrite. Human AMs lack nitric oxide synthetase, and no nitric oxide formation is observed in them. This species-specific difference in the oxidative capacity of AMs is suggested to contribute to the observed inflammatory responses in rats.

Morfeld et al. (97) have written an extensive review on the translation of impaired clearance in rats to humans. They reviewed mortality studies of coal workers and other dust-related industry cohorts and concluded that it seems too simplistic to assume that what occurs in the rats occurs in humans, too, after adjusting for some anatomical and physiological differences (97). Differences in lung physiology, characteristics of AMs, and the absence of increased tumor incidences after exposure to dusts in epidemiological studies suggest that humans are less sensitive to PSLT particle–induced impaired clearance compared to rats, but no firm conclusions can be drawn with the available information.

In line with the above, published epidemiology studies show no correlation between TiO_2 exposure and lung cancer or mortality (98–105). Measurements in human exhaled breath condensate demonstrated that markers of lipid peroxidation were elevated in production workers exposed to TiO_2 compared to the controls, suggesting lung injury at a molecular level via oxidative stress (106). In this recent study, TiO_2 exposure was well characterized and estimated to range between 0.40 and 0.65 mg/m^3 (106). The well-characterized exposure is in contrast to the TiO_2 exposure reported in the other human studies: The reported levels of exposure were not clear or were inconclusive, and all studies had methodological limitations. In addition, details on the crystal structure, particle size, and internal or retained dose were lacking. IARC and RAC members discussed epidemiological data, and a summary of the studies can be found in the RAC report (15, annex 3). IARC concluded that there is inadequate evidence in humans for the carcinogenicity of TiO_2 (26). RAC similarly concluded that the epidemiological data do not consistently suggest an association between exposure to TiO_2 and risk of lung cancer (15). In a recent study by Thompson et al.

(5), a summary was given of lung cancer risk estimates from the epidemiological studies of TiO_2, including an assessment of the internal and external validity of these investigations. The authors concluded that the findings of TiO_2 epidemiologic studies are likely to be impacted by exposure misclassification, confounding, and other extraneous factors. Nevertheless, the authors stated that the data (showing no correlation between TiO_2 exposure and lung cancer or mortality) support a moderate level of confidence for the human evidence (5). Overall, the human data consistently reports a lack of significantly elevated risk of lung cancer associated with TiO_2 exposure, though it should be acknowledged that these studies have limitations.

2.5. Conclusion on Carcinogenic Potential After Inhalation

From the available data, we conclude that the suggested AOP leading to lung tumors is operative in rats (**Figure 3**; **Supplemental Table 3**). For mice, the data are too limited to conclude whether the AOP is operative or not; especially robust data are lacking on impaired clearance, preneoplastic lesions, and the induction of lung tumors. As discussed above, there are several differences between rats and humans regarding lung physiology such as the site of deposition and clearance and retention mechanisms. Therefore, the carcinogenic effects observed in rats after long-term, high-dose exposure, which results in impaired clearance, might be less or even not relevant for humans. It is noted, though, that whereas the available data indicate a lower sensitivity of humans to PSLT particle–induced lung inflammation, data on TiO_2 or PSLT particle–related lung inflammation in humans are essentially missing. In any case, the evidence is insufficient to conclude that impaired clearance and subsequent inflammation cannot occur in humans. Dose-response information on earlier KEs such as oxidative stress, inflammation, and epithelial proliferation in humans after inhalation of TiO_2 and other PSLT particles is necessary to judge whether the MOA in rats is at least partly operative in humans or not (15).

Supplemental Material >

2.6. Mechanism of Action After Oral Exposure

Compared to the inhalation route, there is less information available on each of the KEs for oral exposure to TiO_2 (**Supplemental Tables 7** and **8**). The available data indicate that TiO_2 can induce some of the KEs in the AOP leading to intestinal tumors [e.g., uptake in the intestinal tract (19, 107–110), ROS generation, oxidative stress (109, 111), inflammation (18, 19, 110, 112, 113), and hyperplasia (18–20)]. The quantitative analysis (**Figure 4**) shows that the KEs in the AOP are induced at similar cumulative doses, indicated by overlapping confidence intervals. The point estimates of the ED50 values do show a trend, indicating that earlier KEs such as ROS generation, oxidative stress, and inflammation might be induced at lower cumulative doses compared to later KEs such as regenerative cell proliferation and preneoplastic lesions.

