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Updated assessment of the genotoxic potential of titanium dioxide based on reviews of *in vitro* comet, mode of action and cellular uptake studies, and recent publications

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ABSTRACT

In 2021 the European Food Safety Authority (EFSA) concluded that "*A concern for genotoxicity of TiO2 particles that may be present in E 171 could therefore not be ruled out.*". A detailed review of the genotoxicity of titanium dioxide (TiO_2) was subsequently published by Kirkland et al. (2022) using a comprehensive weight of evidence (WoE) approach in which test systems and endpoints were allocated different levels of relevance. At that time only 34 publications met the reliability and quality criteria for being most relevant in the evaluation of genotoxicity, and based on these it was concluded that the existing evidence did not support a direct DNA damaging mechanism for TiO_2. Recently a number of regulatory opinions have been published, in which papers were cited that described *in vitro* DNA damage (mainly comet), mode of action, and cellular uptake studies that were not discussed in Kirkland et al. (2022). Furthermore, a number of additional papers have been published recently or have been identified from the regulatory opinions as a result of using extended search criteria. A total of 70 publications not previously reviewed in Kirkland et al. (2022) have been reviewed here, and again show that the published data on the genotoxicity of TiO_2 are inconsistent, often of poor quality, and in some cases difficult to interpret. The cellular uptake studies show some evidence of cytoplasmic uptake, particularly in cells treated *in vitro*, but there is no convincing evidence of nuclear uptake. In terms of genotoxicity, the conclusions of Kirkland et al. (2022) that existing evidence does not support a direct DNA damaging mechanism for titanium dioxide (including nano forms), and that the main mechanism leading to TiO_2 genotoxicity is most likely indirect damage to DNA through generation of reactive oxygen species (ROS), are still valid.

1. Introduction

Titanium dioxide (TiO_2) is widely used across many industries, as a pigment in paints and cosmetics (Pigment White 6 or CI 77891), and as a food colorant (E171). TiO_2 is also found in many other products such as sunscreens, printer inks and medicines.

In 2020, the European Commission requested a review of the safety profile of food-grade TiO_2 (E171), which EFSA concluded in mid-2021. In the 2021 EFSA opinion (EFSA, 2021), genotoxicity was raised as a safety issue, concluding that a genotoxic concern could not be ruled out for TiO_2, and that TiO_2 particles have the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations. No clear correlation was observed between the physico-chemical properties of TiO_2 particles and the outcome of either *in vitro* or *in vivo* genotoxicity assays. A concern for genotoxicity of the TiO_2 nanoparticles in E171 could therefore not be ruled out. In addition and in contrast to the previous EFSA assessment, the negative carcinogenicity study with E171 was considered as being not relevant. It was also stated that several modes of action for the suspected genotoxicity may operate in parallel

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Abbreviations

8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine
BALT	bronchus-associated lymphoid tissue
BEGM	Bronchial Epithelia Growth Media
BET	Brunauer, Emmet and Teller
BIN	binucleated cell
BSA	bovine serum albumin
CA	chromosomal aberrations
CLS	centrifugal liquid sedimentation
COL	colchicine
CP	cyclophosphamide
ctDNA	calf thymus DNA
CytoB	cytochalasin B
DCFDA	2′,7′–dichlorofluorescein diacetate
DCFH-DA	2′,7′–dichlorodihydrofluorescein diacetate acetyl ester
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDX	energy-dispersive X-ray
EFSA	European Food Safety Authority
EndoIII	endonuclease III
epCC	epithelial co-cultures
ESR	electron spin resonance
FBS	foetal bovine serum
FDA	US Food and Drug Administration
FEG-SEM	field-emission-gun SEM
Fpg	formamidopyrimidine-DNA glycosylase
FT-IR	Fourier Transform Infrared
H_2O_2	hydrogen peroxide
HPMC	hydroxypropyl methylcellulose
Hprt	hypoxanthine-guanine phosphoribosyl transferase
ICP-MS	inductively coupled plasma mass spectrometry
IP	intraperitoneal
IV	intravenous
JRC	Joint Research Centre
LC	lethal concentration
LD	laser diffraction
LDH	lactate dehydrogenase
LDV	laser Doppler velocimetry
MDA	malondialdehyde
mES cells	mouse embryo stem cells
MMS	methyl methanesulfonate
MN	micronucleus/micronuclei
MP	microparticle
NB	nuclear bud
nm	nanometres
μm	micrometre
NO	nitric oxide
npSCOPE	nanoparticle-scope
NRU	neutral red uptake
NTA	nanoparticle tracking analysis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP	nanoparticle
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate buffered saline
PCE	polychromatic erythrocyte
PCS	photon correlation spectroscopy
PHA	phytohaemagglutinin
RBC	red blood cell
RICC	relative increase in cell count
ROS	reactive oxygen species
SEM	scanning electron microscope
SHE	Syrian hamster embryo
SOD	superoxide dismutase
spICP-MS	single particle ICP-MS
TDMA	Titanium Dioxide Manufacturers Association
TEER	Transepithelial/transendothelial electrical resistance
TEM	transmission electron microscopy
TG	test guideline
TI	tail intensity
TM	tail moment
UV	ultraviolet
WoE	weight of evidence
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate
XRD	X-ray diffraction

and the relative contributions of different molecular mechanisms elicited by TiO_2 particles are not known. Consequently, a non-thresholded mode of action (MoA) cannot be ruled out. In addition, a cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified. EFSA concluded that it was not possible to set an acceptable daily intake (ADI), and the use of E171 was no longer considered safe as a food additive.

A detailed review of the genotoxicity of titanium dioxide (TiO_2) was subsequently published by Kirkland et al. (2022). In that review a different approach to that used by EFSA was employed. A comprehensive weight of evidence (WoE) approach was used in which test systems and endpoints were allocated different levels of relevance. Consistent with the recommendations of OECD (2017), studies investigating permanent genetic changes (gene mutations, micronuclei, chromosomal aberrations) were given more weight than DNA damage endpoints (e.g. comet assay), which may be lethal or reversible. In addition, the hierarchical nature of testing schemes implies that *in vivo* results with evidence of tissue exposure should carry more weight than *in vitro* results. Each relevant dataset was therefore reviewed in detail for robustness of study design, appropriate and acceptable negative and positive controls, and the quality and plausibility of the data. Of the 192 relevant datasets identified, only 34 met the reliability and quality criteria for being most relevant in the evaluation of genotoxicity. It was concluded that the existing evidence did not support a direct DNA damaging mechanism for TiO_2.

Since the Kirkland et al. (2022) review a number of regulatory opinions have been published, and are summarised below:

- The Japanese Ministry of Health, Labour and Welfare of Japan (MHLW) published a report (https://www.mhlw.go.jp/content/11130500/001125207.pdf) confirming the safety of titanium dioxide (TiO_2) as a food additive. This was based on 3 key points:
  - The results from an independent 90-day study (Akagi et al., 2023) carried out by the Japanese National Institute of Health Sciences (NIHS) that shows extremely low absorption and no adverse effects from the oral ingestion of TiO_2. This was undertaken to address the concerns raised by the European Food Safety Authority (EFSA) in their opinion from May 6, 2021.
  - A literature review that revealed that since 2021 no new genotoxicity studies had been identified that would raise concerns for the genotoxicity of TiO_2.
  - The consistency in the opinions published by Health Canada (HC, 2022), Food Standards Australia New Zealand (FSANZ, 2022), the US Food and Drug Administration (FDA, 2024) and the interim

findings of the United Kingdom's Food Standards Agency (FSA, 2022) which do not support the conclusions of the EFSA.

- The World Health Organisation (WHO) Joint Expert Committee on Food Additives (JECFA), after reviewing research from all available sources, concluded that previously raised safety concerns for E171 (also known as INS 171) are not supported by the available evidence, which was reinforced by the very low oral absorption rate and absence of any identifiable hazards (JECFA, 2023). JECFA also evaluated human dietary exposure to TiO_2, estimating the maximum 95th percentile to be 10 mg/kg bw/day, which was used for the risk evaluation of E171 in the diet. They noted that "*Although there were uncertainties in the genotoxicity data, the Committee took into account the fact that INS 171 was not carcinogenic in adequately conducted 2-year studies in mice and rats at doses of up to* 7500 mg/kg *bw per day for mice and* 2500 mg/kg *bw per day for rats, the highest doses tested.*" It should be noted that JECFA independently reviewed the genotoxicity studies for relevance, robustness, and reliability. In addition, JECFA recommended more research to address the current uncertainty about the distribution of TiO_2 particle sizes in food and to develop genotoxicity tests that are more appropriate for nanoparticles.
- The EU Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) published an Opinion on the safety of titanium dioxide in toys (SCHEER, 2023) and concluded that "*considering there are several uncertainties regarding the genotoxicity of TiO_2 particles (existence of both positive and negative results with different endpoints, varying quality of the studies in terms of the characterisation of the TiO_2 used in studies) the WoE for genotoxicity is weak to moderate. For inhalation exposure, the WoE of genotoxic hazard of TiO_2 is moderate, based on the high quality, but relatively low consistency of the results. However, for oral exposure, the WoE of genotoxic hazard of TiO_2 is weak.*" They also commented "*it can be observed that in studies on TiO_2 in nanosize the results (mainly in vitro studies) show higher probability of positive response than in studies on microsize or with sizes slightly above* 100 nm. *It is possible that a probability of a genotoxic effect diminishes as the size of TiO_2 increases, and the observed positive effects can depend on the presence of a nanofraction. The potential genotoxicity of pigmentary fine TiO_2, including the demonstration of the absence of a nanofraction, remains uncertain. Overall, based on the results of in vitro and in vivo genotoxicity studies, the SCHEER is of the opinion that the pigmentary fine TiO_2 grades can be considered to have no genotoxic potential after oral and inhalation exposure, provided the presence of a nanofraction can be excluded*". It should be noted that SCHEER considered many *in vitro* DNA damage (notably comet) studies to be relevant, which Kirkland et al. (2022) did not, and they also claimed evidence for uptake into the nucleus of cells and direct binding of TiO_2 with DNA in several *in vitro* studies and in two *in vivo* studies for ultrafine TiO_2 particles.
- The EU Scientific Committee on Consumer Safety (SCCS) published Scientific Advice on Titanium Dioxide (SCCS, 2024) and concluded that whist the previous SCCS Opinions on dermally applied cosmetic products remain unchanged "*the available evidence is not sufficient to exclude the genotoxicity potential of almost all of the types of TiO_2 grades used in oral cosmetic products. The only exception are two nano grades (RM09 and RM11) for which the provided genotoxicity data indicate no genotoxicity concern. More information is, however, needed on the potential uptake and cellular effects of the nano grades in the oral mucosa to consider them safe for use in oral-care products.*" Also, "*More experimental data are needed from studies carried out under valid protocols and appropriate testing guidelines to exclude the genotoxicity potential of the selected representatives of the other grades of TiO_2 (both pigmentary and nano) used in oral cosmetic products.*"

