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REVIEW ARTICLE

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Systematic review of mechanistic evidence for TiO_2 nanoparticle-induced lung carcinogenicity

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ABSTRACT

Nano-sized titanium dioxide particles (TiO_2 NPs) are a high-production volume nanomaterial widely used in the paints, cosmetics, food and photovoltaics industry. However, the potential carcinogenic effects of TiO_2 NPs in the lung are still unclear despite the vast number of *in vitro* and *in vivo* studies investigating TiO_2 NPs. Here, we systematically reviewed the existing *in vitro* and *in vivo* mechanistic evidence of TiO_2 NP lung carcinogenicity using the ten key characteristics of carcinogens for identifying and classifying carcinogens. A total of 346 studies qualified for the quality and reliability assessment, of which 206 were considered good quality. Using a weight-of-evidence approach, these studies provided mainly moderate to high confidence for the biological endpoints regarding genotoxicity, oxidative stress and chronic inflammation. A limited number of studies investigated other endpoints important to carcinogenesis, relating to proliferation and transformation, epigenetic alterations and receptor-mediated effects. In summary, TiO_2 NPs might possess the ability to induce chronic inflammation and oxidative stress, but it was challenging to compare the findings in the studies due to the wide variety of TiO_2 NPs differing in their physicochemical characteristics, formulation, exposure scenarios/test systems, and experimental protocols. Given the limited number of high-quality and high-reliability studies identified within this review, there is a lack of good enough mechanistic evidence for TiO_2 NP lung carcinogenicity. Future toxicology/carcinogenicity research must consider including positive controls, endotoxin testing (where necessary), statistical power analysis, and relevant biological endpoints, to improve the study quality and provide reliable data for evaluating TiO_2 NP-induced lung carcinogenicity.

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Background

Titanium dioxide nanoparticles (TiO_2 NPs) are among the most produced nanomaterials worldwide, with increasing global use in many applications, e.g. inks and paints, photocatalysts, food and plastic colorants, drug delivery applications, sunscreens, and cosmetic products, owing to their unique physicochemical properties (Shi et al. 2013; Wang, Sanderson, and Wang 2007). Given the predicted increase in demand in the future (Research Markets 2021), occupational and environmental exposure to these NPs is also anticipated to increase. Human exposure routes for NPs include oral, pulmonary, and dermal exposure. In the workplace, the most relevant route of exposure to NPs is via inhalation, which can result in lung inflammation and fibrosis and, over time, potentially lead to lung cancer development.

TiO_2 particles have traditionally been considered low-soluble, low-toxicity particles, which is why they have been included as 'negative control' in many early *in vitro* and *in vivo* studies (Dankovic, Kuempel, and Wheeler 2007; Shi et al. 2013). However, studies investigating *nano-sized* TiO_2 have commonly shown cytotoxic effects via oxidative stress responses, DNA damage, apoptosis (Brandão et al. 2020; Grande and Tucci 2016; Wani and Shadab 2020), changes in the cell cycle (Chang et al. 2022) and inflammation (Schanen et al. 2009). Furthermore, epidemiological studies on workers handling TiO_2 (nano)particles suggest that TiO_2 exposure may lead to

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inflammation, DNA damage and oxidative stress responses in the lungs of the workers (Bergamaschi et al. 2022; Liou et al. 2017; Liou et al. 2016; Pelclova, Zdimal, Kacer, et al. 2016; Pelclova, Zdimal, Fenclova, et al. 2016). These studies, however, have often poor characterization of the TiO_2 exposure. Thus, it is unclear whether the exposure includes other materials than TiO_2 and which size distribution and purity the particles have.

While there is substantial research on the toxicity of TiO_2 NPs, there still needs to be consensus on the mechanisms of action following pulmonary exposure to TiO_2 NPs. The *in vitro* and *in vivo* studies present contradictory results, most probably due to differences in the physicochemical parameters of the TiO_2 NPs tested, the exposure system, and the study design/experimental protocol. It is well known that the physicochemical parameters of NPs, such as particle size, shape, chemical composition, surface area, agglomeration/aggregation state, and purity, influence the reactivity of the particles when in contact with biological systems (Nel et al. 2006; Nel et al. 2009; Oberdörster, Oberdörster, and Oberdörster 2005).

In 2006, the International Agency for Research on Cancer (IARC) classified TiO_2 as possibly carcinogenic to humans (Group 2B) (IARC 2010). However, the classification does not distinguish between bulk material and nano-sized forms. Based on the IARC monograph for evaluating carcinogenic risks to humans from carbon black, titanium dioxide, and talc, this classification concluded that 'there is inadequate evidence in humans for the carcinogenicity of titanium dioxide'. At the same time, there is 'sufficient evidence from experimental animals for the carcinogenicity of titanium dioxide' (IARC 2010). IARC compiled existing data from epidemiological and animal studies. Critical for the evaluation by IARC were two animal studies that observed lung tumors in rats after two years of chronic exposure to 250 mg/m^3 of fine-sized rutile TiO_2 (Lee, Trochimowicz, and Reinhardt 1985) and to 10 mg/m^3 of P25 (mixture of anatase/rutile) TiO_2 NPs (Heinrich et al. 1995).

Similar to IARC, in 2017, the European Chemicals Agency (ECHA) classified certain forms of TiO_2 powder, where 1% (w/w) or more of the particles have an aerodynamic diameter of ≤ 10 µm, as a Carc 2, H351 (inhalation) category 2 suspected human carcinogen (RAC 2017). Despite the classification from both IARC and ECHA, the mechanisms by which TiO_2 causes carcinogenicity in the lung are not fully understood, and there are uncertainties regarding the carcinogenic potential of *nano-sized* TiO_2 following inhalation. There is major concern that NPs, including TiO_2 NPs, can induce genotoxic outcomes, which are associated with an increased risk of cancer development (DeMarini 2019). However, it is also important to characterize the non-genotoxic effects contributing to carcinogenesis. In 2016, IARC introduced a 'key characteristics of human carcinogens' approach. This approach is used within the larger framework of IARC to evaluate the mechanistic evidence of carcinogenicity for chemicals and other agents. The key characteristics (KCs) are a set of 10 chemical (agent) specific properties for cancer hazard identification, as outlined by Hanahan and Weinberg (Hanahan and Weinberg 2011). These properties include 'is electrophilic or can be metabolically activated to an electrophilic', 'is genotoxic', 'alters DNA repair or causes genomic instability', 'induces epigenetic changes', 'induces oxidative stress', 'induces chronic inflammation', 'is immunosuppressive', 'modulates receptor-mediated effects', 'causes immortalization', 'alters cell proliferation, cell death, or nutrient supply' (Smith et al. 2016).

The use of KCs of carcinogens by IARC may prove helpful in identifying and classifying carcinogens. The lack of a systematic literature evaluation and consideration of a study's scientific quality and reliability present a weakness in evaluating carcinogens by IARC using the KCs (Goodman and Lynch 2017). Using the concept of KCs alone as a tool for assessing cancer hazards cannot predict cancer better than chance alone (Becker et al. 2017). Mechanistic evidence is critical to understanding human cancer. With the international effort to reduce the use of animals in toxicity testing and reliance on developing high-throughput *in vitro* assays, cancer hazard evaluations and risk assessments done by regulatory bodies are likely to rely more on mechanistic data. Mechanistic studies have increased in volume, diversity and relevance to cancer hazard evaluation. For *in vitro* and *in vivo* mechanistic studies to be useful for cancer hazard evaluation, they must be of high quality, reliable and have significant biological endpoints relevant to cancer development. There are existing frameworks for the study quality assessment of *in vitro* and *in vivo* studies, such as the Klimisch system (Klimisch, Andreae, and Tillmann 1997), the Toxicological Data Reliability Assessment Tool (ToxRTool) (Schneider et al. 2009), and the Science in Risk Assessment and Policy (SciRAP) tool (Roth, Zilliacus, and Beronius 2021). Likewise, weight-of-evidence approaches can be used to assess the reliability and relevance of the existing evidence. No

published analysis has examined the *in vitro* and *in vivo* evidence for TiO_2 NP lung carcinogenicity using the KCs of carcinogens. Utilizing a systematic evaluation of the scientific peer-reviewed literature, a quality and reliability assessment, and a weight-of-evidence approach, we evaluated the mechanistic data from *in vitro* and *in vivo* studies associated with pulmonary exposure to TiO_2 NPs. Specifically, the efforts were to (a) identify the KCs TiO_2 NPs exhibit and (b) identify knowledge gaps regarding *in vitro* and *in vivo* mechanistic evidence and future research needs to decrease the uncertainties regarding the carcinogenic potential of TiO_2 NPs in the lung. Thus, the major objective of this systematic review was to compile the existing *in vitro* and *in vivo* evidence on TiO_2 NP toxicity and carcinogenicity with the intent to address the following Population, Exposure, Comparator, Outcome and Study (PECOS) framework question: 'Using the 10 key characteristics of carcinogens, is there mechanistic evidence from *in vitro* and *in vivo* studies between 2006 and 2023 that supports TiO_2 NP carcinogenicity in the lung?' Considerations regarding the TiO_2 NP physicochemical characteristics, and study design/experimental protocols are discussed.

Systematic literature search

A systematic literature review, based on PECOS and Preferred Reporting Items for Systematic Reviews and Meta-Analysis (Prisma) Methodology (Page et al. 2021a; Page et al. 2021b), was performed in order to identify existing *in vitro* and *in vivo* data on the mechanisms of TiO_2 NP toxicity relevant to the onset of lung carcinogenesis. Four databases were considered for the literature search: PubMed, Embase, Web of Science and TOXicology information onLINE (TOXLINE). Based on the ten KCs of carcinogens (Smith et al. 2016) and the search terms for these provided by Guyton et al. (2018), our literature search included peer-reviewed literature from 2006 to April 2023 and was divided into six search strings using the modified KCs after Guyton et al. (2018): (1) Genotoxicity (the agent is genotoxic, and/or alters DNA repair or causes genomic instability); (2) Epigenetics (induces epigenetic alterations); (3) Oxidative stress (induces oxidative stress); (4) Chronic inflammation (induces chronic inflammation and/or is immunosuppressive); (5) Receptor-mediated effects (modulation of receptor-mediated effects and/or hormones); (6) Cell proliferation/Apoptosis (alters cell proliferation, cell death or nutrient supply, and/or causes immortalization). The search terms for each KC are listed (Additional file 1 in Supplementary Appendix). The general inclusion criteria for the literature search were: (a) not a review and (b) limited to 2006–2023. The exclusion criteria were (a) abstracts only, (b) articles preceding 2006, (c) manuscript in a language other than English, (d) manuscripts that did not meet the scoring criteria. The references were collected, managed and screened using Covidence [Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia] and Microsoft Excel software. Duplicate studies were removed automatically by Covidence and manually by the reviewers during the different stages of processing and screening of the references.