There are insufficient data available on all of the later KEs (**Supplemental Tables 7** and **8**). For epithelial injury, only a single study measured and observed apoptosis in the colon (114). Only two studies measured DNA damage in the intestine, and they detected no genotoxicity of TiO_2 in Peyer's patches (PPs) of rats (measured by Comet assay) (19) or in the colons of mice (measured by micronucleus assay) (114). Epithelial proliferation was measured in only two studies in mice, which showed inconsistent results (20, 114). These KEs require more thorough investigation, especially since results of many studies show a DNA-damaging potential of TiO_2 after oral exposure in blood (112, 113), bone marrow (112–116), and liver (112, 114, 116, 117). This in itself is not predictive for the formation of tumors, but it does argue for caution.

The available data show inconsistent results regarding the induction of hyperplasia by TiO_2, indicated by the large confidence interval in **Figure 4**. Two studies in which rodents were exposed

to relatively high concentrations of TiO_2 (up to 1,000 and 2,500 mg/kg bw/day) reported no hyperplasia (21, 23), while three more recent studies using lower concentrations (5 and 10 mg/kg bw/day) did report the induction of hyperplasia in the colon (18–20). A potential explanation for these contradicting observations is that, at high concentrations, large agglomerates are formed in the stomach, thereby reducing the absorption of TiO_2 in the gut. This increased agglomeration in gut conditions has been shown for silica particles, which is in line with observations in a rat study that showed that the lowest concentration resulted in the highest absorption (118). The difference in findings between the high-dose, 2-year studies and the lower-dose, subchronic and subacute studies with regard to the intestine is cause for concern that the observations for other organs might also be different at lower doses. At lower doses, perhaps more TiO_2 can pass through the intestines and reach these organs, where it can then accumulate. Unfortunately, except for the study by Bettini et al. (19), none of the above studies measured the internal absorption of TiO_2. Bettini et al. showed transepithelial passage in the jejunum and the colon after one week of exposure to TiO_2 and that the titanium reached the liver. Their results indicate that the daily consumption of TiO_2-containing food may constitute a persistent source for systemic passage of TiO_2.

A recent study in which both lower and higher concentrations of TiO_2 (1.3 up to 374 mg/kg bw/day) were included revealed no induction of hyperplasia in the colon after 100 days (22). The authors suggested that differences in the administration of TiO_2 might explain the differences between study results. Some studies show induction of hyperplasia in rodents upon exposure to TiO_2 dispersed in water given via drinking or oral gavage (18–20). Interestingly, dietary exposure studies in rodents showed no adverse effects (21, 22), including a study published by Warheit et al. (23), who exposed animals via oral gavage and found no effects of TiO_2. By dispersing TiO_2 in water versus mixing it with food, the characteristics of the particles can change and differ greatly, and thus the bioavailability can differ significantly between such studies. As discussed above, most of these studies did not measure TiO_2 uptake.

2.6.1. Species differences. There are considerable physiological and anatomical differences between the gastrointestinal tracts of rodents and humans such as physiological factors [e.g., forestomach occurrence in rodents and number and location of PPs; rodents have about 5–15 PPs along the entire small intestine, while humans have over 200 PPs, which are located mainly in the ileum and jejunum (119, 120)], pH, bile, pancreatic juice, mucus and fluid volume and content, and microbiome (119). These differences influence the dissolution rate, absorption, distribution, and excretion of TiO_2. In rodent studies, when ingested, TiO_2 is mainly excreted via the feces (>99.7% after 7 days) (107). In the gut, TiO_2 can be taken up by M cells, which are differentiated epithelial cells that are specialized in transcytosis of macromolecules and particles (108). M cells are mainly present in the gut-associated lymphoid tissue (GALT) such as PPs. The size, shape, and overall distribution of PPs vary from person to person. After transport by M cells, TiO_2 accumulates in macrophages present in the GALT. In human intestinal tissue, pigmented cells (macrophages) containing TiO_2 were identified in the PPs in the ileum and in colonic lymphoid aggregates (121). Similarly, in rats, TiO_2 particles were found in the PPs along the small intestine as well as in the colonic mucosa (19).