Many of the publications referred to in these opinions were reviewed by Kirkland et al. (2022). However, within these opinions, numerous papers were cited that described *in vitro* DNA damage (mainly comet) studies that were not considered of sufficient relevance, in terms of endpoint or test system, to contribute to the overall assessment of genotoxic activity and so were not reviewed in Kirkland et al. (2022). Also some papers were cited in these opinions discussing mode of action and cellular uptake that were not discussed in Kirkland et al. (2022). In addition, some papers describing relevant genotoxicity studies were cited in these opinions that were not identified at the time of the Kirkland et al. (2022) review presumably because different search criteria were used to support these opinions. Finally, a number of papers have been published since the Kirkland et al. (2022) review was completed, additional publications were cited in the above regulatory opinions but not previously identified or reviewed, or have been identified as a result of using extended search criteria. This paper therefore considers these publications and the impact they have on the overall assessment of the genotoxic potential of TiO_2.

1.1. Data sources

As mentioned above, the SCHEER (2023) and SCCS (2024) opinions cited many publications describing *in vitro* DNA damage assays. These publications have been identified and have now been reviewed.

Mode of action studies were cited in the SCHEER (2023) opinion. These publications have been identified and have now been reviewed.

Cellular uptake studies were cited in the SCCS (2024) opinion. These publications have been identified and have now been reviewed.

Also in the SCHEER and SCCS opinions some publications were cited describing more relevant genotoxicity endpoints (gene mutations, micronuclei, chromosomal aberrations) which had not been found at the time of the Kirkland et al. (2022) review. This is presumably because the search criteria used for the SCHEER and SCCS opinions, and described therein, were different from those used for the Kirkland et al. (2022) review, described in Table S1 in that publication. These papers have now been reviewed.

Papers describing genotoxicity tests with TiO_2 but published since the literature searches for the review of Kirkland et al. (2022) were closed in 2021 have been located during regular monitoring of journals by EBRC Consulting GmbH (Hannover, Germany) using PubMed, as well as STN International (which was used previously for Kirkland et al., 2022). The search criteria are described in supplementary Table S1 and Table S2 respectively. The publications found during the searches completed between 2021 and the end of February 2024 have now been reviewed.

In total 63 publications are reviewed herein. Most were not previously reviewed at all in Kirkland et al. (2022), but in a few of the 63 publications some relevant datasets (gene mutations, micronuclei, chromosomal aberrations *in vitro* and *in vivo*, and comets *in vivo*) were previously reviewed. These have been noted in the Annexes. In this paper some of the publications are discussed under more than one category since several different properties were investigated.

2. Methods

In the 2022 publication of Kirkland et al. only those endpoints with a default weighting of "moderate" or "high" were reviewed in detail. This amounted to 192 datasets within the various publications and study reports. The remaining 145 datasets (with default "low" or "negligible" weightings) were not reviewed, a "low" or "negligible" default weighted study did not contribute meaningfully to the assessment of genotoxic or carcinogenic hazard.

In this analysis, such a weighting was performed and presented in the data tables but no studies were excluded and all identified publications were reviewed, this allowed a critical analysis of the data used in some of the more recent regulatory opinions to be evaluated. There were *in vitro* comet publications as well as mode of action studies and cellular uptake studies that were included. Each dataset was given an initial weighting according to the criteria in Kirkland et al., (2022), but then the "weights" (for both positive and negative studies) were adjusted (if

necessary) according to the reliability of the study and the quality of the data. Examples of the questions to be considered include, but are not limited to, source of TiO_2 being tested, experimental design and "closeness" to OECD guidance (where appropriate), coding of slides, cytotoxicity measurement, statistical evaluation of data, use of historical control ranges, evidence of tissue exposure, inclusion of positive controls and other pertinent details that could help determine the "robustness" of a study. Data tables differ slightly in format from the Kirkland et al., (2022) publication as the publications themselves did not contain data from standard genotoxicity studies.

Since multiple experts were working across several different endpoints, consistency was addressed by peer review by at least 2 of the listed authors. In some cases, reliability scores and WoE assessments were changed. Thus, by internal peer review and discussion it was possible to achieve a high level of consistency.

3. Results

3.1. General observations

Throughout the publications reviewed there were many variables:

- Many different types of TiO_2 particles were tested (anatase, rutile, coated, uncoated), of different sizes ranging from nanoparticles6-100 nm) to microparticles (>1 μm).
- Not all particles were characterised on receipt to verify their authenticity.
- Suspensions of particles were prepared in different ways. Some used wetting and/or stabilising agents, and some used sonication (mainly bath sonication but in a few studies the authors used a probe).
- Particle size distributions in stock suspensions and/or culture media were determined in some studies but not all, and often using different techniques.
- Many different cell types were used in the *in vitro* tests, ranging from primary cells (e.g. Syrian hamster embryo) to established cell lines, some of which are p53-deficient and therefore susceptible to misleading positive genotoxic responses (Fowler et al., 2012).
- Concentrations of particles tested varied from very low (e.g. 10 μg/mL) to very high (e.g. 1000 μg/mL) and therefore in some cases the OECD recommended upper limit for nanoparticles of 100 μg/mL (OECD, 2022) was exceeded.
- Treatment times were variable ranging from short (e.g. 1 hr.) to long (e.g. 72 hrs.).

With such a heterogenous mixture of particles tested, preparation methods, cell types, concentrations, and treatment times, it is difficult to draw any conclusions regarding the impact of type or size of particle, or treatment conditions, on genotoxic activity.

3.2. In vitro DNA damage assays

Although there are numerous publications on *in vitro* damage assays with TiO_2, we have focussed only on the 27 publications investigating DNA damage *in vitro* that were identified from the SCHEER and SCCS opinions, plus 2 papers (Vignard et al., 2023; Ferrante et al., 2023) that were identified from the recent literature searches described above. These are reviewed in detail in Annex 1 (Supplementary file) and summarised in Table 1. Ten of these papers contained data on cellular uptake (reviewed in Annex 3 and summarised in Table 3) and/or other genotoxicity data (reviewed in Annex 4 and summarised in Table 4A)

It can be seen from Table 1 and Annex 1 that within these studies there were many deficiencies and limitations that raised questions regarding the robustness, relevance, and reliability of the results. For example:

- In almost all studies it was unclear whether slides were coded before analysis and therefore scorer "bias" cannot be excluded.
- In most studies it was unclear whether treated cultures and/or slides were protected from light and therefore photocatalytic activation to produce reactive oxygen species cannot be excluded.
- In most studies severely damaged cells (hedgehogs) were not reported so it is not known whether they were included in or excluded from calculations of tail intensity or tail moment.

In summary, all of the DNA damage studies, including some of those reporting negative outcomes, have limitations which means reaching a clear conclusion is difficult. Reported positive results cannot be confirmed because of the deficiencies, in particular lack of coding of slides, lack of protection from light and the failure to report on severely damaged cells and whether they were included in or excluded from tail intensity calculations. Some comets may be due to oxidative damage. The most robust study with the fewest limitations (Jalili et al., 2018) was negative for DNA damage at high concentrations.

3.3. Mode of action studies

From the SCHEER and SCCS opinions, and from literature searches described above, there were 7 publications investigating DNA binding/interaction *in vitro*, 2 publications investigating DNA binding/interaction *in vivo*, and 1 publication each investigating direct formation of reactive oxygen species (ROS) leading to mitochondrial dysfunction, downregulation of some genes involved in DNA repair pathways, and interaction with proteins involved in the control of chromosome segregation. These are reviewed in detail in Annex 2 (Supplementary file) and all except 2 review papers, which do not contain new data (summarised below), are summarised in Table 2.