Screening

In the screening process (i.e. title/abstract and full-text screening), relevant publications were identified by two independent reviewers. At the end of the screening, any conflicts were resolved through discussion between the two reviewers or by having a third reviewer, if necessary. Before starting the title/abstract screening, a decision tree (Figure 1) for the screening criteria was defined and applied. Articles not meeting the inclusion criteria (as outlined in Figure 1) were excluded when the two reviewers agreed upon the decision. Articles that fulfilled the inclusion criteria were collected for full-text screening. At that stage, it was decided to group the articles based on their exposure route and the target organ affected. Pulmonary exposure and the upper and lower airways, including immune cells in the airways, were relevant in the current review. References reporting other exposure routes like oral, dermal, or systemic routes were excluded. The following additional criteria were defined for qualification of the full-text articles. They must include information on: 1) TiO_2 NPs (pristine, no doping or coating that would change the surface properties); 2) aerodynamic particle size of starting material (needed to be <100 nm) and hydrodynamic diameter of the material in suspension or aerodynamic measurements; 3) cell type (if *in vitro*); 4) route of administration (if *in vivo*); 5) number of replicates or number of animals; 6) particle dose; 7) post-exposure time point(s); 8) method(s) for assessing the biological effect(s)/KC(s); and 9) statistical analysis with appropriate tests. Publications lacking information on one or more of the above criteria were excluded when two reviewers agreed upon the decision. References that fulfilled the criteria were included in a reference database. Duplicate

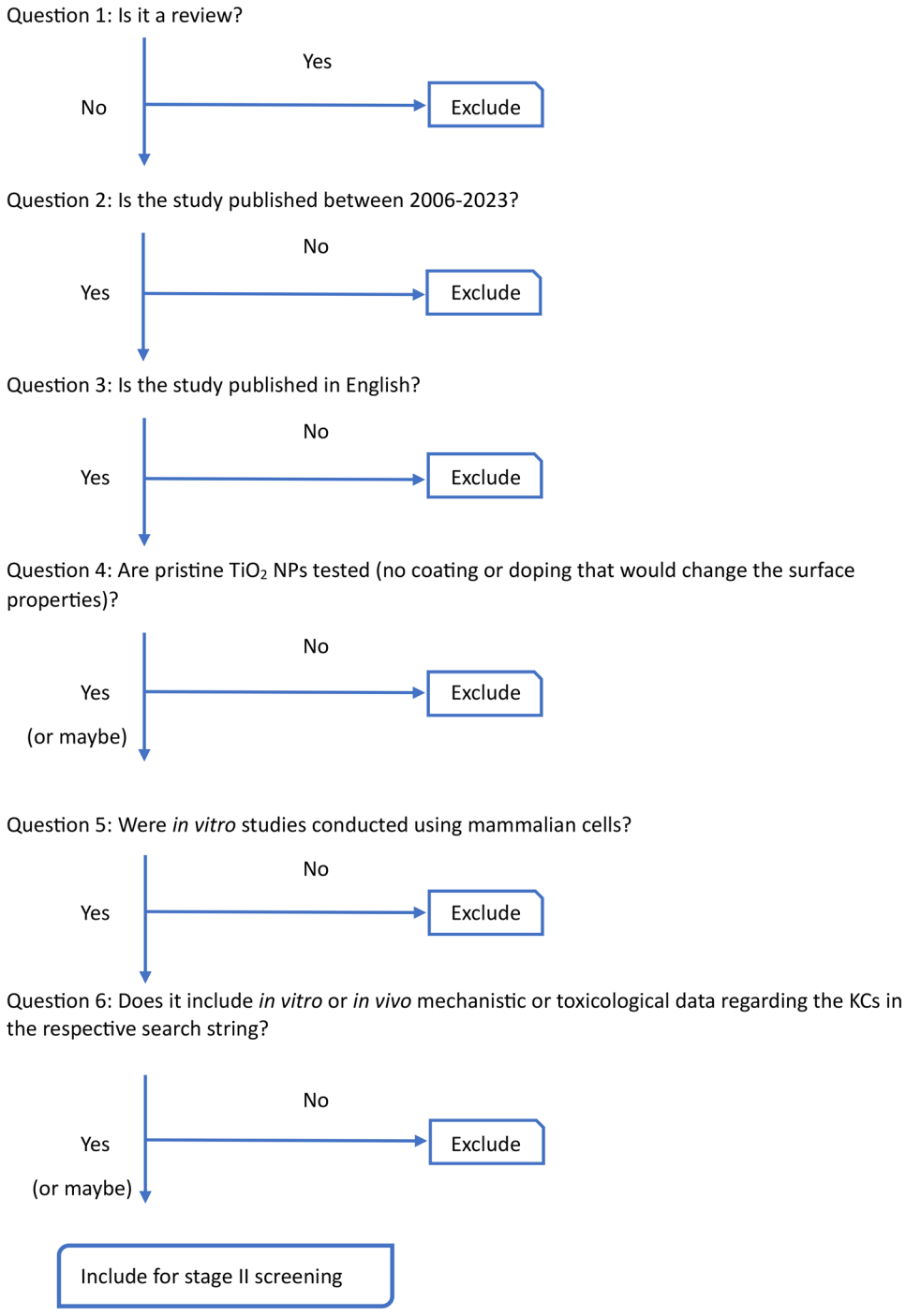

Figure 1. Decision tree for title/abstract screening.

references overlapping the six search strings were manually removed.

Quality and reliability assessment

The quality and reliability of the full-text articles were assessed by two independent reviewers using the Toxicological data Reliability Assessment Tool (ToxRTool) software (Schneider et al. 2009), which helps assign Klimisch reliability categories (Klimisch, Andreae, and Tillmann 1997). Following the principles of the ToxRTool, together with expert judgment, each study was assigned a reliability category 1 (reliable without restrictions), 2 (reliable with restrictions), or 3 (unreliable). The ToxRTool consists of two parts, one for evaluating *in vitro* data and one for *in vivo* data. Publications reporting both *in vitro* and *in vivo* data were separately scored. In that sense, a publication could be assigned two distinct Klimisch categories if it presented both *in vitro* and *in vivo* data.

The ToxRTool template (excel format) was modified to include information on NP characterization (adapted and modified from (Card and Magnuson 2010; Fernández-Cruz et al. 2018); see Additional file 2 in Supplementary Appendix). Considering various recommendations on which NP parameters should be evaluated when conducting toxicological studies (Card and Magnuson 2010), changing expectations of reviewers/journals over time (more comprehensive NP characterization in recent studies) and a wide time frame from 2006 to 2023 included in the present review, it was decided to include publications reporting a 'minimum set' of NP parameters relevant to TiO_2 NPs to be assigned a Klimisch category 1 or 2. Information on NP parameters could be given in a publication (provided by the supplier or the authors) or in a recent paper (e.g. published within 3–5 years) that is cited in the publication. These NP parameters were particle size and size distribution (in water/buffer/cell culture medium or air); particle shape; and crystalline phase (anatase, mixture of anatase/rutile or rutile). This decision was based on evaluating all qualified full-text articles to minimize selection bias.

In addition, the measurement of endotoxin contamination of the TiO_2 NP formulations was considered very important and mandatory in question 2 of the ToxRTool ('Is the purity of the substance given?'). Including relevant positive controls for the assays and the TiO_2 NPs was considered important but could not be taken into account in the quality assessment as many studies would have been excluded (Klimisch category 3) as 'positive controls' were considered indispensable by the ToxRTool. Similar to the above screening process, any conflicts regarding the Klimisch category were resolved through discussion between the two reviewers or using a third reviewer, if necessary.

Data extraction

Data extraction, adapted from (Rolo et al. 2022), was performed on studies assigned a Klimisch category 1 or 2, by two reviewers: one doer and one reviewer. Any conflicts were resolved by discussion. A complete list of studies used in the *in vitro* and *in vivo* databases is provided in additional tables (Additional file 3 and 4 in Supplementary Appendix). A list of excluded *in vitro* and *in vivo* studies assigned a Klimisch category 3 is also provided in additional tables (Additional file 5 and 6 in Supplementary Appendix). The extraction database was curated for consistency.

Weight-of-evidence approach

A significant challenge, especially with the *in vitro* assays, is the question of biological relevance to humans. A recent study by Smith et al. (2020) described current and emerging *in vitro* assays to measure the KCs, which are reflected in most studies included in the current review. To 'score' the biological relevance in humans, the assays/endpoints investigated to study the respective KCs were evaluated by a weight-of-evidence approach regarding their association with carcinogenic hazard. Evidence-weighting assumptions for genotoxicity endpoints were based on a previously published review (Kirkland et al. 2022), while assumptions for the other KCs were based on expert judgment. The tables with the default weighting for the different KCs are provided (Additional file 7 in Supplementary Appendix). The general weight descriptors are as follows: *negligible level of confidence* (the endpoint is not linked to an adverse effect relevant to the respective KC); *low level of confidence* (the endpoint is indicative of the KC but not directly linked to mechanisms associated with carcinogenicity); *moderate level of confidence* (the endpoint is potentially relevant for carcinogenicity or subject to secondary cytotoxicity); *high level of confidence* (the endpoint has been shown to play a significant role in the process of carcinogenicity). All extracted references were reviewed for their assays/endpoints and the default weight-of-evidence of the respective endpoint. Only those publications reporting assays/endpoints with a default weighting of 'moderate' or 'high' (Additional file 8 and 9 in Supplementary Appendix) were reviewed in detail and considered in synthesizing evidence for TiO_2 NP lung carcinogenicity. A list of *in vitro* and *in vivo* studies with a negligible or low confidence was also compiled (Additional file 10 in Supplementary Appendix). These studies were not considered for data synthesis.

It should be noted that some publications contained assays/endpoints with 'moderate' or 'high' confidence that were reviewed in detail. However, the same publication could contain endpoints (for the same or different KC) with 'low' or 'negligible' confidence that were not reviewed. Due to the high number of studies considered in the current review, a wide range of study protocols, various assays/endpoints, and differences in the physicochemical properties of the NPs, the *in vitro* and *in vivo* studies were not evaluated on a single level by the weight-of-evidence approach. The reliability and relevance were discussed for each key characteristic.

Based on this analysis, a summary of the outcome for each KC, grouped by crystalline phase of the TiO_2 NPs, was compiled. It was outside the scope of this narrative systematic review to make in-depth weight-of-evidence assessments.

Results

The systematic literature search yielded 20,722 articles among the four databases (PubMed, Embase, Web of Science and TOXLINE) with 15,489 articles screened for title/abstract. An overview of the articles obtained, screened and excluded is shown in the PRISMA chart (Figure 2). In the full-text screening, 1,842 articles were assessed for eligibility, with 932 articles excluded based on the exclusion criteria: review ($n = 15$), abstract only ($n = 16$), wrong intervention/KC ($n = 85$), wrong study design ($n = 88$), not English language ($n = 4$), wrong exposure/not pristine TiO_2 NPs ($n = 95$), wrong route of administration/cell type ($n = 612$), wrong date ($n = 14$), withdrawn study ($n = 3$). Thus, 910 articles from the six search strings were eligible for further processing. After removing overlapping studies in the six search strings, 346 articles were included in the quality and reliability assessment using the ToxRTool (Figure 2), of which 262 articles reported *in vitro* data and 103 articles reported *in vivo* data. As stated above, some articles included both *in vitro* and *in vivo* data.