2.6.2. Relevance to humans. Additional information on interspecies differences between rodents and humans regarding oral exposure to (nano)particles is lacking, hampering a conclusion on specific differences in the absorption and effects of TiO_2 in the gut. Hyperplasia of the colon in rodents is considered relevant for hazard assessment, as intestinal tumors are rare in both rats and mice (122). In addition, aberrant crypt foci can also occur in humans. As there is insufficient

information on the species differences between rodents and humans regarding oral exposure to TiO_2, human relevance of the observed hyperplasia of the colon is assumed.

2.7. Conclusion on Carcinogenic Potential After Ingestion

Compared to the inhalation route, there is less information available on each of the KEs for oral exposure to TiO_2 (**Figure 4**; **Supplemental Tables 7** and **8**). The available data indicate that TiO_2 can induce some of the KEs in the AOP leading to intestinal tumors (e.g., ROS generation, oxidative stress, inflammation, and hyperplasia). The actual tumor formation was not observed in an available 2-year oral carcinogenicity study in rats and mice (21). Potentially, the high doses of up to 2,500 mg/kg bw/day might have induced the formation of large agglomerates and thereby reduced the absorption of TiO_2. In addition, the study lacked determination of internal tissue concentrations of TiO_2 and a thorough characterization of the particles. Recent studies showed that TiO_2 induced histopathological changes in the colons of rats and mice and even induced tumor formation in a chemically induced, colitis-associated, colorectal cancer mouse model (18–20). As humans are exposed daily to TiO_2 via food, toothpaste, and oral medication (14) at levels close to those leading to the observed hyperplasia in rodents, the observed hyperplasia in the gut in the more recent studies is a reason for concern. Furthermore, we lack an Organisation for Economic Co-operation and Development (OECD) guideline oral carcinogenicity study with well-characterized TiO_2 at both lower and higher doses that investigates whether the observed TiO_2-induced hyperplasia can progress into intestinal tumors. Preferably, several forms of TiO_2 (e.g., differing in crystallinity and size) should be tested, as it remains unclear whether there are differences in hazard associated with different forms of TiO_2. Determination of the Ti or TiO_2 concentration in several organs and in the different cell types and locations in the organs would be highly recommended in such a study to allow for consideration of the relevance of the study outcome for humans. Although there are physiological and anatomical differences in the gastrointestinal tracts between rodents and humans, there are no indications that intestinal effects in rodents are irrelevant for humans, and thus human relevance of the observed hyperplasia must be assumed.

Supplemental Material >

3. RECOMMENDATIONS

Below we give recommendations to reduce the uncertainty regarding the carcinogenic potential of TiO_2. For each of the KEs in the suggested AOPs, information needs are identified (**Table 1**). The recommended steps can be performed in parallel to speed up the hazard assessment of TiO_2.

From the available rodent studies, there is evidence that TiO_2 can induce lung tumors in rats after inhalation via impaired clearance and persistent inflammation. This is consistent with the KEs suggested in the AOP (**Figure 1**). Probably, the suggested AOP is operative in rats upon long-term exposure to high concentrations (>10 mg/m^3 per day for 2 years) of TiO_2. The lack of data from chronic inhalation studies in species other than rat forces the community to rely on the rat inhalation studies. However, uncertainty remains over whether this AOP could also be operative in humans. Differences between rats and humans regarding lung retention of the particles, differences in ROS production by AMs, and the absence of associations observed between occupational exposure to TiO_2 and lung cancer in epidemiological studies suggest that humans are less sensitive to TiO_2-induced persistent inflammation and carcinogenicity in the lungs. Nevertheless, the evidence is insufficient to conclude that impaired clearance and subsequent inflammation cannot occur in humans. In addition, a direct genotoxic effect of TiO_2 cannot be excluded. To reduce these uncertainties, we recommend investigating some of the earlier KEs of the AOP in