The review by Braakhuis et al. (2021) discusses mode of action data for inhalation and oral exposures to TiO_2 via two adverse outcome pathway (AOP) approaches that focus on ROS generation leading to oxidative stress, and persistent inflammation leading to proliferation of epithelial tissue and preneoplastic lesions. The 2 pathways are similar in terms of ROS generation and oxidative stress leading to persistent inflammation and eventual injury to target cells. They postulate several key events (KEs) that may be related to mechanisms that drive toxicity through exposures delivered via the inhalation route that have been linked to tumorigenicity. An additional key event has been postulated which is direct damage of DNA in gut epithelial cells.

The majority of the KEs from the inhalation AOP have little corresponding evidence in the AOP via the oral route. It is likely that common factors such as generation of ROS, oxidative stress and inflammation may lead to similar concerns via the oral route however there are critical differences between oral and inhalation exposures, not least the lack of overload via the oral route where excess TiO_2 will pass through the gut without being absorbed.

In the review by Magdolenova et al. (2014), the authors performed a meta-analysis of *in vitro* and *in vivo* genotoxicity studies with engineered nanoparticles. From a search of 4346 articles in the open literature on NP toxicity there were 112 publications with genotoxicity data. In terms of MoA, 1 paper (Huang et al., 2009) discusses interactions with proteins involved in the control of chromosome segregation and the spindle apparatus. They showed *in vitro* that TiO_2 NPs interfered with mitosis causing multipolar spindles. However, treatment of NIH 3T3 and NHW cells was for 13 weeks, which is incredibly long to culture immortalised cells such as these, and the possibility of genomic "drift" cannot be excluded. Furthermore, there were seemingly no time-matched negative controls. There is no clear evidence of intracellular TiO_2 exposure via TEM. There were increases in ROS that would be expected with NP's that have a large surface area.

Of the MoA publications reviewed there is a lack of evidence for many of them being biologically relevant. TiO_2 can bind to naked DNA via intercalation (see Annex 2, Supplementary file), however there is no

Table 1
In vitro DNA damage assays.

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Gurr et al. (2005)	Comet *in vitro* (±Fpg)	Anatase TiO_2 (10 nm), sourced from Yang, Taipei (not independently characterised) Anatase TiO_2 (20 nm), sourced from Yang, Taipei (not independently characterised) Anatase TiO_2 (≥200 nm), purchased from Sigma–Aldrich (not independently characterised) Rutile TiO_2 (200 nm) purchased from Kanto Chem (not independently characterised). Anatase TiO_2 (200 nm) purchased from Kanto Chem (not independently characterised).	TiO_2 was "sterilised" by heating and suspended in PBS, but no mention of dispersion methods.	BEAS-2B cell line single conc. 10 μg/mL for 1 h ±Fpg	Positive (at least 2-fold increases) but data not clear. Charts are stacked bars and difficult to read.	• Single concentration tested for only 1 h. • Data only presented graphically – unclear responses. • Some experimental conditions e.g. electrophoresis are unclear. • Unclear whether slides coded. • Number of cells scored unclear. • No positive controls • Hedgehogs not scored. • Background seems high in negative controls. • Malonaldehyde (MDA), nitric oxide (NO) and H_2O_2 were also measured. All particles except (Sigma) Anatase (200 nm) gave greater positive response with Fpg. • 10 and 20 nm Anatase samples induced MDA, NO and H_2O_2	Evidence of increases in DNA strand breakage but data uninterpretable for all types of TiO_2 tested for the reasons stated.	Low
Demir et al. (2015)	Comet *in vitro* (±Fpg)	Non-ultrafine (microparticulate) TiO_2 purchased from Sigma. Not characterised and no details given. Nano TiO_2 (21 nm), purchased from Sigma. TEM used to confirm morphology and mean size. Nano TiO_2 (50 nm) purchased from MK Impex Corp. TEM used to confirm morphology and mean size.	Nanogenotox protocol followed (water containing 0.05% BSA and ultrasonication). DLS and LDV used to characterise formulated samples.	HEK293 and NIH/3T3 cell lines, treated for 1 h. Concentrations unclear, since 1, 10 and 100 μg/mL and 10, 100 and 1000 μg/mL are both mentioned.	Negative for microparticulate form. positive for NP's at top concentration but unclear what this is. No sig. difference with Fpg	• Nanogenotox dispersion protocol followed. • Cells treated for 1 h, 200 scored/ dose. • No concurrent cytotoxicity measures. • Concentrations tested are unclear could be 100 or 1000 μg/mL top conc. • No mention of hedgehogs • Unclear whether cultures for standard comet were protected from light, although slides treated with Fpg were kept in the dark or red light.	Negative for microparticulate form but uninterpretable for both TiO_2 NPs.	Low

(*continued on next page*)

Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Brown et al. (2019)	Comet *in vitro* (±Fpg) Toxtracker•	E171 TiO_2 purchased on-line but not independently characterised.	TiO_2 dispersed in water with 2% FBS and sonicated. Characterisation of formulated samples by DLS and Zeta potential	Caco-2, HepG2 and mES (ToxTracker) cell lines. 3–5 concentrations tested ± Fpg for 4 h.	E171 induced significant increases in comets in mES cells at highest concentration only but not in HepG2 and Caco-2. Positive when data from different cell lines is pooled.	• Only 100 cells scored for comets. • Coding of slides unclear. • No hedgehogs scored/reported. • Data pooled across treatment groups. • For ToxTracker no reporters induced but glutathione levels reduced in HepG2 and Caco-2 cells. • Strand breaks expressed as lesions per 10^6 bases. • No historical range • Inconsistent results across cell lines • Cytotoxicity/ viability measured but after 24 h when treatment was only for 4 h.	Comet induction noted but considered inconclusive due to issues noted No increases noted in any of the reporters tested	Low Moderate
Karlsson et al. (2009)	Comet *in vitro* (±Fpg)	Nano TiO_2 (20–100 nm) obtained from Sigma-Aldrich. Bulk material characterised by TEM. Microparticle TiO_2 (0.3–1 μm) obtained from Sigma-Aldrich. Bulk material characterised by TEM.	TiO_2 suspended in DMEM containing 10% FBS and sonicated. DLS used to characterise formulations.	A549 cell line ± Fpg tested at 40 and 80 μg/mL for 4 h.	Nano TiO_2 induced positive responses in % Tail Intensity (TI; >2 fold for NP and >3 fold for microparticles at the highest concentrations.	• No positive controls • Only 70 cells scored for comets. • Hedgehogs/ clouds not scored or mentioned. • Unclear whether cells were protected from light. • Unclear whether slides were coded. • Toxicity not concurrent (trypan blue at 18 hours) • No difference with Fpg treatment	Positive with limitations	Low
Zijno et al. (2020)	Comet *in vitro* (±Fpg)	Non ultrafine TiO_2 NM100 (100 nm dia) obtained from JRC Nanomaterials Repository, which does not require independent characterisation. Nano TiO_2 NM101 (6 nm dia) obtained from JRC Nanomaterials Repository, which does not require	TiO_2 dispersed in water containing 0.05% BSA and sonicated according to Nanogenotox protocol. DLS used to characterise samples once formulated.	BEAS-2B cell line tested at 0.1, 1,10 and 100 μg/mL for 24 h ±Fpg	NM100 and NM101 increased %TI to approximately 4x controls in a dose dependant manner. Increase in IL-6 only for NM101. Fpg treatments also resulted in significant increases in % tail DNA	• Hedgehogs not measured. • Cytotoxicity via trypan blue exclusion • % TI low in solvents (1%) • 100 cells/ concentration scored for comets. • Unsure whether slides were coded prior to scoring.	Positive with limitations	Low

(*continued on next page*)

Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		independent characterisation.						
Kermanizadeh et al. (2012, 2013)	Comet *in vitro* (±Fpg)	NM 101 – (Anatase uncoated) two sizes 4–8 and 50–100 nm. obtained from JRC Nanomaterials Repository. NRCWE001 – (Rutile uncoated) 80–400 nm obtained from JRC Nanomaterials Repository. NRCWE002 – (Rutile with positively charged coating) 80–400 nm obtained from JRC Nanomaterials Repository NRCWE003 – (Rutile with negatively charged coating) 80–400 nm obtained from JRC Nanomaterials Repository. NRCWE004 – (rutile, coating not mentioned) 5 types ranging from 1 to 4 to 1000–2000 nm obtained from JRC Nanomaterials Repository	TiO_2 dispersed in MilliQ water and sonicated. XRD and TEM used to characterise bulk samples. DLS used once formulated.	In the 2012 publication C3A (human hepatoma cells). 2013 publication used HK-2 (human renal proximal tubule epithelium cells). 4-hr. treatments ± Fpg. Concentrations tested were fractions of the LC_{20} (2xLC_{20}, LC_{20}, 0.5x LC_{20}	Positive in C3A cells ± Fpg. Positive in HK-2 ±Fpg. Positive in C3A cells ± Fpg• Positive in C3A cells ± Fpg. Positive in HK-2 cells ± Fpg• Negative in C3A cells ± Fpg• Positive in C3A cells ± Fpg•	• Cytotoxicity (LC_{20}) measured via WST-1 proliferation in HK-2 cells, unknown for C3A cells. • Hedgehogs not measured. • For C3A %TI less than 5% in negative controls but higher and more variable in HK-2 • Cells may not have been protected from light. • Unclear whether slides were coded prior to scoring. • Results across the 2 studies are inconsistent. • Inflammatory markers induced in C3A and HK-2 cells.	Positive with limitations for all except NRCWE003.	Low
Hamzeh and Sunahara (2013)	Comet *in vitro*	MT15 (anatase nano, 5.9 nm) obtained commercially. Not independently characterised. P25 (anatase/ rutile, 34.1 nm) obtained commercially. Not independently characterised). Nanofilament (rutile, 15.5 nm) obtained commercially. Not independently characterised. Hombitan LW-S (anatase, 169.4 nm) obtained commercially. Similar to E171. Not independently characterised. Viva Nano Titania (rutile, 1–10 nm, coated) obtained commercially. Not independently characterised.	TiO_2 suspended in culture medium with serum and sonicated. DLS used to characterise formulated samples. All particles agglomerated resulting in particle sizes from 365 to 600 nm.	V79 cell line (Chinese hamster, p53 deficient), 24-hr. exposures with 10 and 100 μg/mL ROS measured via DCFDA	%Tail Intensity (TI) and Tail Moment (TM) were significantly increased with MT15, P25 and nanofilament, TM was significantly increased with Hombitan. ROS was significantly induced by MT15, P25 and nanofilament, to a lesser extent with Hombitan and Viva Nano	• Toxicity measured via MTT • Positive controls H_2O_2 and Allosperse-A (polymer control) • % TI and TM were measured • Slides may not have been coded or protected from light • Hedgehogs not reported • V79 are p53 deficient and susceptible to misleading positive results • Coated NP's less toxic and negative for comets possibly due to larger agglomerates.	Positive with limitations (MT15, P25). Negative with limitations (Nanofilament, Hombitan and Viva Nano.)	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Di Bucchianico et al. (2017)	Comet *in vitro* (±Fpg)	NM100 (anatase, uncoated, 20–28 nm), NM101 (anatase, coated, 5–8 nm).and NM103 (rutile coated, 20–28 nm) obtained from JRC Nanomaterials Repository.	TiO_2 suspended in water containing 0.05% BSA and sonicated. DLS used to characterise samples once formulated, sizes of agglomerates between 224 and 531 nm.	BEAS-2B cells treated for 3 and 24 h with 1, 5 and 15 μg/mL TiO_2 ±Fpg. Some 24-hr. cultures also exposed to UV light (3 min at 65 $\mu W/cm^2$)	Negative (%Ti) in standard comet assay. Fpg treatment resulted in significant increases however, error bars overlapped with controls so considered unreliable to draw conclusions on oxidised bases. UV irradiation gave clear increases in %TI.	• Cytotoxicity assessed using Alamar Blue (Resazurin), very little toxicity seen. • Hedgehogs not mentioned or measured. • Higher concentrations could have been tested. • Slides may not have been coded prior to scoring. • UV irradiated slides showed large increases and the photo reactivity of TiO_2. • Apoptosis and necrosis measured by microscopy and flow cytometry	Negative with limitations in the standard comet assay.	Low
Murugadoss et al. (2020)	Comet *in vitro*	NM1020a (17 nm) and NM10200a (117 nm) obtained from the JRC Nanomaterials Repository.	Dispersion using modified method of Guiot and Spalla (2013). pH adjusted prior to addition of BSA and sonication. TEM and DLS used to characterise samples once formulated.	Human Bronchial Epithelium (HBE) and Caco-2 cells treated for 24 h with 5, 25, 50 and 100 μg/mL THP-1 (monocytic leukaemia cell line) treated with large and small agglomerations of the above.	Significant increases in %TI in at least one concentration tested from all materials.	• Around 50% of agglomerates settle out over 24 h. • Hedgehogs not scored • Cytotoxicity estimated from LDH measurements • Data only presented as graphs • Negative controls seem to be zero for %ITI • No historical data on any cell line • Comet methodology not well described • Positive control data absent • Unclear whether slides were coded	Increases in % TI noted at least at one concentration for all materials, however data is considered uninterpretable for the reasons discussed	Low
Murugadoss et al. (2021)	Comet *in vitro*	NM1020a (17 nm) obtained from the JRC Nanomaterials Repository. NM10200a (117 nm) obtained from the JRC Nanomaterials Repository.	TiO_2 dispersed using a method in which pH was adjusted to reduce agglomeration. TEM and DLS used to characterise samples once formulated.	Human Bronchial Epithelium (HBE) treated for 4 h to aerosols of TiO_2 HBE's grown and exposed to aerosols on transwells.	Negative Positive•	• Details on characterisation, formulation and pH adjustment as per Murugadoss et al. (2020). • Cytotoxicity measured with LDH and WST-1 assay • Glutathione levels measured • Unclear whether cultures or slides were protected from light • Unclear	Negative with limitations Positive with questionable biological relevance for the reasons discussed	Low Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						whether slides were coded • Positive responses only where significant membrane damage was induced • Hedgehogs not measured		
Proquin et al. (2017)	Comet *in vitro*	E171 – food grade TiO_2 donated by Sensient Technologies Co. Characterised by SEM. TiO_2 microparticles (average 535 nm) were custom made by PlasmaChem. Characterised by TEM and SEM. TiO_2 NP's (10–25 nm) were purchased from Io-Li-Tech. Characterised by SEM.	TiO_2 dispersed in DMEM containing 0.05% BSA or McCoys 5a medium containing 10% FBS and then sonicated. DLS was used to characterise formulations.	Caco-2 cell line treated with 0.143 μg/cm^2 for all TiO_2 types and 1.43 μg/cm^2 for NP's for 24 h. ROS measured using ESR, significant ROS induced but not in Caco-2 cells	Positive	• Concentrations given as μg/cm^2 • Toxicity estimated with Trypan Blue • H_2O_2 included as positive control (only treated for 30 min) • Increases in % TI noted (3-fold) but comet assay methods not described • ROS measured via electron spin resonance (ESR) • Hedgehogs not measured • Comet assay conditions not described • Caco-2 are p53 null • Unclear whether slides were coded	Increases in strand breaks noted however methods not well described so considered Unreliable/ Uninterpretable	Low
Guichard et al. (2012)	Comet *in vitro*	Anatase nano TiO_2 (14 nm) purchased from Sigma-Aldrich. Samples characterised by TEM. Rutile nano TiO_2 (62 × 10 nm) purchased from Sigma-Aldrich. Samples characterised by TEM. Anatase micro TiO_2 (160 nm) purchased from Sigma-Aldrich. Samples characterised by TEM. Rutile Micro TiO_2 (530 nm) purchased from Sigma-Aldrich. Samples characterised by TEM.• P25 TiO_2 (25 nm) purchased from Evonik-Degussa. Samples characterised by	Particles suspended in DMEM with 20% FBS and sonicated., once formulated DLS and LD used to characterise formulation.	SHE cells (primary hamster cells) treated with 10, 25 and 50 μg/cm^2 TiO_2 for 24 h protected from light. Intracellular ROS measured by DCFDA at lower concentrations (1, 5 and 10 μg/cm^2 after 72 h exposure.	Positive – increases in intracellular ROS noted Negative– increases in intracellular ROS noted• Positive– increases in intracellular ROS noted•	• RICC used to estimate toxicity however unsure if this was applied correctly • Cytotoxicity may have been from separate experiments • Hedgehogs not measured • Unclear whether slides were coded	Induction of comets in all samples other than rutile nano TiO_2 considered to be of questionable biological relevance due to induction of ROS under all conditions.	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		TEM.•						
Falck et al., (2009)	Comet *in vitro*	Nano rutile TiO_2 (coated with <5% SiO2) 10 × 40 nm, purchased from Sigma-Aldrich. Morphology confirmed by TEM but not size. Nano anatase (<25 nm), purchased from Sigma-Aldrich. Morphology confirmed by TEM but not size. Fine rutile (<5 μm), purchased from Sigma-Aldrich. Morphology confirmed by TEM but not size.	TiO_2 suspended in BEGM culture media and sonicated. TEM used to characterise samples before and after formulation.	BEAS 2B cell line treated for 24, 48 and 72 h with 1,5,10,20, 40, 60, 80, 100 μg/cm^2 TiO_2 (100 μg/cm^2 is equivalent to 360 μg/mL)	Positive Positive• Positive•	• Concentrations chosen from previous toxicity experiment using Trypan Blue • %TI values in controls were variable (3–10%) • Results for all particle types were inconsistent and variable • Slides coded • Hedgehogs not measured • Primary particle size not checked • Unclear whether cultures were protected from light	Increases in comets noted however data considered uninterpretable due to reasons noted.	Low
Andreoli et al., (2018)	Comet *in vitro*	Anatase TiO_2 NP (20–60 nm), rutile TiO_2 NP (30 × 100 nm), anatase/ rutile NP mix (45–262 nm), anatase TiO_2 microparticles (50–270 nm) and rutile TiO_2 microparticles (50–3000 nm). All particles purchased from Sigma-Aldrich and characterised by TEM.	TiO_2 dispersed in MilliQ water or RPMI containing 15% FBS and sonicated. SEM used to characterise formulated samples.	Human peripheral blood mononuclear cells treated with 10, 50, 100, 200 μg/ mL TiO_2 for 24 h. 8-oxodG levels measured using HPLC in cells treated for 6 or 24 h.	Positive, significant increases in %TI compared to controls, and significant increases in ROS	• Cultures kept in the dark • Cytotoxicity estimated by Trypan Blue – none seen • % TI in controls were very low • Unsure whether slides were coded prior to scoring • Hedgehogs were identified but unclear whether they were included in %TI analysis.	Inconclusive – increases in %TI noted but could be due to ROS	Low
Stoccoro et al. (2017)	Comet *in vitro* (±EndoIII and Fpg)	Citrate coated TiO_2 NPs prepared by the authors. Not independently characterised. Silica coated TiO_2 NPs prepared by the authors. Not independently characterised. Uncoated TiO_2 NPs obtained from Colorobbia Italia. Not independently characterised. P25 TiO_2. Source not specified. Not independently characterised.	TiO_2 sonicated then added to 0.05% BSA in PBS. TEM used to characterise samples once formulated.	A549 (lung epithelial) cell line treated with TiO_2 types for 48 h at concs. of 10, 20 and 40 μg/cm^2 (32, 64 and 128 μg/mL) including EndoIII and Fpg. Apoptosis and necrosis also measured	Weakly positive (2-3-fold) Negative• Weakly positive (2-3-fold)• Positive•	• Apoptosis and necrosis measured – levels as high as comet responses • Slides may not have been coded prior to scoring • Hedgehogs not reported • Primary DNA damage could have been due to oxidised pyrimidines, apoptosis or both. • Similar responses seen from 2016 study in Balb/3T3 cells with ethe same materials but different concentrations and treatment times.	Questionable biological relevance – comet increases could have been DNA fragmentation from apoptosis/ necrosis	Low
Dorier et al. (2019)	Comet *in vitro* (±Fpg)	E171 Food grade TiO_2, obtained from a French food	TiO_2 sonicated in water for 30 min immediately	Caco-2 co-cultured with HT29-MTX	Negative	• Viability assessed in separate	Negative with limitations	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		supplier and characterised as per Dorier et al. (2017). A12 Anatase TiO_2 (12 nm) Synthesised in authors lab, characterised as per Dorier et al. (2017).	prior to treatment. Samples were characterised in Dorier et al. (2017)	(mucus secreting cells) in a 70:30 ratio treated with a single 50 μg/mL concentration of TiO_2 for 24 h with and without Fpg.	Negative• NM105 (anatase/rutile 24 nm) obtained from the JRC Nanomaterials Repository but characterised as per Dorier et al. (2017).	experiment using MTT up to 200 μg/mL for 6 and 48 h – no toxicity observed • Hedgehogs not scored • Higher concentrations up to recommended 100 μg/mL could have been tested • 8-oxodG measured by HPLC – no increases • Double strand breaks measured by p53 foci – no increases. • Intracellular ROS measured by DCFH-DA increases noted from all TiO_2 treated cultures. • Unclear whether slides were coded prior to scoring • Unclear whether cultures and slides were protected from light		
Negative•								
Vignard et al. (2023)	Comet *in vitro* H2AX phosphorylation	E171 Food grade TiO_2. obtained from a French on-line food supplier. Characterisation was by SEM (performed on same sample in previous publications). Anatase TiO_2 NP (21 nm) obtained from Sigma -Aldrich. Not independently characterised. NM102 (115 nm) obtained from the JRC Nanomaterials Repository.	TiO_2 sonicated in ultrapure water (on ice) at 40% amplitude for 1 min. DLS used to characterise formulated samples.	TR146 cell line (human head/ neck cancer cell line with defective p53) treated with 5, 50 and 100 μg/mL of each particle type for 2 h (with 72 h recovery)	Positive for induction of comets, Induced ROS and γH2AX markers Negative – no ROS or γH2AX• Positive for induction of comets, Induced ROS and γH2AX markers•	• Cytotoxicity estimated using trypan blue – toxicity observed but less than 50% • Treatments were only for 2 h. • TEM and confocal microscopy showed agglomerated TiO_2 particles in the cytoplasm • γH2AX and 53BP1 measured as well as ROS via the CellROX green reagent. • Cultures and slides not protected from light • Slides may not have been coded prior to scoring	Questionable biological relevance - increases in DNA strand breaks noted but may be secondary to ROS	Low
Ferrante et al. (2023)	Comet *in vitro*	Rutile TiO_2 NP's (60 nm) obtained from Nanovision (Italy). Not independently characterised.	TiO_2 weighed into vials, suspended in cell culture media and sonicated (final concentration	HCT116 (human colon adenocarcinoma) and Caco-2 cell lines treated with TiO_2 from 0.1 to	Positive in both cell lines	• Cytotoxicity was measured by MTT assay – 50% toxicity seen at 14.29 μg/mL for NP's and 1.88 μg/mL for E171	Increases in strand breaks noted but considered unreliable due to reasons noted.	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		E171 Food grade TiO_2 obtained from an Italian food supplier. Not independently characterised.	1000 μg/mL). spICP-MS used to characterise samples once formulated but not done in final treatment medium.	500 μg/mL for 72 h.	Positive in both cell lines•	in Caco-2 cells. 41.13 and 3.447 μg/mL in HCT116 cells • Positive for comets in both cell lines, higher magnitude of response in HCT116 • Western blot analysis showed no apoptotic markers from either cell line. • Caco-2 cells are p53 null and susceptible to misleading positive results • Particle size in final culture media not measured • Cultures and slides may not have been protected from light • No positive control or coding of slides • Hedgehogs not scored		
Gea et al. (2019)	Comet *in vitro* (±Fpg)	P25 TiO_2 (20 ± 5 nm) obtained from Evonik-Degussa. Characterised by SEM Food grade TiO_2 (similar to E171) 150 ± 50 nm obtained from Faravelli Group. Characterised by SEM TiO_2 Bipyramids (50 ± 9 nm) synthesised by authors. Characterised by SEM TiO_2 Rods (108 ± 47 nm) synthesised by authors. Characterised by SEM TiO_2 Platelets (75 ± 25 nm) synthesised by authors. Characterised by SEM	TiO_2 dispersed in MilliQ water containing 1% DMSO and sonicated. Formulated samples were characterised using DLS.	BEAS-2B cell line treated with TiO_2 for 1 h under laboratory light followed by 23 h in darkness. Food grade and platelet forms were also tested for 24 h in darkness. With and without Fpg.	Positive with Fpg only Positive ± Fpg response greater with Fpg• Negative• Negative• Positive ± Fpg•	• Cytotoxicity assessed with LDH release and WST-1 cell proliferation reagent – little or no cytotoxicity induced. • Unclear whether slides were coded prior to scoring • Hedgehogs not scored • Greater comet responses with Fpg suggest a mode of action based on ROS.	Positive with limitations. May be due to oxidative damage.	Low
Kang et al. (2011)	Comet *in vitro*. MN *in vitro* summarised in Table 4A.	P25 TiO_2 – 20 nm purchased from Degussa. Not independently characterised.	TiO_2 suspended in sterile PBS and sonicated for 30 min (conditions not stated). No characterisation performed on	Human peripheral lymphocytes (PHA stimulated for 24 h) treated with 1 or 5 μg/mL TiO_2 for 24 h in the presence and	Positive for comet induction, greater magnitude of response with UV. However, positive MN	• Period and conditions of UV irradiation unclear • 5 μg/mL TiO_2 was below recommended maximum so	Comet assay uninterpretable due to lack of detail on methodology and assay timings.	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			formulated samples.	absence of UV light.	response only seen in the presence of UV (see Table 4A).	higher concs. could have been tested • No positive control • Toxicity measured with Trypan blue – little/no toxicity observed • Apoptosis and mitochondrial depolarization measured – no effects noted • Lack of detail on methodology • Tail moment rather than %TI used • Slides not coded prior to scoring • Hedgehogs not measured. • MN test also performed – only significant increases noted in P25 with UV light which could have been due to ROS or apoptosis		
		"normal" TiO_2 – 99% rutile, 1 μm pigmentory grade purchased from Sigma-Aldrich. Not independently characterised.			Negative for comet induction, and negative for MN in presence and absence of UV (see Table 4A).•		Comet assay uninterpretable due to lack of detail on methodology and assay timings.	Low
Petkovic et al. (2011)	Comet *in vitro* (±Fpg)	TiO_2-A (anatase 18 nm) purchased from Sigma-Aldrich. Characterised using FEG-SEM, and TEM TiO_2-B (anatase 105 nm) purchased from Sigma-Aldrich. Similar in size to E171. Characterised using FEG-SEM, and TEM	TiO_2 dispersed in PBS followed by 30 min sonication in a water bath. Formulated samples were characterised using XRD and measurement of Zeta potential.	HepG2 cells treated with unirradiated or pre-irradiated TiO_2 for 2, 4 and 24 h with 1, 10, 100 and 250 μg/mL of each TiO_2 type ± Fpg.	Positive for comets; greater response with pre-irradiated TiO_2. All responses greater with Fpg Positive for comets at higher concentrations but likely not biologically relevant. Greater response with pre-irradiated TiO_2. All responses greater with Fpg•	• MTT assay used to estimate toxicity ~40% noted at highest concentrations. • Testing concentration limit for NP's exceeded recommended maximum. • Slides not coded prior to scoring. • Hedgehogs not scored	Equivocal for both non-irradiated samples. Positive with limitations for pre-irradiated TiO_2-A. Weak positive for pre-irradiated TiO_2-B. Comets are likely due to oxidative damage or dead/dying cells.	Low
Jalili et al. (2018)	Comet *in vitro* (±Fpg) γH2AX foci MN *in vitro* summarised in Table 4A	NM103 – Rutile, hydrophobic, 25 nm, obtained from the JRC Nanomaterials Repository.	Nanogenotox dispersion followed, TiO_2 wetted in ethanol (0,5%) then suspended in 0.05% BSA in water before probe sonication on ice. Formulated samples characterised by DLS	Caco-2 and HepaRG cell lines treated with between 1 and 80 μg/cm^2 (equivalent to >250 μg/mL) for 3 or 24 h ± Fpg	Negative for comet. No γH2AX increases. Also no significant increases in MN (see Table 4A).	• Nanogenotox protocol used for dispersion. • Cytotoxicity estimated using neutral red uptake (NRU) – no toxicity observed; cell counts also confirm no toxicity. • Cellular uptake confirmed by TEM. • H2AX endpoint measured – no significant response.	Negative for comet and γH2AX endpoints.	Low
		NM104 – Rutile hydrophilic, 25 nm, obtained from the JRC Nanomaterials Repository.			Negative for comet. No γH2AX increases. Also no significant increases in MN (see Table 4A).•		Negative for comet and γH2AX endpoints.	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						• ROS measured using CellROX deep red reagent at 3 hours exposure – no significant ROS noted. • Slides were coded. • Hedgehogs were counted		
Vila et al. (2018)	Comet *in vitro* (±Fpg) Monolayer integrity, permeability, penetration, cellular uptake also measured (see Annex 3 and Table 3)	NM100 obtained from the JRC Nanomaterials Repository	Nanogenotox protocol used. Particles showed spherical morphology with a mean size of 104.01 nm. After dilution into Dulbecco's modified Eagle's high glucose medium without pyruvate containing 10% FBS for cell treatment, some aggregates or agglomerates seen, and DLS showed mean diameter of 244.70 nm.	Differentiated Caco-2 cells treated for 24 h, with 10, 25 and 100 μg/mL.	Negative for comets both with and without Fpg.	• Nanogenotox protocol used for dispersion. • No cytotoxicity induced up to 100 μg/mL. • Cell treatments were apparently not protected from light, which could be a confounding factor. • Slides not coded before scoring.	Negative with limitations	Low
Nakagawa et al. (1997)	Comet assay *in vitro* (±UV)	P-25 (anatase, 21 nm) was obtained from Nippon-Aerosil, WA (anatase, 255 nm) and WR (rutile, 255 nm) were obtained from Wako, and TP-3 (rutile, 420 nm) was obtained from Fuji Titan. They were not characterised by the authors.	Not described.	L5178Y mouse lymphoma cells. P-25 from 3.1 to 800 or 200 μg/mL (-and + UV respectively), For the other 3 particle types ranged from 50 to 3000 μg/mL (±UV). Treatment was for 24 h either in the dark or following 50 min exposure to 5J/cm^2 UV light.	P-25 most cytotoxic -UV (50% at 800 μg/mL) but no increase in comets. Only WA induced comets -UV. WR was not cytotoxic and did not induce comets. P-25, WA and TP-3 induced comets + UV but in the presence of significant cytotoxicity.	• Particles not characterised on receipt. • Method of dispersion not described. • OECD recommended upper concentration limit exceeded. • Only 50 cells/ treatment scored for comets. • Comets assessed by tail length instead of recommended % tail DNA or tail moment. • Slides not coded before analysis. • No measurement of ROS which might be responsible for the comet responses + UV.	Very difficult to interpret due to many limitations.	Low
Kumar et al. (2020)	Comet assay *in vitro*	Nano- and bulk-TiO_2 obtained from Sigma-Aldrich. Unclear whether supplied particles were characterised. Nano-TiO_2 could	Particles suspended in PBS at 1 mg/mL and then probe sonicated. Diluted in DMEM/F12 medium containing 10%	Human mammary epithelial (MCF-7) cells treated for 12 h with concs, ranging from 1 to 100 μg/mL	Positive for comets at 100 μg/mL for both particles. Nano-TiO_2 also positive at 10 μg/mL. However, significant	• Unclear whether characterisation of nano-TiO_2 by DLS and SEM was for supplied particles, suspensions in PBS or	Positive with serious limitations. DNA damage probably due to oxidative stress or dead/dying cells.	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		be either 56.08 (from SEM images) or 107.3 nm (DLS), but no size detail for bulk-TiO_2.	FBS for cell treatment. Unclear whether suspensions in PBS or medium were characterised.		induction of ROS and apoptosis at all concs. showing induction of comets	suspensions in culture medium. • No characterisation data for bulk-TiO_2. • No positive control included. • Concentrations inducing >30% cytotoxicity should have been excluded. • Not stated how many cells were scored. • Not stated that slides were coded before analysis. • Unclear whether severely damaged (hedgehogs) cells were counted, and whether they were included in or excluded from calculations of comet area and % tail DNA.		
Barillet et al. (2010)	Comet *in vitro* γH2AX foci Cytotoxicity, ROS induction & cellular uptake also investigated.	TiO_2-CEA NPs synthesised by the authors by laser pyrolysis and annealed under air at 400 °C for 3 h to remove free carbon impurities. TiO_2–P25 (Aeroxide P25, 75% anatase) obtained from Degussa AG. TiO_2-Sigma and TiO_2-Sigma-R (637262, 100% rutile; ≤100 nm) obtained from Sigma-Aldrich. Size and shape were characterised by TEM. Particles were loosely agglomerated (confirmed by photon correlation spectroscopy). Other characteristics were determined by XRD. However, unclear whether these data were from a previous publication.	Stock suspensions prepared in ultrapure sterile water at 10 mg/mL and dispersed by sonication for 30 min at 4 °C using a pulsed mode. Stock suspensions were further diluted for treatment in DMEM culture medium. In medium NPs agglomerated as 200–400 nm clusters except for TiO_2-Sigma particles which agglomerated to >3 μm clusters – data from a previous publication.	A549 human lung carcinoma cells, NRK-52E rat kidney proximal cells, LLC-PK1 pig kidney proximal cells, WIF-B9 rat hepatoma/human fibroblast hybrid cells and Can-10 rat hepatoma cells. Only NRK-52E cells used to study comets (only with TiO_2-CEA) and γH2AX foci (TiO_2-CEA, P25 & TiO_2-Sigma) Concs. tested for both endpoints were 50–200 μg/mL. Treatment was for 24 h.	Dose-dependent increase in tail moment with TiO_2-CEA. No induction of γH2AX foci with any of the 3 NPs tested yet all 3 NPs induced ROS. Cellular uptake reported for all NPs in 3 cell types but results only for TiO_2-CEA in NRK-52E cells (24 h) or A549 cells (48 h) NPs isolated or localised in cytoplasmic vesicles. NPs "rarely observed in cell nuclei" but no images of nuclear uptake presented.	• Unclear if characterisation of NPs in DMEM was done for this study or based upon previous results. • MTT assay showed little cytotoxicity but can underestimate ability of treated cells to proliferate. • Comparison of cytotoxicity between cell lines only performed at single conc. • No positive or negative control data for the comet assay. • Unclear whether treated cultures were protected from light • Unclear how many cells were scored for γ-H2AX foci. • Unclear whether slides were coded before scoring. • Severely damaged cells (hedgehogs) not	Uninterpretable	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						reported, so unknown if they were included or excluded from tail moment calculations. • Comet response may be due to ROS generation, but no correlation between ROS, cytotoxicity or comet responses. • Details on cellular uptake lacking or conflicting; no evidence of nuclear uptake was presented. • The chemical composition of the internalised particles was not confirmed. • Poor presentation conflicting methods & results make interpretation difficult.		
Brzicoba et al. (2019)	Comet *in vitro* (±Fpg) Cytotoxicity, ROS induction & cellular uptake also investigated.	NM-100 (190 nm) obtained from the JRC Nanomaterials Repository	Stock dispersions prepared at a conc. of 2.56 mg/mL according to the Nanogenotox protocol. Sonicated dispersions were diluted to the required concs. in RPMI1640 culture medium containing 10% FBS and vortexed for 10 s immediately before treatment. Particle size distribution and Zeta potential determined by DLS. In water & culture medium average sizes increased to 235 & 254 nm respectively indicating aggregation & agglomeration.	THP-1 human monocytic leukaemia cells differentiated into macrophage-like cells before treatment. 24 h treatment with 1, 10 & 25 μg/mL	No cytotoxicity up to 100 μg/mL. Cellular uptake into cytoplasmic vesicles. None reported in the nucleus. No induction of ROS. No significant induction of comets either + or – Fpg. No effects on immune response markers, cell cytokines, growth factors or surface markers.	• The MTS assay can underestimate cytotoxicity and the ability of treated cells to proliferate. • The chemical composition of the internalised particles was not confirmed. • Unclear whether slides were coded before scoring. • Unclear whether treated cultures and/or slides were protected from light. • Severely damaged cells (hedgehogs) were not reported so not known whether they were included in or excluded from calculations of DNA damage lesions. • Higher concentrations should have been tested.	Negative with some limitations	Low