Of the 262 *in vitro* studies assessed with the ToxRTool, nearly a third of the studies (36.6%, 96/262) were assigned Klimisch category 1, while 14.9% (39/262) were assigned Klimisch category 2. Almost half of the studies (48.5%, 127/262) were assigned Klimisch category 3, of which the majority of studies (62.2%, 79/127) lacked TiO_2 NP characterization in the dispersion/delivery medium (Figure 3**, left**). Other reasons for Klimisch category 3 (i.e. exclusion) were: too few biological replicates (13.4%, 17/127); missing number of replicates (10.2%, 13/127); lack of statistical method for the respective KC (9.4%, 12/127); and substantial flaws in study design, data presentation and/or interpretation (4.7%, 6/127) (Figure 3**, left**).

Of the 103 *in vivo* studies, 59.2% (61/103) were assigned Klimisch category 1 and only 9.7% (10/103) were assigned Klimisch category 2. A third of studies (31.1%, 32/103) were assigned Klimisch category 3 and were thus excluded. Like the *in vitro* studies, most *in vivo* studies (75%, 24/32) lacked the TiO_2 NP characterization in the dispersion/delivery medium or aerodynamic measurements (Figure 3, **right**). Other reasons for Klimisch category 3 and exclusion were: number of animals/replicates too low (15.6%, 5/32); missing number of animals (3.1%, 1/32); and lack of statistical method for the respective KC (6.3%, 2/32) (Figure 3, **right**).

Most of the 135 included *in vitro* studies reported on oxidative stress, chronic inflammation and genotoxicity (Figure 4). Fewer studies investigated proliferation/apoptosis/cell cycle/transformation and epigenetic effects of TiO_2 NP exposure, while only one *in vitro* study reported receptor-mediated effects. From the 71 *in vivo* studies, a clear majority reported chronic inflammation, while some studies looked at oxidative stress, genotoxicity and proliferation/apoptosis/cell cycle/transformation (Figure 4). Two studies investigated epigenetic changes due to TiO_2 NP exposure, whereas only one *in vivo* study reported receptor-mediated effects.

About 70% of the *in vitro* studies reporting chronic inflammation (47/65) and oxidative stress (48/68) were assigned Klimisch category 1 (Figure 5), while for genotoxicity it was 83% (39/47) of the studies. For proliferation/apoptosis/cell cycle/transformation and epigenetics, about 55% (15/27 and 6/11, respectively) of the *in vitro* studies were assigned Klimisch category 1. The sole *in vitro* study on receptor-mediated effects was assigned Klimisch category 2 (Figure 5). For the *in vivo* studies, between 84% and 92% of the studies reporting chronic inflammation (56/66), oxidative stress (16/19), proliferation/apoptosis/cell cycle/transformation (6/7) and genotoxicity (12/13) were assigned Klimisch category 1. The sole *in vivo* study on receptor-mediated effects was assigned Klimisch category 1, while the two studies on epigenetic changes were assigned Klimisch category 1 and 2 (Figure 5).

Regarding the TiO_2 NP characteristics, several physicochemical parameters including particle size, crystalline phase, aerodynamic diameter, hydrodynamic diameter, surface area, and surface charge were provided to a different degree in these publications. Most *in vitro* studies assessed the anatase (56.3%, 76/135) and mixture of anatase/rutile (45.2%, 61/135) with a primary particle size below 25 nm (69.6%, 94/135) (Table 1).

Only 10.4% (14/135) of publications studied rutile TiO_2 NPs. In addition, TiO_2 NPs with primary particle sizes of 25–50 nm and 50–100 nm were studied in 28.1% (38/135) and 23% (31/185) of the publications, respectively. The hydrodynamic diameter of the TiO_2 NPs, most often measured using DLS, was over 100 nm in most *in vitro* studies. Although 35.6% (48/135) of studies lacked information about the hydrodynamic diameter in water or buffer solution, these studies measured the hydrodynamic diameter

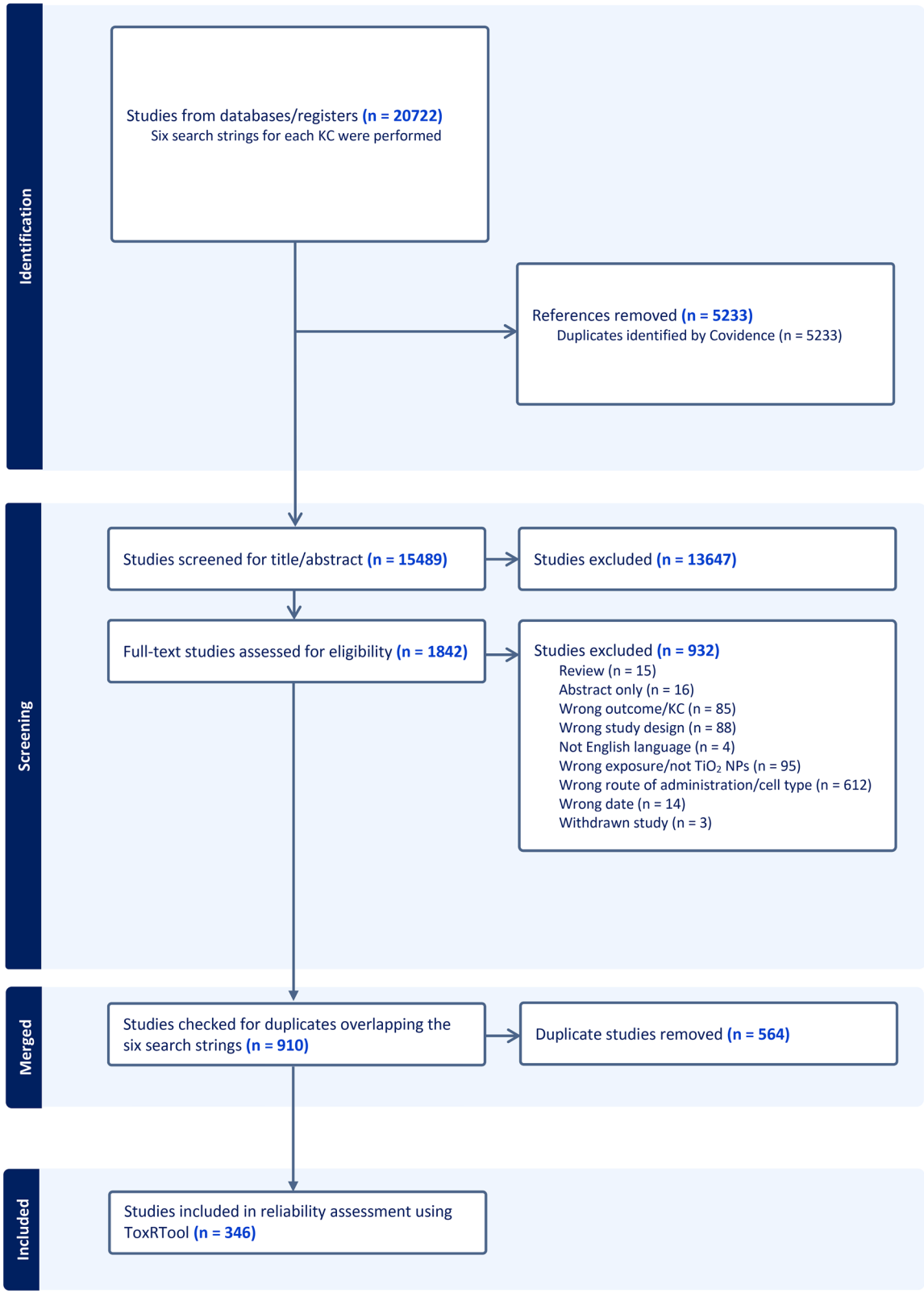

Figure 2. PRISMA Chart showing the number of articles identified, screened and included during the systematic literature search and screening (title/abstract and fulltext). Reasons for exclusion during fulltext screening are given.

in cell culture exposure medium as it was a criterion to include these measurements in either water, buffer solution or cell culture exposure medium. Information about the specific surface area and charge was missing in about 41% (56/135) of *in vitro* studies. Most studies observed a negative

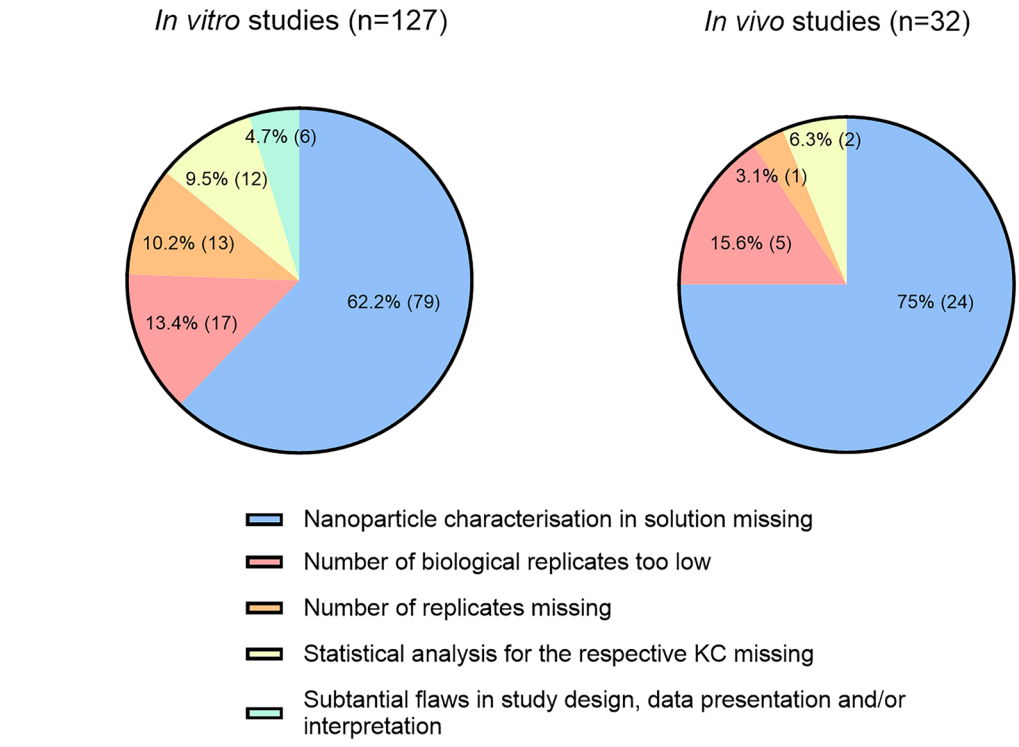

Figure 3. Reasons for exclusion of *in vitro* and *in vivo* studies in the quality and reliability assessment process using the ToxRTool. These studies were assigned Klimisch category 3 and were not included in the subsequent synthesis of data. The total number and percentages (in parenthesis) of the *in vitro* studies (left) and *in vivo* studies (right) excluded for the respective reason are presented in the pie chart.