Table 1 Current scientific status and information needs for TiO_2

Adverse outcome pathway	Description	Inhalation exposure	Oral exposure
Initiating event	Distribution and uptake of TiO_2	Known in rats and mice	Need for information on distribution of TiO_2 within and around the intestinal tissue in both rodents (e.g., available tissues from previously published studies) and humans (postmortem tissues)
KE1 and KE2	ROS generation and oxidative stress	Limited information available from studies in rats and mice	Limited information available from studies in rats and mice
KE3	Inflammation	Known in rats and mice Need for information on inflammation potential in humans	Limited information available from studies in rats and mice Need for information on inflammation potential in humans
KE4	Epithelial injury	Limited information available from studies in rats and mice	Limited information available from studies in rats and mice
KE5	DNA damage	Contradicting findings Need for method optimization and harmonization Need for reliable genotoxicity assays in lung cells	Contradicting findings Need for method optimization and harmonization Need for reliable genotoxicity assays in colon and other relevant tissues and cells based on distribution of TiO_2
KE6	Cell proliferation	Information available from studies in rats and mice	Limited information available from studies in rats and mice Need for proliferation assays incorporating BrdU, Ki67, or PCNA
KE7	Preneoplastic lesions	Known in rats, limited data in other species	Contradicting findings in rats and mice indicating a cause for concern
Adverse outcome	Carcinogenicity	Known in rats, limited data in other species	Need for a guideline 2-year carcinogenicity study using well-characterized food-grade TiO_2, including toxicokinetics and assessment of early key events of the MOA of TiO_2
	Human relevance	Need for dose-response information on preneoplastic lesions and other, earlier KEs (oxidative stress, inflammation, proliferation) in humans after inhalation of TiO_2	Need for information on distribution and accumulation of TiO_2 in human intestine (focus on GALT) Need for in vitro assays with human tissues to investigate early KEs (ROS generation oxidative stress, DNA damage) in the MOA

Abbreviations: GALT, gut-associated lymphoid tissue; KE, key event; MOA, mechanism of action; ROS, reactive oxygen species; TiO_2, titanium dioxide.

more detail. Information on early KEs in humans such as oxidative stress, inflammation, and, importantly, cell proliferation after inhalation exposure to TiO_2 would especially help to assess whether the AOP leading to lung tumors is at least partly operative in humans and thus whether the effects observed in rats are relevant to humans. This could be done by, for example, measuring biomarkers in exhaled breath condensate, as described by Pelclova et al. (106), or measuring inflammatory cell counts and differentiation in bronchoalveolar lavage samples of workers exposed to TiO_2.

For oral exposure, there is uncertainty whether TiO_2 can induce intestinal tumors in rats and mice. The available data indicate that some of the KEs in the suggested AOP can be induced by TiO_2 in both rats and mice (e.g., intestinal uptake, ROS generation, oxidative stress, inflammation, and hyperplasia). As people are exposed daily to TiO_2 via food and other consumer products, there is urgency to address the question of whether TiO_2 can induce intestinal tumors after prolonged oral exposure. To conclude on the carcinogenic potential of TiO_2 after oral exposure, a 2-year carcinogenicity assay should be performed according to OECD test guideline 451 or 453 and using (preferably several forms of) well-characterized food-grade E171. In this study, toxicokinetics should be included to relate the internal exposure to the observed effects and to allow us to extrapolate to the longer exposure durations and accumulation in humans. In addition, this study would preferably analyze the distribution of TiO_2 within and around the intestine and earlier KEs in the MOA given in this review such as ROS formation, oxidative stress, DNA damage, and inflammation.

As indicated above, there is insufficient information on interspecies differences between rodents and humans regarding kinetics and hazard after oral exposure to (nano)particles. If intestinal tissues are available from the previously published rodent studies, as such samples are often stored, they could be used to analyze the internal dose and localization of TiO_2 in the tissues, especially in the intestine. In addition, postmortem intestinal tissue in humans may be analyzed to determine the distribution of TiO_2 over different tissues such as GALT and within specific cell types such as macrophages and epithelial cells. This could aid in (*a*) linking observed effects to internal exposure, (*b*) comparing the distribution within the intestinal tissues between rodents and humans, (*c*) obtaining further insight in the MOA of TiO_2, and (*d*) optimizing in vitro mechanistic studies by including information on cellular uptake of TiO_2. The comparison between TiO_2 intestinal distribution in rodents and humans will give insight into the relevance of rodent studies for human risk assessment. A limitation of measuring TiO_2 in human tissues is that the source and the characteristics of the TiO_2 particles that people were exposed to are not known. Subsequently, in vitro assays with human tissues, where possible, might provide insight into whether the early KEs in the MOA, seen in the in vivo studies described above, can also be induced by TiO_2 in humans.