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Table 1 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Toyooka et al. (2012)	γH2AX foci Cytotoxicity, ROS induction and cellular uptake also investigated.	TiO_2 nanoparticles (NPs; <5 nm) obtained from Sigma-Aldrich and microparticles (MPs; <5000 nM) obtained from Wako Pure Chemicals. They were not independently characterised.	Particles were suspended in DMEM at a conc. of 20 mg/mL and sonicated for 1 min in a bath sonicator. BSA-coated NPs were also prepared at 20 mg/mL in 5 mg BSA/mL, sonicated for 1 min and washed three times in distilled H_2O. These suspensions were diluted to 10 μg/mL in DMEM and the size distribution was determined using a Zetasizer Nano. Size distributions in DMEM were 250–650 nm (average of 378 nm) for the NPs, and 600–1050 nm (average of 773 nm) for the MPs. BSA-coated NPs showed a downward shift in average particle size to 280 nm, respectively.	A549 human lung carcinoma cells. Treatment was for 0.5–48 h (depending on several different methods to identify γH2AX induction) with concs. ranging from 1 to 1000 μg/mL, clearly exceeding OECD recommended upper limit.	Dose-dependent increases in γ-H2AX for NPs (1–1000 μg/mL) and MPs (≥75 μg/mL) by Western blotting. Not cell cycle dependent but proportional to cellular uptake. BSA-coated NPs induced less γH2AX. NPs and MPs induced slight (<20%) but significant cytotoxicity only at ≥500 μg/mL. Cellular uptake (1-hr. treatment) shown by flow cytometric analysis using forward and side scatter, and was greater for NPs than MPs. Uptake reduced by BSA coating. ROS induction was slight (1.5-fold). Particles shown to adsorb to naked DNA. N-acetyl cysteine inhibited DNA double strand breaks induced by H_2O_2, but not TiO_2 NPs, suggesting γ-H2AX generation by TiO_2 NPs is independent of ROS formation.	• No characterisation of the primary particles was reported. • There was no positive control for most of the assays. • Unclear whether cultures were protected from light. • Measurement of side scatter by flow cytometry may not identify whether TiO_2 particles are inside or outside the cells, or their location if inside. • Adsorption of TiO_2 particles to naked DNA is not biologically relevant. • Most of the treatments were far above the concentration limit recommended by OECD (100 μg/mL) and not biologically relevant.	Uninterpretable	Low