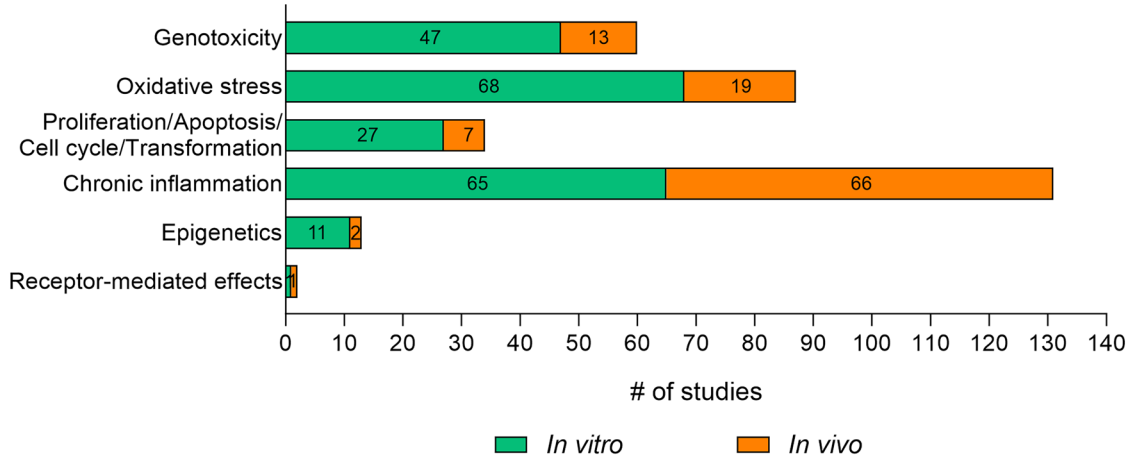

Figure 4. Overview of the number of *in vitro* (green) and *in vivo* (orange) studies reporting TiO_2 NPs and the respective KC, that were assigned Klimisch category 1 or 2 and were thus included in the data synthesis for evaluating the carcinogenic potential of TiO_2 NPs in the lung.

surface charge of the TiO_2 NPs investigated (Table 1). In the *in vivo* studies, the anatase (52.9%, 37/70) and mixture of anatase/rutile (37.1%, 26/70) crystalline phase of TiO_2 NPs were frequently studied. All studies included information about the primary particle size, and the majority (94.3%, 66/70) investigated TiO_2 NPs less than 25 nm. However, when in solution, the hydrodynamic diameter of the TiO_2 NPs increased above 100 nm in most of the studies. The specific surface area of the TiO_2 NPs was indicated in 27.1% (19/70) of the studies, whereas information about surface charge was missing in 72.8% (51/70) of the publications (Table 2).

Sorted by KC, most *in vitro* and *in vivo* studies did not report the endotoxin level in the TiO_2 NP formulations (Figure 6). About 80%–90% of *in vitro* studies

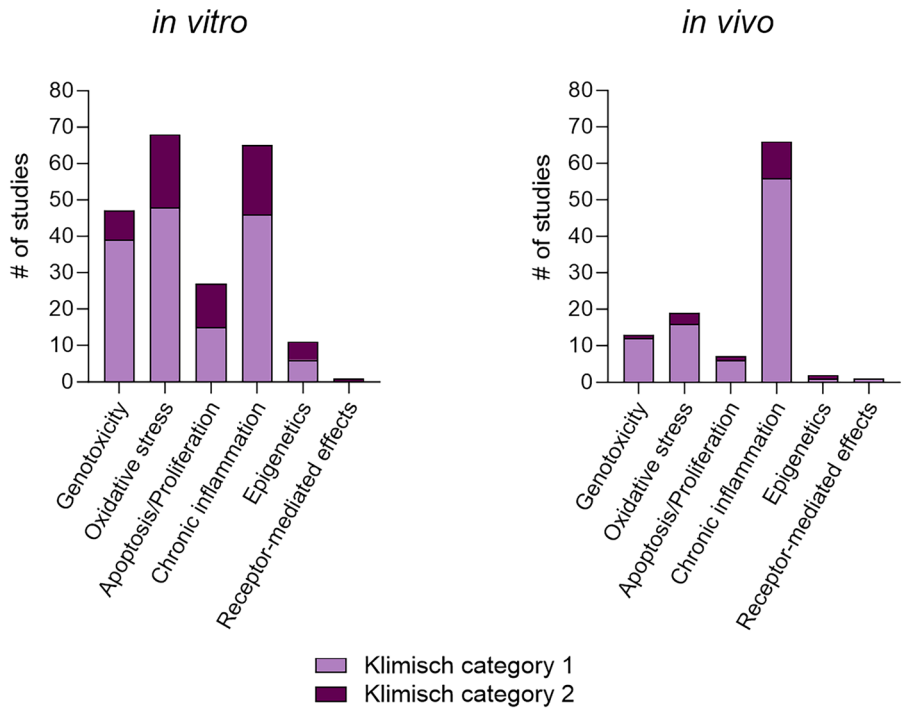

Figure 5. Overview of the number of *in vitro* and *in vivo* studies in Klimisch category 1 or 2, sorted by KC.

Table 1. Physicochemical properties of the TiO_2 NPs used in the *in vitro* studies in Klimisch category 1 or 2.

TiO_2 NP characteristic	Categories	# of studies
Crystalline phase	Anatase	76
	Mixture	61
	Rutile	14
	Other	2
	NA	13
Primary size (nm)	<25	94
	25–50	38
	50–100	31
	NA	11
Hydrodynamic diameter (nm)	<25	3
	25–50	3
	50–100	13
	>100	73
	NA	48[a]
Specific surface area (SSA, m^2/g)	<50	36
	50–100	39
	>100	40
	NA	57
Surface charge (Zeta potential, mV)	Negative	92
	Positive	19
	NA	55

[a]These studies lacked information about the hydrodynamic diameter in either water or a buffer solution. They included information about the hydrodynamic diameter in cell culture exposure medium.

reporting genotoxicity (43/47), oxidative stress (54/68) and proliferation/apoptosis/cell cycle/transformation (22/27) did not include endotoxin measurement, whereas none of the studies investigating epigenetic changes and receptor-mediated effects mentioned this information. For chronic inflammation, two third (66.2%, 43/65) of the *in vitro* studies did not report the level of endotoxin in the TiO_2 NP formulations. Similarly, roughly 70%–85% of *in vivo* studies investigating genotoxicity (11/13), oxidative stress (16/19), proliferation/apoptosis/cell cycle/transformation (5/7) and chronic inflammation (55/66) did not include endotoxin measurement, whereas none of the epigenetic studies did. The sole *in vivo* study on receptor-mediated effects reported the endotoxin level in the TiO_2 NP formulations (Figure 6).

Mechanistic evidence

Based on a weight-of-evidence approach, the assays/endpoints investigated for each KC were assigned a

Table 2. Physicochemical properties of the TiO_2 NPs used in the *in vivo* studies in Klimisch category 1 or 2.

TiO_2 NP characteristic	Categories	# of studies
Crystalline phase	Anatase	37
	Mixture	26
	Rutile	12
	Other	1
	NA	8
Primary size (nm)	<25	66
	25–50	16
	50–100	7
	NA	0
Hydrodynamic diameter (nm)	<25	5
	25–50	12
	50–100	10
	>100	59
	NA	0
Specific surface area (SSA, m^2/g)	<50	12
	50–100	33
	>100	31
	NA	19
Surface charge (Zeta potential, mV)	Negative	9
	Positive	15
	NA	51

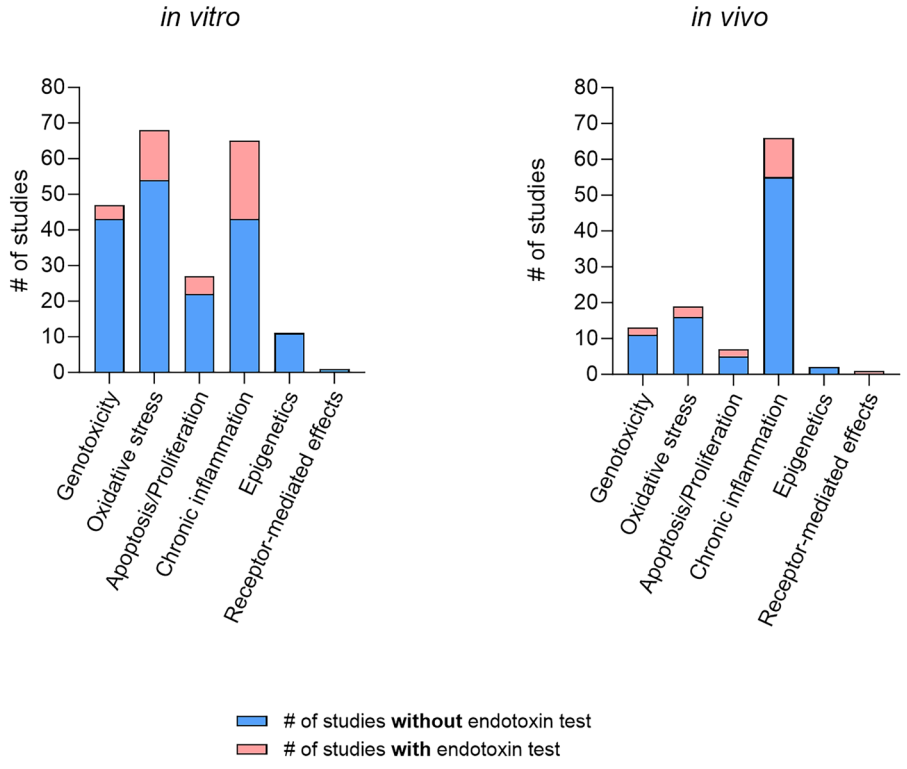

Figure 6. Overview of the number of *in vitro* and *in vivo* studies in Klimisch category 1 or 2, that included endotoxin measurement of the TiO_2 NPs, sorted by KC.

level of confidence. A list of *in vitro* and *in vivo* studies with moderate or high confidence in the endpoints investigated, sorted by KC and including the Klimisch category and the level of confidence, is tabulated (Additional file 8 and 9 in Supplementary Appendix). These tables also include the crystalline phase of the TiO_2 NPs, the endpoints and the main findings. A list of *in vitro* and *in vivo* studies with negligible or low confidence was also compiled (Additional file 10 in Supplementary Appendix). These studies were not considered for the data synthesis.