For both inhalation and oral exposure, genotoxicity is important, as currently there is some indication for a mechanism of indirect DNA damage via oxidative stress, and a direct DNA-damaging effect of TiO_2 cannot be excluded (24). The available in vivo genotoxicity observations are diverse, and this may be explained (in part) by the fact that each study tested a different type of TiO_2 and there were differences in exposure route, concentration and duration, and the type of genotoxicity assay used (e.g., Comet assay, micronucleus, pig-a mutation assay, *hprt* mutation assay, 8-OHdG, and γ-H2AX assay). In addition, the internal distribution of TiO_2 has rarely been reported, making the comparison of studies even more difficult. Especially when genotoxicity testing is focused on specific tissues, one should measure whether TiO_2 indeed reaches those tissues.

Previous review papers also stated that there are still too few reliable studies to assess the genotoxic potential of nano-sized TiO_2 in animal models (34, 40). Our recommendation follows the conclusion presented in the recent paper by the Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute on genotoxicity assessment of nanomaterials (32) to optimize and harmonize genotoxicity assays for testing nanomaterials before further testing TiO_2. In fact, harmonization of these assays is ongoing (e.g., Malta initiative). Once genotoxicity assays are optimized for testing (nano)particles, and measurement of their internal distribution is included in the study, those assays can be used to test the genotoxicity of food-grade TiO_2. The Comet assay, with and without the addition of FPG, is probably a suitable assay to detect both ROS-induced DNA damage and total DNA damage in relevant tissues such as the colon.

Next to genotoxicity, epithelial cell proliferation is an important KE ultimately leading to tumor formation. For the oral route, proliferation assays using BrdU, Ki-67, or PCNA are needed to investigate whether TiO_2 can induce this KE and to unravel whether the AOP leading to intestinal tumors is operative.

Finally, the currently available information does not allow for conclusions on the relationship between specific physicochemical characteristics of TiO_2 and the induction of the KEs and AO, mainly because the particle characteristics are poorly reported. Future studies should characterize particles thoroughly and focus on specific characteristics such as crystal structure, size, surface coating, and reactivity of TiO_2 in relevant cell types to investigate whether all forms of TiO_2 should be considered to be of equal toxic potential.

SUMMARY POINTS

1. Upon chronic inhalation exposure, TiO_2 can induce lung tumors after inhalation in rats similar to other PSLT particles via impaired clearance, persistent inflammation, and persistent regenerative epithelial cell proliferation.
2. Insufficient information is available to conclude that there is a carcinogenic potential of TiO_2 after oral exposure, though the MOA may be similar to the MOA in lung.
3. A genotoxic MOA of TiO_2 cannot be excluded and should be investigated in relevant tissues for both inhalation and oral exposure.
4. Knowing the kinetics and biodistribution of TiO_2 is key to understanding its MOA related to genotoxicity and carcinogenicity.

FUTURE ISSUES

1. There is need for a 2-year oral carcinogenicity study with well-characterized (food-grade) TiO_2 at the lower and higher dose ranges, including assessment of biodistribution and earlier KEs in the MOA.
2. Regarding inhalation exposure, information on the occurrence of earlier KEs such as oxidative stress and inflammation in humans is needed to assess whether the MOA of TiO_2 is (at least partly) operative in humans.
3. Future studies should characterize TiO_2 thoroughly and focus on specific characteristics such as crystal structure, size, surface coating, and reactivity to investigate whether all forms of TiO_2 should be considered to be of equal toxic potential.
4. Optimization and harmonization of genotoxicity assays are needed for testing nanoparticles.

DISCLOSURE STATEMENT

This work was funded by the Dutch Ministries of Infrastructure and Water Management; Health, Welfare and Sport; and Social Affairs. All authors participated in preparation of the paper as part of their regular employment and did not receive any special compensation from any source for preparation of the paper. The authors have sole responsibility for the writing and content of the

paper. The authors declare that they had no competing interests during the writing of this paper. M.B.H. joined the company Reckitt Benckiser (RB), which markets products containing TiO_2, only after this paper was nearly completed; recommendations and conclusions were not changed and do not necessarily reflect RB's position.

ACKNOWLEDGMENTS

We would like to thank Dr. Jack R. Harkema for optimizing the adverse outcome pathway figures and for critically reviewing the manuscript. In addition, we would like to thank Dr. Jan van Benthem, Dr. Adrienne Sips, and Dr. Joke Herremans for critically reviewing this manuscript.

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