convincing evidence of intranuclear exposure to TiO_2 particles. While it has been suggested that TiO_2 particles could theoretically interact with the genetic material during mitosis when the nuclear membrane disappears, one would have to postulate that all of the particles also leave the nucleus before the nuclear membrane reforms. Otherwise TiO_2 particles would be found inside the nucleus of interphase cells, and as noted above, there is no convincing evidence of this. It is also unlikely that particles could access DNA in its supercoiled state during mitosis to achieve intercalation. Therefore, while the possibility of binding to cellular DNA exists, it is not biologically plausible without definitive proof of intranuclear exposure. Downregulation of genes involved in DNA repair is unlikely to occur as the methodology in Pogribna et al. (2020) is flawed, and changes in gene regulation can also be a consequence of general toxicity or hypoxia for adherent cell lines. For the same reasons stated above (lack of evidence of intranuclear exposure) this is also biologically implausible. Interaction with chromosome segregation machinery was seen where *in vitro* cell cultures had been grown for an extended period of time to the point at which they probably suffered spontaneous genetic drift. The study of Huang et al. (2009), which is the only relevant MoA study in the meta-analysis of Magdolenova et al. (2014), was methodologically flawed and therefore unreliable. At up to 7 weeks of exposure to TiO_2 there were no signs of interference with cell division machinery.