The following paragraphs give a summary of the *in vitro* and *in vivo* studies in Klimisch category 1 or 2 investigating the respective KCs. Further, in an attempt to group the effects of TiO_2 NPs, the studies were sorted by the crystalline phase of the TiO_2 NPs, and a summary of the outcome for each KC is presented.

Genotoxicity

It is well-established that genotoxicity is linked to the development of cancer (Smith et al. 2016). An agent is considered genotoxic when it causes DNA damage (including DNA adducts, DNA strand breaks, DNA crosslinks and DNA alkylation), induces mutations (alterations to the genome), or both. The genotoxicity endpoints were assigned confidence based on a previously published study (Kirkland et al. 2022). For *in vitro* studies, the micronucleus formation and HPRT gene mutation were considered as relevant endpoint for carcinogenesis. For *in vivo* studies, mainly the comet and DNA peroxidation assay were considered a high level of confidence.

Anatase TiO_2 NPs were the most investigated TiO_2 NP type in genotoxicity studies, with a similar amount of *in vitro* and *in vivo* studies. While some *in vitro* studies did not observe any changes in micronucleus formation (García-Rodríguez et al. 2019; Ghosh et al. 2017; Louro et al. 2019), the *in vitro* evidence favors increased DNA damage, i.e. micronucleus formation and HPRT gene mutation following anatase TiO_2 NP exposure (Chen et al. 2014; Di Bucchianico et al. 2017; Falck et al. 2009; Jain et al. 2017; Kurzawa-Zegota et al. 2017; Medina-Reyes et al. 2019; Srivastava et al. 2013). However, two studies reporting increased DNA damage were assigned Klimisch category 2 (Di Bucchianico et al. 2017; Falck et al. 2009). *In vivo*, there is inconclusive evidence regarding the genotoxic potential of anatase TiO_2 NPs. Using DNA strand breaks as an endpoint assessed by comet assay, two short-term studies using intratracheal injection of TiO_2 NPs in rats and head-only inhalation in mice found increased DNA damage (Han et al. 2020; Larsen et al. 2016). However, the particle doses used in these studies were quite high, which might not be relevant to human occupational exposure scenarios. Another study observed a dose-dependent increase in DNA peroxidation in mice following repeated intratracheal instillation with TiO_2 NPs for 90 d (Li et al. 2013). This study was assigned Klimisch category 2 and must be interpreted cautiously. Naya et al. (Naya et al. 2012) showed that single and repeated intratracheal instillation of TiO_2 NPs in rats did not change the % tail DNA in lung epithelial cells. Similarly, no DNA damage in mice was observed after 3 d (Murugadoss et al., 2020) and 180 d (Danielsen et al. 2020) following single exposure to TiO_2 NPs and after repeated whole-body inhalation exposure (Lindberg et al. 2012).

In vitro, a mixture of anatase/rutile TiO_2 NPs at 10–40 µg/cm^2 induced micronucleus formation in A549 cells after 72 h (Stoccoro et al. 2017). However, the most *in vitro* genotoxicity studies did not find any effect on micronucleus formation or HPRT gene mutation (Brandão et al. 2020; Ghosh et al. 2017; Kazimirova et al. 2020; Prasad et al. 2013; Tavares et al. 2014). For mixture (anatase/rutile) TiO_2 NPs, only one *in vivo* study in Klimisch category 1 and with a moderate confidence was identified (Relier et al. 2017). Using a repeated (3 doses once every four days) endotracheal instillation paradigm, the study found a statistically significant increase in DNA lesions in the rat lung after 35 d after exposure to the two highest doses (2.5 and 10 mg/kg) of TiO_2 NPs tested. Double-strand breaks increased 2 h after exposure at the highest dose (Relier et al. 2017).

Only one *in vitro* study investigating rutile TiO_2 NPs was identified (Corradi et al. 2012). However, results for the micronucleus assay using A549 cells were unavailable as NP agglomeration obscured the analysis. *In vivo*, three studies on rutile TiO_2 NPs were identified, each reporting a different outcome (Hadrup et al. 2017; Li et al. 2018; Wallin et al. 2017), rendering the mechanistic evidence for rutile TiO_2 NP genotoxicity inconclusive. Measuring 8-OHdG levels in lung DNA showed no significant difference between the TiO_2 NP inhalation group (up to 1.84 mg/m^3) and controls after 6 months (Li et al. 2018). On the other hand, using the comet assay as endpoint for genotoxicity, one study using intratracheal instillation (67 µg/mouse) found a lower level of DNA strand breaks as compared to the controls after 24 h (Hadrup et al. 2017), while another instillation study showed increased levels of DNA strand breaks in the lung tissue of TiO_2-exposed mice (18–162 µg/mouse) after 1 and 28 d post-exposure (Wallin et al. 2017).

Overall, there is inconclusive evidence for genotoxicity following exposure to anatase, mixture anatase/rutile and rutile TiO_2 NPs, with a tendency to 'increased effect' following exposure to anatase TiO_2 NPs (Figure 7).

Epidemiological studies suggest increased 8-OHdG levels in exhaled breath condensates (Pelclova, Zdimal, Fenclova, et al. 2016) and white blood cells (Liou et al. 2017). However, these findings are difficult to compare to the mechanistic evidence due to the poor TiO_2 exposure characterization (i.e. size distribution and purity).

Oxidative stress

In healthy cells, there is a balance between generating reactive oxygen species (ROS) and

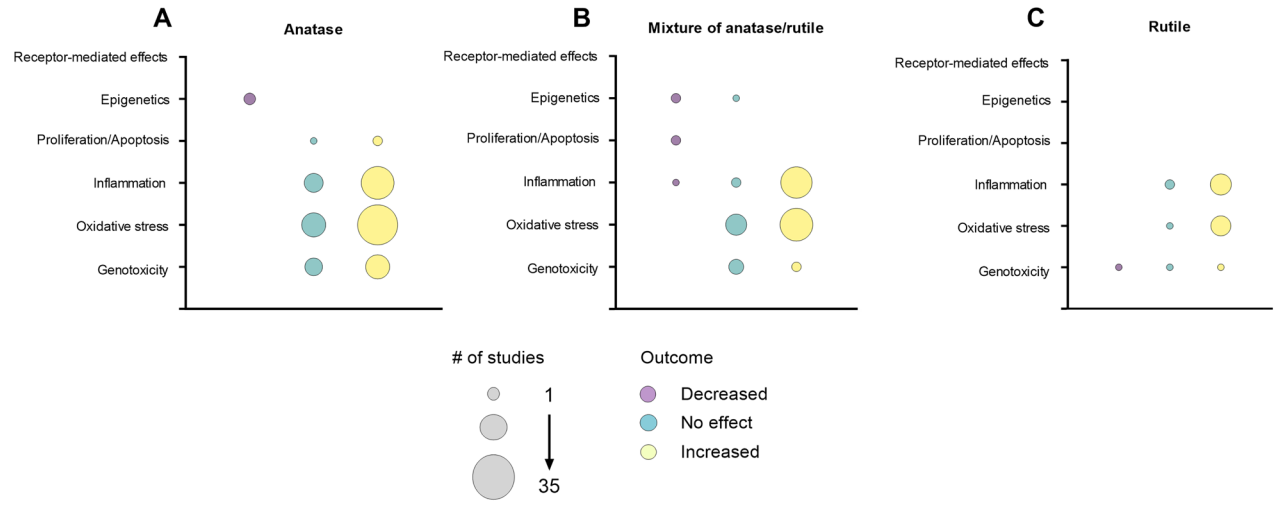

Figure 7. Bubble charts indicating the outcome 'decreased effect' (violet), 'no effect' (blue) and 'increased effect' (yellow) for the respective KC reported in TiO_2 NP *in vitro* and *in vivo* studies with moderate or high confidence in the biological endpoints assessed. The bubble size indicates the number of studies reporting the respective outcome for at least one dose and time point following anatase TiO_2 NP (A); mixture anatase/rutile TiO_2 NP (B); and rutile TiO_2 NP (**C**) exposure.

counteracting anti-oxidant mechanisms. Following chemical exposure or other cell stress/injury, this balance can be disturbed in favor of the generation of ROS, which can eventually result in oxidative stress. The generation of ROS has been implicated as a major mechanism of carcinogens. However, as non-carcinogens can also induce oxidative stress, which has been associated with several (non-cancer) chronic diseases and pathological conditions such as cardiovascular disease, neurodegenerative disease, and chronic inflammation, this KC has to be interpreted with caution unless other KCs accompany it.

For the i*n vitro* evidence, only intracellular ROS levels and lipid peroxidation were considered relevant endpoints for carcinogenesis. In contrast, *in vivo* studies investigating intracellular ROS levels, lipid peroxidation, protein levels of oxidative stress-related genes and anti-oxidant mechanisms were considered to have a moderate or high confidence level.

Anatase and a mixture of anatase/rutile TiO_2 NPs were the most investigated TiO_2 NPs in studies regarding oxidative stress, with more than three- to six-fold more *in vitro* data than *in vivo* studies. For rutile TiO_2 NPs, only one *in vivo* study was identified, whereas nine *in vitro* studies investigated this particle type. Although quite some evidence shows no effect on oxidative stress responses, overall, most studies reported increased oxidative stress following TiO_2 NP exposure. The *in vitro* evidence supports increased oxidative stress responses following TiO_2 NP exposure. Several studies have shown that short-term, submerged exposure of bronchial and alveolar epithelial cells with anatase TiO_2 NPs resulted in increased intracellular ROS and/or lipid peroxidation (Ahmad et al. 2018; Andersson et al. 2011; Auveriyavit et al. 2012; Bai, Chen, and Gao 2015; Chen et al. 2022; De Matteis et al. 2016; Ekstrand-Hammarström et al. 2012; Hussain et al. 2009; Hussain et al. 2010; Ma et al. 2017; Park, Lee, Lee, et al. 2014; Shi et al. 2010; Srivastava et al. 2011; Srivastava et al. 2013; Sweeney et al. 2015; Yuan et al. 2021). Air-liquid-interface (ALI) TiO_2 NP exposure of a co-culture of A549/THP-1 cells has also been demonstrated increased intracellular ROS production after 24 h (Loret et al. 2016; Loret et al. 2018). Furthermore, increased oxidative stress responses have been observed following anatase TiO_2 NP exposure of lung fibroblasts (Hamzeh and Sunahara 2013; Jain et al. 2017) and various immune cell-models (Chen et al. 2018; Dinesh et al. 2017; Kolling et al. 2020; Schanen et al. 2013; Tada-Oikawa et al. 2016; Xiong et al. 2013), while others have not reported any changes regarding oxidative stress responses in lung epithelial cells and immune cell-models (Alinovi et al. 2017; Ahamed, Akhtar, and Alhadlaq 2019; Belade et al. 2015; Danielsen et al. 2015; Ghosh et al. 2017; Johnston et al. 2015; Kose et al. 2020; Kose et al. 2021; Spigoni et al. 2015; Vergaro et al. 2016). *In vivo*, several studies showed increased oxidative stress responses following anatase TiO_2 NP exposure. Single intratracheal injection of 200–1000 mg/kg TiO_2 NPs increased ROS production and lipid peroxidation (Han et al. 2020). However, this study used very high particle doses,

which might not be relevant in real-life exposure scenarios. Other studies using single nose-only inhalation reported increased lipid peroxidation after exposure to 7–10 mg/m^3 TiO_2 NPs (Noël et al. 2012; Noël et al. 2013), and elevated ROS and reactive nitrogen species (RNS) level in lung tissue of mice were found after repeated intranasal instillation of 2.5–10 mg/kg anatase TiO_2 NPs (Zhou et al. 2019). Repeated intratracheal instillation was found to result in increased lipid peroxidation (Li et al. 2013; Horváth et al. 2018; Papp et al. 2020; Sun, Tan, Zhou, et al. 2012), increased ROS production (Li et al. 2013), and increased levels of heme oxygenase-1 (HO-1) protein (Sun, Tan, Ze, et al. 2012). However, other studies did not observe changes in oxidative stress effects following a single intratracheal or oropharyngeal exposure to anatase TiO_2 NPs (Loret et al. 2018; Murugadoss et al., 2020; Roulet et al. 2012).

For mixture TiO_2 NPs, an increase of intracellular ROS and/or lipid peroxidation was described following short-term, submerged exposure of lung epithelial cells (Andersson et al. 2011; Armand, Tarantini, et al. 2016; Ekstrand-Hammarström et al. 2012; Gandamalla, Lingabathula, and Yellu 2019; Guadagnini et al. 2015; Hussain et al. 2009), lung fibroblasts (Hamzeh and Sunahara 2013; Nica et al. 2022) and various macrophage cell-models, including THP-1 cells (Tada-Oikawa et al. 2016; Hanot-Roy et al. 2016; Pavlin et al. 2022; Tada-Oikawa et al. 2020), RAW264.7 cells (Dhupal et al. 2018; Hu et al. 2019; Xiong et al. 2013), bone marrow-derived macrophages (Kolling et al. 2020; Tsugita, Morimoto, and Nakayama 2017) and alveolar macrophages (Park, Lee, Shim, et al. 2014; Scherbart et al. 2011). Single exposure of a co-culture of A549/THP-1 cells at ALI to a mixture of TiO_2 NPs (3–20 µg/cm^2) resulted in increased intracellular levels of ROS after 24 h (Loret et al. 2016), while continuous exposure of the same co-culture model to low doses of TiO_2 NPs (0.1–3 µg/cm^2) induced significant oxidative stress responses (Loret et al. 2018). However, another study using A549 cells at ALI could not detect changes in intracellular levels of ROS following exposure to a mixture of TiO_2 NPs (0.7–25.8 µg/cm^2) (Hufnagel et al. 2020). Likewise, some studies could not detect changes in oxidative stress responses following short-term submerged exposure of lung epithelial cells and immune cells to a mixture of TiO_2 NPs (Bacova et al. 2022; Ghosh et al. 2017; Hufnagel et al. 2020; Kose et al. 2020; Kose et al. 2021; Poon et al. 2020; Vergaro et al. 2016; Wan et al. 2012). The *in vivo* evidence for oxidative stress responses after exposure to a mixture of TiO_2 NPs is inconclusive. Both single whole-body inhalation (1 mg/m^3) and intratracheal instillation (800 µg/rats) of rats with mixture TiO_2 NPs resulted in increased levels of HO-1 in lung tissue and bronchioalveolar lavage fluid (BALF) (Baisch et al. 2014; Yoshiura et al. 2015), whereas single nose-only inhalation of rats to 20 mg/m^3 mixture TiO_2 NPs showed increased lipid peroxidation (Noël et al. 2013). On the other hand, single and repeated intratracheal instillation with mixture TiO_2 NPs had no effect on intracellular ROS production (Loret et al. 2018) and glutathione levels in the lung tissue (Relier et al. 2017).

Using rutile TiO_2 NPs, one *in vitro* study showed no effect on oxidative stress responses after short-term, submerged exposure of A549 cells (Andersson et al. 2011). In contrast, the majority of studies observed increased intracellular ROS levels after exposure of lung epithelial cells (Aueviriyavit et al. 2012; De Matteis et al. 2016; Ekstrand-Hammarström et al. 2012; Pearce, Okon, and Watson-Wright 2020; Sweeney et al. 2015), lung fibroblasts (Hamzeh and Sunahara 2013) and immune cells (Danielsen et al. 2015; Tada-Oikawa et al. 2016) to TiO_2 NPs. *In vivo*, intratracheal instillation of rutile TiO_2 NPs increased HO-1 in BALF of rats up to 7 d post-exposure (Morimoto et al. 2016).

In summary, there is much evidence that exposure to anatase TiO_2 NPs increased oxidative stress (Figure 7). Similar to anatase, for the mixture of anatase/rutile TiO_2 NPs, there is substantial evidence for increased induction of oxidative stress. Increased oxidative stress has also been observed for rutile TiO_2 NPs, although the number of studies was far less than for anatase or mixture of anatase/rutile TiO_2 NPs (Figure 7).

The few epidemiological studies available support oxidative stress as a possible mechanism of TiO_2 exposure. Accordingly, one study investigating TiO_2-handling workers suggest that TiO_2 exposure may lead to lower antioxidant enzyme activity (Liou et al. 2016). Another study investigating workers handling nanomaterials, including TiO_2, found increased lipid peroxidation in exhaled breath condensate (Liou et al. 2017). These studies are, however, difficult to compare to the mechanistic evidence due to poor or lacking characterization of TiO_2 exposure.

Chronic inflammation

Chronic inflammation is a long-term reaction to an inflammatory stimulus that involves the continuous

recruitment of mononuclear leukocytes (monocytes and lymphocytes) and is accompanied by tissue injury due to a sustained inflammatory environment. Chronic inflammation can last several weeks, months, or even a lifetime in the case of some chronic inflammatory disorders and plays a central role in the development and progression of several chronic diseases, including diabetes, asthma, cardiovascular diseases and cancer (Zhong and Shi 2019). Epidemiological studies on TiO_2-handling workers suggest that TiO_2 exposure may lead to a subtle alteration of lung pathobiology (Bergamaschi et al. 2022), inflammation and fibrotic changes in the lungs (Pelclova, Zdimal, Fenclova, et al. 2016). The mechanistic evidence for chronic inflammation is only derived from *in vivo* data as the *in vitro* endpoints most commonly used to study inflammation, such as gene expression of inflammation-related markers, cytokine release or nitric oxide production, together with short post-exposure time points and missing feedback loops/regulatory mechanisms, might not be of relevance for 'chronic inflammation' and *in vivo* carcinogenesis, and was given a negligible or low level of confidence. Thus, these studies were not included in the data synthesis. For the *in vivo* studies, protein levels of cytokines, BALF cell count, and lung histology were considered endpoints of moderate or high confidence. It is to be further noted that the *in vivo* studies differed amongst each other concerning the physicochemical particle characteristics, formulation method, particle doses tested, exposure scenario (single vs. repeated exposure), and post-exposure time points, which made it nearly impossible to compare the study findings in a meaningful manner. However, in an attempt to group the results, exposure scenarios and the main outcomes were considered.

For anatase TiO_2 NPs, most studies showed evidence that exposure to these TiO_2 NPs results in chronic inflammation. Single intratracheal instillation/nebulization/injection in rat and mice has been shown to result in dose-dependent short-term neutrophil influx which declined after several days/weeks, with histological changes showing inflammatory cell infiltration, thickening of the alveolar wall, and increased protein levels of cytokines such as TNFα, IL-6, MCP-1, IL-1, IL-12 and IL-10 (Aragao-Santiago et al. 2016; Danielsen et al. 2020; Han et al. 2020; Hashizume et al. 2016; Kobayashi et al. 2009; Loret et al. 2018; Liu et al. 2010; Park, Lee, Shim, et al. 2014; Rahman et al. 2017). Short-term increase in neutrophils in BALF was also described following single nose-only inhalation of rats (Noël et al. 2013; Noël et al. 2012). An increased number of total bronchioalveolar lavage (BAL) cells and macrophages was observed following single whole-body inhalation in mice (Grassian, Adamcakova-Dodd, et al. 2007; Grassian, O'Shaughnessy, et al. 2007). A study using repeated intranasal exposure of mice to 20 mg/kg TiO_2 NPs for 30 d described changes in the morphology and histology in the lungs (Ma et al. 2019). Similar pathological findings, including infiltration of inflammatory cells and thickening of the pulmonary interstitium and edema, were observed following repeated intranasal exposure of mice for 90 days (Li et al. 2013). Additionally, another study reported similar histopathological changes, an increased number of cells in BAL, and increased levels of inflammatory cytokines after repeated intranasal exposure for 90 d (Yu et al. 2014). Repeated intranasal exposure for 6 and 9 months showed increased inflammatory cytokine levels (Hong et al. 2015; Zhou et al. 2019), indicating a sustained inflammatory response. Repeated whole-body inhalation in mice with 28.5 mg/m^3 for 5 d resulted in increased neutrophil influx, whereas repeated whole-body inhalation with 25-50 mg/m^3 for 13 weeks and 32 mg/m^3 for 26 weeks led to increased neutrophil influx, increased number of lymphocytes and enlarged particle-laden macrophages (Yamano et al. 2022a; Yamano et al. 2022b). However, some studies did not show changes in inflammation following single oropharyngeal aspiration (Kim et al. 2014; Murugadoss et al., 2020), intratracheal instillation (Okada et al. 2016; Roulet et al. 2012; Roursgaard et al. 2011; Rushton et al. 2010), nose-only (Scarino et al. 2012) or head-only inhalation (Larsen et al. 2016), which could be due to different particle characteristics, the formulation method, various exposure doses or other experimental parameters.