In summary, there is no clear evidence of direct interaction of TiO_2 with intranuclear DNA, or with non-DNA targets such as mitochondria or the cell division apparatus. The most biologically plausible MoA demonstrated by a large amount of data is generation of ROS. Increased ROS leads to indirect formation of DNA damage and a nonlinear response. The generation of ROS from TiO_2 and other large surface nanoparticles is well documented. On careful review of the data, ROS generation could be considered as the only MoA for TiO_2 exposure that is supported by robust data.

Table 2
Mode of action (MOA) studies including *in vitro* and *in vivo* studies on DNA interactions.

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Ali et al. (2018)	DNA binding (calf thymus DNA)	TiO_2 NP's (Sigma-Aldrich). No independent characterisation of bulk material.	TiO_2 formulated in water & sonicated for 15 min at 40 W. Formulated samples were characterised using XRD and TEM	Acellular – 15 μM naked ctDNA treated with 0.62–6.26 μM TiO_2 NP's	TiO_2 binds to naked ctDNA intercalating into the minor groove	• Binding of NP to ctDNA measured spectrophotometrically • Binding of TiO_2 to ctDNA possibly due to electrostatic effects • Molecular docking analysis showed DNA minor groove binding by TiO_2 • Acellular system with naked DNA unclear whether TiO_2 would penetrate the mammalian cell and enter the nucleus.	Limited biological relevance due to artificiality of test system	Low
Patel et al. (2016)	DNA binding (human DNA extracted from peripheral blood)	TiO_2 NP's (Sigma-Aldrich). No independent characterisation of bulk material.	Dispersion not stated. Limited characterisation including density and activation energy measurements.	Acellular – 13.71 μg naked human DNA extracted from peripheral blood leukocytes treated with 0–200 μM TiO_2 NP's	TiO_2 binds to naked human DNA via intercalation	• Hyperchromicity noted, indicative of TiO_2 binding to DNA • Fluorescent quenching added evidence that TiO_2 intercalates DNA	Limited biological relevance due to artificiality of test system	Low
Patel et al. (2017)	DNA binding (human DNA extracted from peripheral blood) Fluorescent displacement.	TiO_2 NP's (Sigma-Aldrich, India) anatase/ rutile mix 20.25 nm. Characterised by XRD.	TiO_2 suspended in deionized water and sonicated for 10 min. XRD used to characterise bulk material, DLS used to characterise formulated samples (particle size in water 255–650 nm)	Acellular – Naked human DNA (peripheral blood leucocytes) fixed concentration (not stated) tested with 24.8–744.68 μM TiO_2	TiO_2 binds to naked human DNA via intercalation	• Displacement of ethidium bromide by TiO_2 indicative of intercalation into DNA	Limited biological relevance due to artificiality of test system	Low
Hekmat et al. (2013)	DNA binding (ctDNA) fluorescent displacement	TiO_2 NP's (Nano Pars Lima Co.) <100 nm. No independent characterisation performed.	TiO_2 formulated in sterile water and sonicated (conditions not stated). Samples stored at 4 °C prior to use.	Acellular – Naked ctDNA (fixed concentration of 4 mMb) tested with 0–80 μM TiO_2 with/without doxorubicin (0–146 μM). MCF7 and T47D breast cancer cells treated with different concs. of TiO_2 and doxorubicin.	TiO_2 binds to ctDNA via intercalation. Synergy between TiO_2 and doxorubicin on cancer cell line growth.	• TiO_2 tested with naked ctDNA with doxorubicin – synergistic effect. • MCF7 and T47D breast cancer cell growth (measured using MTT) reduced by a combination of UV photo-activated TiO_2 and doxorubicin. Non-UV-irradiated TiO_2 had no effect on cell growth. • No evidence of cellular uptake in MCF7 or T47D cells	Limited biological relevance – artificial test system, effects on cell growth only seen when TiO_2 is photo activated	Low
Hekmat et al. (2020)	DNA binding (ctDNA) and growth inhibition of cancer cell line	TiO_2 NP's (Sigma-Aldrich). No independent characterisation performed.	TiO_2 formulated in sterile water and sonicated for 10 min Stored at 4 °C prior to use.	Acellular – ctDNA (8.32 μM) exposed to TiO_2 (3.1–46.5 μM) with and without paclitaxel. Cancer cell line MDA-MB-231 exposed to TiO_2 with and without paclitaxel	Binding of TiO_2 to DNA, synergistic effect with paclitaxel. Growth inhibition of cancer cell line with TiO_2; also synergy with paclitaxel on growth inhibition.	• Naked DNA used for binding experiments, limited biological relevance. • Synergistic effect on DNA binding with TiO_2 and paclitaxel. • MDA-MB-231 growth inhibition with TiO_2 and paclitaxel using MTT showed a synergistic response in growth reduction.	Limited biological relevance – artificial test system, effects on cell growth do not include any cellular uptake measurement.	Low