The *in vivo* mechanistic evidence for chronic inflammation following exposure to a mixture of TiO_2 NPs favors increased inflammatory responses. Single intratracheal instillation with mixture TiO_2 NPs has been reported to result in elevated levels of eosinophils and neutrophils, increased total number of BAL cells, and an early and transient increase in several inflammatory cytokines (Gustafsson et al. 2011; Hashizume et al. 2016; Kobayashi et al. 2016; Loret et al. 2018; Okada et al. 2016; Park et al. 2009; Rahman et al. 2017; Rushton et al. 2010; Sager, Kommineni, and Castranova 2008; Warheit et al. 2007; Yoshiura et al. 2015). An increased number of neutrophils, macrophages and eosinophils has also been reported following single nose-only inhalation

in mice and rats (Jonasson et al. 2013; Noël et al. 2013). In contrast, elevated protein levels of IL-1β and IL-6 and increased neutrophil influx have been found after single whole-body inhalation of mixture TiO_2 NPs (Baisch et al. 2014; Grassian, Adamcakova-Dodd, et al. 2007). Repeated intratracheal exposure with mixture TiO_2 NPs resulted in increased number of neutrophils and elevated levels of inflammatory cytokines (Abdulnasser Harfoush et al. 2020; Relier et al. 2017) and extensive disruption of alveolar septa, macrophage accumulation, and slight alveolar thickness (Chang et al. 2014). An acute inflammatory response was reported after nose-only inhalation of rats with 10 mg/m^3 mixture TiO_2 NPs, which decreased over 180 days post-exposure (Chézeau et al. 2019; Chézeau et al. 2018). Another nose-only inhalation study with rats showed an increased neutrophil influx, which was still elevated 70 d post-exposure (Gustafsson et al. 2014). Repeated whole-body inhalation in rats with 4.1 mg/m^3 TiO_2 NPs increased the number of alveolar macrophages and showed mild inflammation (Okada et al. 2019). In contrast, two other whole-body inhalation studies did not observe any changes in inflammatory responses (Rossi et al. 2010; Scuri et al. 2010). One study using repeated nose-only inhalation was identified that reported reduced white blood cell count after exposure to mixture TiO_2 NPs (Eydner et al. 2012).

Although fewer studies on rutile TiO_2 NPs regarding chronic inflammation were found, the evidence suggests that intratracheal instillation/nebulization/spraying with rutile TiO_2 NPs increased the total number of cells in BAL, led to acute neutrophil influx which decreased over time, and increased levels of CCL3 and IL-6, while repeated exposure to rutile TiO_2 NPs for several weeks resulted in an increased number of neutrophils and macrophages, and increased levels of CINC1 and CINC2 in BAL of mice (Hadrup et al. 2017; Hashizume et al. 2016; Morimoto et al. 2016; Roursgaard et al. 2011; Saber et al. 2019; Sagawa et al. 2021; Tomonaga et al. 2020; Wang et al. 2021; Wallin et al. 2017). Two studies that showed no inflammatory responses due to rutile TiO_2 NP exposure were identified (Aragao-Santiago et al. 2016; Morimoto et al. 2016).

In conclusion, for anatase TiO_2 NPs, there is much evidence that exposure to these TiO_2 NPs resulted in increased inflammation (Figure 7). However, some studies could not detect inflammatory responses. Substantial evidence exists for increased inflammatory responses for a mixture of anatase/rutile and rutile TiO_2 NPs. However, there were fewer studies for rutile TiO_2 NPs than for a mixture of anatase/rutile TiO_2 NPs (Figure 7).

The *in vivo* evidence for TiO_2-induced chronic inflammation supports the findings from epidemiological studies (Bergamaschi et al. 2022; Pelclova, Zdimal, Fenclova, et al. 2016). However, direct comparison of the experimental data with the epidemiological studies is hampered by the often poor characterization of TiO_2 exposure in these studies.

Proliferation/apoptosis/cell cycle/transformation

Alterations in replication and/or cell cycle control, and evasion of apoptosis have been implicated in the development of cancer (Smith et al. 2016). *In vitro*, population doubling and BrdU incorporation assay were considered relevant for carcinogenesis, while, for *in vivo* studies, DNA fragmentation, apoptotic and anti-apoptotic protein levels (apoptosis), and Ki67 staining (proliferation) were considered relevant endpoints with moderate to high confidence. More studies investigating transformation and cell cycle with relevant endpoints are needed as the mechanistic evidence for proliferation/apoptosis/cell cycle/transformation is limited.

For anatase TiO_2 NPs, there is *in vivo* evidence that repeated intratracheal instillation of rats for 5 days per week for 6 weeks resulted in a dose-dependent increase in TUNEL-positive (that is, apoptotic) cells at 10 mg/kg and 18 mg/kg TiO_2 NPs (Papp et al. 2020). Using the RasH2 mouse model, a 26-week inhalation study did not find evidence for carcinogenicity of TiO_2 NPs in the lung (Yamano et al. 2022a). The results from this study indicated that the cell proliferative ability (i.e. proliferative marker Ki67 index) of alveolar epithelial cells type 2 was not increased by 2–32 mg/m^3 anatase TiO_2 NPs (Yamano et al. 2022a). In another long-term (13 weeks) inhalation study using rats, the same authors reported an increased cell proliferative ability of alveolar epithelial cells type 2 following 50 mg/m^3 TiO_2 exposure (Yamano et al. 2022b). Together, these results indicate a potential for increased cell proliferation in the alveoli following long-term inhalation to higher doses of anatase TiO_2 NPs.

For the mixture anatase/rutile TiO_2 NPs, two *in vitro* studies investigating proliferation have been identified (Armand, Tarantini, et al. 2016; Armand, Biola-Clier, et al. 2016). Using population doubling and the BrdU incorporation assay as measures for proliferation, both studies showed that continuous exposure for 2 months of A549 cells to TiO_2 NPs (1–50 µg/ml) led to a dose-dependent decrease in

the proliferation rate of the A549 cells (Armand, Tarantini, et al. 2016; Armand, Biola-Clier, et al. 2016).

In summary, for anatase TiO_2 NPs, the evidence for proliferation/apoptosis/cell cycle/transformation is inconclusive, with a tendency to 'increased effect' (Figure 7). Decreased proliferation was observed following the exposure to a mixture of TiO_2 NPs. No mechanistic evidence was identified regarding 'proliferation/apoptosis/cell cycle/transformation' for rutile TiO_2 NPs (Figure 7).

Epigenetic changes

Epigenetic alterations, including changes in DNA methylation levels, chromatin compaction states, and histone modifications, can impact cancer development as these alterations affect gene expression and DNA repair dynamics (Herceg et al. 2013; Smith et al. 2016). Epigenetic alterations can be a primary mechanism, but also be induced by other biological responses, such as chronic inflammation. For both *in vitro* and *in vivo* epigenetic studies, global (or overall) DNA methylation and RNA methylation (m6A levels) were considered moderate to high confidence. Similar to the KC 'proliferation/apoptosis/cell cycle/transformation', the mechanistic *in vitro* and *in vivo* evidence for epigenetic changes is relatively scarce.

Nevertheless, for anatase TiO_2 NPs, the available evidence collectively points to a DNA hypomethylation. After 24 h, significant DNA hypomethylation in BEAS-2B cells was observed for anatase TiO_2 NPs at 3.25 and 25 µg/ml (Ghosh et al. 2017). Another study with A549 and 16HBE cells showed that anatase TiO_2 NPs reduced genomic DNA methylation levels in these cells at 1–100 µg/ml after 48 h (Ma et al. 2017). Repeated exposure to TiO_2 NPs at 20 mg/kg for 30 days resulted in significant global hypomethylation in young mice (5 weeks old), whereas no significant changes were observed in adult mice (10 weeks old) (Ma et al. 2019).

The evidence for epigenetic alterations for a mixture of TiO_2 NPs is inconclusive. It is to be noted that only *in vitro* evidence for mixture TiO_2 NPs was identified. One study observed no changes in the overall DNA methylation level in A549 cells exposed to TiO_2 NPs for 4 h, 24 h or 48 h (Biola-Clier et al. 2017). In contrast, two other studies observed DNA hypomethylation after 24 h exposure of bronchial epithelial cells to 25 µg/ml TiO_2 NPs (Ghosh et al. 2017) and after 72 h exposure of A549 cells to 10–40 µg/cm^2 TiO_2 NPs (Stoccoro et al. 2017).

Even though these studies indicate a similar pattern for anatase TiO_2 NPs, and partially for mixture TiO_2 NPs regarding epigenetic changes, more studies are warranted to confirm the observed changes in the DNA methylome.

Overall, a decreased effect following anatase TiO_2 NP exposure was found in studies investigating epigenetic changes (Figure 7). The findings regarding epigenetic changes were inconclusive for the mixture of anatase/rutile TiO_2 NPs. No mechanistic evidence regarding epigenetic changes for rutile TiO_2 NPs could be identified (Figure 7).

Receptor-mediated effects

Receptor activation effects can be divided into activation of cell-surface receptors and intracellular receptor activation. Cell-surface receptor activation induces intracellular signal transduction pathways resulting in a biological response, while the activation of intracellular receptors initiates the translocation of these receptors into the nucleus where they bind to DNA and act as transcription factors for relevant target genes. The most relevant molecular pathways that are regulated through ligand-receptor interaction include cell proliferation, apoptosis, and xenobiotic metabolism (Smith et al. 2016). In our study, only one *in vitro* and one *in vivo* study investigating receptor-mediated effects were assessed to their quality and reliability with the ToxRTool and the weight-of-evidence approach. As both studies (Ho et al. 2017; Jeon et al. 2021) examined only the gene expression levels of AhR/PPAR/LXR, they were considered negligible and low confidence studies, respectively. Thus, no reliable and relevant mechanistic evidence on receptor-mediated effects of TiO_2 NPs in the lung is available.

Level of evidence

Overall, taking the challenges and limitations of the *in vitro* and *in vivo* studies into account, the mechanistic evidence identified in this review suggests that TiO_2 NPs might possess the ability to induce chronic inflammation and oxidative stress. It was therefore rated as sufficient mechanistic evidence (Figure 8, marked in green). For genotoxicity, the *in vivo* data is inconclusive for all the TiO_2 NP types, whereas there is sufficient evidence that anatase TiO_2 NPs induce HPRT gene mutations and micronucleus formation *in vitro*. Nevertheless, the overall evidence was rated as limited (Figure 8, marked in orange) since there is no consensus between the

in vitro and *in vivo* evidence. The mechanistic evidence for the KCs 'epigenetic changes' and 'proliferation/apoptosis/cell cycle/transformation' is inconclusive. There were only a few studies identified investigating the effect of TiO_2 NPs on these KCs. More studies are needed to confirm the observed effects. Thus, the mechanistic evidence was rated inadequate (Figure 8, marked in grey). For receptor-mediated changes, there is no reliable and relevant mechanistic evidence for TiO_2 NPs available. It was therefore also rated inadequate (Figure 8, marked in grey).

Discussion

The present study identified the existing *in vitro* and *in vivo* mechanistic evidence, from 2006-2023, of TiO_2 NP lung carcinogenicity using the ten key characteristics of carcinogens for identifying and classifying carcinogens.

Regarding the evidence presented in this review, there is quite some variation in (a) the relevance and quality/reliability of studies concerning TiO_2 NPs and the respective KCs; (b) the effect of TiO_2 NPs on each KC; (c) the amount of mechanistic evidence for each KC and d) the number of studies regarding anatase, mixture and rutile TiO_2 NPs. Using a weight-of-evidence approach, we evaluated the studies regarding their biological relevance and reliability. Evidence-weighting assumptions were mainly based on expert judgment regarding the biological relevance of the assays/endpoints for carcinogenesis. This could have led to a more stringent sorting of studies.

For some KCs, the predictability of *in vitro* assays relative to *in vivo* studies represents a challenge, especially for the KC 'chronic inflammation'. The *in vitro* studies investigating chronic inflammation were considered not biologically relevant for *in vivo* carcinogenesis and excluded from data synthesis. The majority of these studies were not designed in a way that they could predict sustained change nor determine the magnitude of change required to initiate carcinogenesis. This makes it difficult to assess whether the observed changes persist over time and are sufficient for the development of cancer. However, it is to be noted that also some *in vivo* studies might not consider relevant exposure scenarios to detect sustained changes.

There are some challenges and limitations to the quality and reliability of the *in vitro* and *in vivo* studies investigating TiO_2 NP toxicity/carcinogenicity. Nearly half of the *in vitro* studies were considered unreliable (Klimisch category 3), whereas a third of studies were assigned Klimisch category 1. Half of the *in vivo* studies were reliable without restriction (Klimisch category 1), while a third was unreliable and thus was excluded. These results indicate a

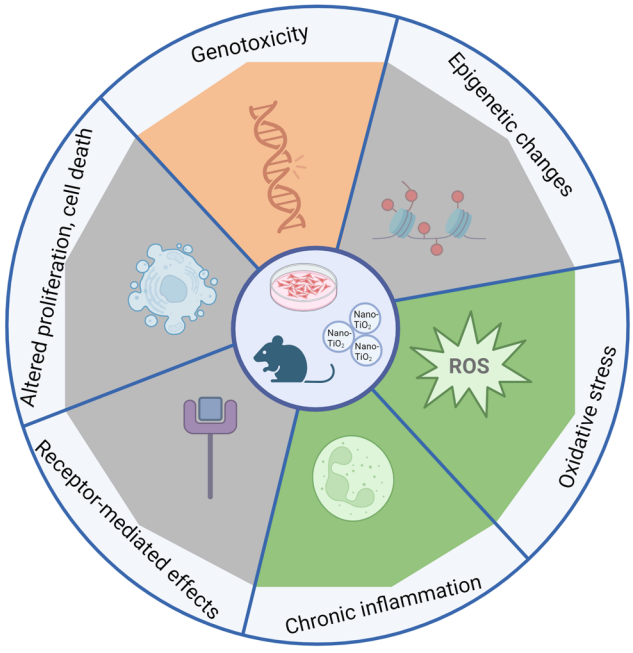

Figure 8. Overview of the level of evidence for the mechanisms of TiO_2 NP lung carcinogenicity, based on the modified key characteristics (KCs) addressed in this study. *Sufficient* evidence (green) could be identified for KC 'chronic inflammation' and 'oxidative stress'. *Limited* evidence (orange) was found for 'genotoxicity'. *Inadequate* evidence was identified for 'epigenetic changes', 'proliferation/apoptosis/cell cycle/transformation' and 'receptor-mediated effects'. Figure created with BioRender.com.

skewness between *in vitro* and *in vivo* studies, which could partly be due to more stringent standards when conducting animal research.

The challenges and limitations of the studies also influence the effects/outcome of TiO_2 NPs on each KC. There are differences in starting material (primary size and surface functionalization vary), various methods for NP dispersion, physicochemical characterization, exposure paradigms (i.e. exposure scenarios/cell systems and post-exposure time points), and a wide range of particle doses, which makes it very challenging to compare these studies. The inconsistent effects observed for the different KCs are likely due to the various physicochemical characteristics of the TiO_2 NPs. This review included only studies that investigated 'pristine' TiO_2 NPs without any doping or coating layer. However, the exact composition of the TiO_2 NPs is not always reported in the studies.

Furthermore, the TiO_2 NP dispersion and sonication methods vary between studies. This might have influenced the observed mechanisms. Using a standardized protocol for dispersion and sonication of TiO_2 NP formulations would aid in the comparison of studies. However, as mechanistic studies usually vary in the study design and hypotheses to be answered, using a standardized protocol for dispersion and sonication might not always be applicable.

We recognize that there might be some limitations regarding the methodological consideration of the physicochemical properties using the ToxRTool, which includes both *in vitro* and *in vivo* studies, but was not explicitly designed to evaluate studies with NPs. As NPs have unique physicochemical properties that vary depending on numerous factors such as temperature, medium composition, solvent, etc., characterizing these properties is very important in toxicity/carcinogenicity studies. We chose to include a minimum set of physicochemical characteristics in the ToxRTool. Setting a lower threshold for exclusion by including more than the minimum set of NP parameters required, e.g. by including a separate nano score (Card and Magnuson 2010), would have resulted in the exclusion of too many articles, which, in our opinion, would have introduced an unreasonably high bias. However, we recognize that these NP parameters are TiO_2 NP-specific and might differ for other NP types.

Another aspect to consider is the inclusion of appropriate positive controls to verify the functionality of the test system, NP assay interference controls and endotoxin measurements. As mentioned above, a positive control for the assay and the TiO_2 NPs is indispensable in the ToxRTool. For genotoxicity, for example, most studies did not include a positive control for the test system, which is particularly important when negative results for TiO_2 NPs were obtained. Further, nanoparticles have been shown to interfere with various assay systems, e.g. fluorescence/absorbance-based assays. However, only very few studies tested the interference potential of the TiO_2 NPs, thus reducing the reliability of these studies.

A significant issue when studying nanoparticle toxicity/carcinogenicity is the potential contamination of the particles with biological components, such as endotoxin or other bacterial components. Strikingly, most studies did not provide information regarding the endotoxin level in the TiO_2 NP dispersion. Endotoxin measurement was included in our modified ToxRTool when evaluating the *in vitro* and *in vivo* TiO_2 NP studies as endotoxin contamination can directly induce immune responses and thus also indirectly affect other endpoints, such as *in vivo* mutagenicity. I*n vitro* evidence shows that endotoxins affect immunological responses (Chapekar et al. 1996; Li et al. 2020; Sweet and Hume 1996). Most studies investigating 'chronic inflammation' did not include and/or report the inclusion of endotoxin measurements. In addition, endotoxins have been shown to induce oxidative stress responses, which is linked to various toxicity endpoints, in several cell types. Thus, it is likely that endotoxin contamination of NPs affects endpoints other than immunotoxicity (Esch et al. 2010). For genotoxicity, very few studies included endotoxin measurements for the TiO_2 NPs, making it difficult to assess if the effect is due to the particles or any potential biological contamination. Although endotoxin is not of highest concern in genotoxicity studies, it has been shown that it can affect inflammatory responses and the induction of oxidative stress. Genotoxicity can be a primary mechanism or a secondary effect due to physiological stress or cytotoxicity (Kirkland et al. 2022). Some studies showed effects for genotoxicity and oxidative stress responses, apoptosis or inflammatory responses (Armand, Tarantini, et al. 2016; Hamzeh and Sunahara 2013; Jain et al. 2017). Thus, the observed genotoxic effects occurred secondary to physiological stress. For studies that solely investigated genotoxic endpoints, it cannot be concluded whether the effect was primary or secondary due to cytotoxic effects. Thus, the measurement of endotoxin levels might also be relevant for genotoxicity studies. Roughly 70% of all studies (i.e. including all KCs) did not include endotoxin measurements, which is concerning regarding the confounding

adverse effects of endotoxin. This could be partly due to the unawareness of the importance of biological contamination in NP formulations in older studies (before 2015). However, newer studies (from 2015 to 2023) lacked endotoxin measurements. As endotoxin contamination may be a significant confounding factor in toxicity studies using NPs, toxicity studies lacking information regarding the endotoxin level in NP formulations should be interpreted cautiously.

Our analysis shows that the mechanistic evidence for each KC is quite variable. Inflammatory responses and oxidative stress, followed by genotoxicity, are the most investigated outcomes in both *in vitro* and *in vivo* studies with TiO_2 NPs. There are several reasons for that: high focus on these endpoints based on earlier research findings; easy availability of assay kits; standardized assays/tests available for some endpoints; high relevance for short-term exposure (which most studies focused on, but which makes these results questionable for carcinogenesis) and other practical/historical reasons (e.g. NPs are supposed to be more reactive than bigger particles, so oxidative stress is the first choice of test). The few epidemiological studies on TiO_2-handling workers also focused on endpoints such as inflammatory responses, oxidative stress and genotoxicity. This could be due to similar reasons as mentioned for the mechanistic studies. On the other hand, epigenetic changes and proliferation/apoptosis/cell cycle/transformation have been less investigated in *in vitro* and *in vivo* studies, which could be due to less focus on these KCs; higher testing costs; more time-consuming methods; unavailability of instrumentation etc. No reliable and relevant mechanistic evidence on receptor-mediated effects of TiO_2 NPs in the lung is available. As the activation of intracellular receptors, such as the AhR, is involved in processes such as inflammation, cell proliferation and differentiation, it is critical to include relevant endpoints investigating receptor-mediated effects in future toxicity/carcinogenicity studies on TiO_2 NPs in order to evaluate the relevance of this KC in TiO_2 NP carcinogenicity. Standardizing and validating methods investigating epigenetic changes, receptor-mediated effects and proliferation/apoptosis/cell cycle and transformation may further contribute to a more robust and bias-free evaluation process and should be included in future research efforts.

In addition to the ten defined KCs used in this study, alternative mechanisms of action may be relevant for TiO_2 NP carcinogenicity in the lung, which were not addressed here. These could include angiogenic effects, non-mutational epigenetic reprogramming, polymorphic microbiomes, senescent cells or unlocking phenotypic plasticity. The last four 'mechanisms' are emerging hallmarks and enabling characteristics of cancer described by Hanahan (2022). More research is needed to see if these mechanisms could play a role in TiO_2 NP lung carcinogenicity.

Conclusion

Our analysis showed that most of the studies investigated oxidative stress, chronic inflammation and genotoxicity following pulmonary exposure to TiO_2 NPs, whereas there is only few data available on other mechanisms of importance in carcinogenesis, such as proliferation and transformation, epigenetic alterations and receptor-mediated effects. Overall, improvements in study quality and reliability, including the consideration of appropriate positive controls, NP interference controls, endotoxin measurement (where necessary), statistical power and relevant assays/endpoints, are needed if mechanistic evidence is to be used in the evaluation of TiO_2 NP carcinogenicity in the lung. Specifically, there is a need for more physiologically relevant, long-term studies using appropriate particle doses, particularly relevant for occupational exposure. Taking the challenges and limitations of the *in vitro* and *in vivo* studies into consideration, TiO_2 NPs might possess the ability to induce chronic inflammation and oxidative stress. Given the limited number of high-quality and high-reliability studies identified in this review, there is a lack of good enough mechanistic evidence for TiO_2 NP lung carcinogenicity.

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Data availability statement

All data supporting the results in this article is included in the main article or as supplementary material.

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