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Table 2 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						• No evidence of cellular uptake (or nuclear uptake) • No link between DNA binding and cellular growth inhibition.		
Zhu et al. (2007)	DNA binding (ctDNA and plasmid DNA)	TiO_2 NP's (Academy for Sciences, China). No characterisation performed.	TiO_2 dispersed in ultra-pure water and ultrasonically dispersed (conditions not stated). Limited TEM characterisation at 100 nm and 10 μm resolutions once formulated and mixed with plasmid and ctDNA.	Acellular – ctDNA and plasmid DNA exposed to 400 μg/mL TiO_2	Evidence of TiO_2 binding to ctDNA and plasmid DNA via phosphate groups on DNA	• TEM used to "visualise" TiO_2 bound to ctDNA – difficult to determine any evidence of single particles or binding rather than association. • IR spectroscopy showed shifts in bands indicative of increasing complexity and DNA binding to TiO_2 • Binding of TiO_2 to phosphate groups of DNA postulated as a mechanism for ROS release.	Limited biological relevance due to artificiality of test system. TEM unlikely to show DNA binding at resolutions used.	Low
Zhang et al. (2014)	Adsorption of DNA oligonucleotides by TiO_2	TiO_2 NP's (Sigma-Aldrich) 20 nm. Characterised by TEM and XRD (confirmed anatase form).	No dispersion protocols used. DLS used to characterise formulated samples, Agglomerates found in water ~300 nm.	Acellular – DNA samples (integrated DNA technologies) i.e. poly A, poly G.	Adsorption of DNA onto TiO_2 is possible, particularly at low (unphysiological) pH but unlikely to occur *in vivo* due to the length of time needed to bind (~90 min)	• Homonucleotides exposed to TiO_2, binding stronger at lower pH possibly due to a positive charge on TiO_2 compared to the negative charge on DNA. • TiO_2 also showed strong binding affinity to double stranded DNA (constructed from single stranded DNA probes). • All experiments conducted with naked, artificially generated DNA.	No biological relevance – adsorption to DNA is unlikely to occur under physiological conditions.	Low
Li et al. (2010)	DNA binding *in vivo*	TiO_2 - prepared in house, size of bulk powder 5 nm. No independent characterisation was performed.	TiO_2 was suspended in HPMC (0.5%), ultrasonically treated for 30 min and vibrated for 5 min.	CD-1 mice dosed i.p. once a day for 14 days with 5, 10, 50, 100 and 150 mg/kg TiO_2. Ti bound to DNA measured	Dose related increases in TiO_2 bound to liver DNA from 50 μg/day and above.	• Unclear whether TiO2 levels controlled in experiment, contamination from equipment and materials cannot be excluded. • Liver weights increased in dose-related manner • No data from TiO_2 in blood or plasma from treated animals. • TiO_2 could be bound to surface of the liver and carried through during processing. • Peritoneal inflammation not evaluated, large agglomerates of TiO_2 could have caused secondary issues	Uninterpretable – high chance of contamination of samples from TiO_2 carried over during necropsy.	Low
Jin et al. (2013)	DNA binding *in vivo*	TiO_2 – Nano anatase <25 nm (Sigma-Aldrich, USA). Characterised by XRD. TiO_2 – Micro rutile <5 μm (Sigma-Aldrich, USA).	No dispersion details given. TEM used to characterise formulated samples.	SD rats dosed intranasally every day for 45 days with a total of 300 μg TiO_2 per individual. Ti bound to liver DNA measured	TiO_2 bound to DNA No interaction with DNA•	• TiO_2 samples characterised by XRD • Single dose of each type of TiO_2 tested (300 μg total) • Contamination of TiO_2 from experimental procedures could not be excluded • TEM of formulated TiO_2 in water showed no	Uninterpretable – high chance of contamination of samples. No direct evidence of covalent binding of TiO_2 to DNA.	Low

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Table 2 (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/ Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		Characterised by XRD. TiO_2 – 5–10% rutile/90–95% anatase mix <10 nm, (Sigma-Aldrich, USA). Characterised by XRD.			TiO_2 bound to DNA•	visible free particles • Unclear whether TiO_2 levels controlled in experiment, contamination from equipment and materials cannot be excluded.		
Pogribna et al. (2020)	Downregulation of DNA repair pathway genes *in vitro*	Aeroxide P25 TiO_2 (Evonik, USA). No independent characterisation was performed on bulk samples.	TiO_2 formulated in pure water and sonicated (conditions not stated). Formulated samples characterised by DLS.	Caco-2, HepG2, NL20 and A-431 cells treated with 100 μg/mL for 24 or 72 h.	Intracellular interactions with TiO_2 from microscopy. Increases in ROS-related stress gene networks.	• Toxicity measured by MTT – no data presented • Dark field microscopy and hyperspectral imaging to visualise TiO_2 associated with cells • Global DNA methylation evaluated; TiO_2 contamination of DNA cannot be excluded • Methylation of 22 gene promoter regions showed methylation of stress-related genes possibly due to hypoxia at 72 h exposures or ROS.	Uninterpretable -low resolution of light microscopy unlikely to be able to visualise TiO_2 in cellular compartments. Contamination likely from experimental procedures	Low

3.4. Cellular and nuclear uptake studies

Seventeen studies investigating cellular and nuclear uptake have been reviewed. Detailed reviews are presented in Annex 3 (Supplementary file) and summarised in Table 3. Some of these papers contained DNA damage data reviewed in Annex 1 and summarised in Tables 1 and 2 contained additional genotoxicity data reviewed in Annex 4 and summarised in Table 4A. Many of the papers contain images of poor resolution and, together with the failure to use EDX to confirm chemical composition, have serious deficiencies. The 2 most robust papers (Stoccoro et al., 2017; Vignard et al., 2023) present convincing evidence of uptake of TiO_2 particles into the cytoplasm of cells exposed *in vitro* but no evidence of nuclear uptake.

3.5. Recent or newly identified publications

Papers published since the review of Kirkland et al. (2022) or older publications that have been identified as a result of extending the searches to include PubMed as well as STN International, are reviewed in detail in Annex 4 (Supplementary file). All except 2 papers, one of which describes both *in vitro* and *in vivo* data and the other is a review which does not contain new data (summarised below), are summarised in Tables 4A, 4B and 4C.

One publication described both *in vitro* and *in vivo* investigations. Kämpfer et al. (2021) examined and compared cytotoxicity, proinflammatory response, and DNA damage induced by polyvinylpyrrolidone-capped silver (AgPVP) and TiO_2 (P25) NPs in four *in vitro* models: Caco-2 and HT29-MTX-E12 monolayer cultures as well as long-term transwell triple cultures ± THP1 macrophages to reproduce healthy versus inflamed human intestinal tissue. These results were compared to the *in vivo* responses to the same NPs in the intestinal tissue of mice in 28-day oral dosing studies.

The TiO_2 NPs and dose formulations were well characterised. Non-proliferating epithelial co-cultures (epCC) were found to be highly comparable to murine intestinal tissue. These epCC were reported to be more resistant to the adverse effects of TiO_2 NPs as compared to proliferating Caco-2 and E12 monocultures. Inclusion of immunocompetent cells and ongoing inflammatory processes did not significantly affect sensitivity.

However, TiO_2 NPs apparently only had significant effects on transepithelial/transendothelial electrical resistance (TEER) in inflamed triple cultures (which were transient), %Tail DNA in Caco-2 monocultures using a 4-hr exposure, and %Tail DNA in inflamed triple cultures using repeated exposures (which represented a *decrease* in DNA damage).

There were no significant effects on any other test system or endpoint measured, including on WST-1 staining in Caco-2 and E12 monocultures; on TEER in epCC or stable triple cultures; on LDH release in Caco-2 and E12 monocultures, epCC, and stable and inflamed triple cultures; on IL8 release in E12 monocultures and epCC; on IL6, TNFα or IL8 release in stable or inflamed triple cultures; on %Tail DNA in E12 monocultures, in epCC, in stable and inflamed triple cultures using a single 24 h treatment, in stable triple cultures using repeated exposures, and in colon cells following 28 days of oral dosing; or on expression of DNA damage genes, an oxidative stress marker, or proinflammatory cytokines in ileal tissue. Thus, the results were almost exclusively negative. However, information was conflicting and often sparse or missing, recommended study designs for the comet assay were not followed, and recommended controls were not included. The results of Kämpfer et al. are therefore of limited reliability and questionable relevance.

Also in addition to the publications summarised in Tables 4A, 4B and 4C a new review was found. Cao et al. (2023) performed an extensive search of the literature (PubMed, web of science, EFSA and China national knowledge infrastructure) searching for data on TiO_2 (published prior to June 30, 2022) using keywords including "genotoxicity", "mutation", chromosome", "gene" and "DNA" as well as other relevant key words associated with TiO_2, nanoparticles and genotoxicity. In total 7916 articles were identified once duplicates were excluded. These studies were filtered on their title and abstract by relevance, removing a further 7588 using a Klimisch-based system the authors termed "toxicological data reliability assessment method (TRAM)" which included

Table 3
Cellular uptake studies.

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Jain et al. (2017)	Flow cytometry (intensity of side- and forward-scatter) & TEM	Titanium dioxide (TiO_2), Nanopowder purchased from Sigma (anatase <25 nm, purity-99.7%). Size & morphology confirmed by TEM.	Dispersed in DMEM containing 10% FBS by probe sonication. DLS showed agglomerates of 176.2 ± 4.9 nm. Zeta potential in culture medium was −12.7 ± 1.7 mV.	V79 cells were treated in the absence of S9 for: • 6 h with 1–100 μg/mLfor flow cytometry analysis • 2, 4, 6 and 24 h with a single dose of 25 μg/mL.for TEM analysis	TiO_2 nanoparticles are internalised by the cells and located in nuclei	• Flow cytometry-based analysis is not appropriate for evaluating cellular uptake; it can be confounded by optical properties of the particles & only provides information on cell association (not internalisation). • EDX was not performed on TEM images; elemental analysis was evaluated on a different instrument (SEM with EDX capability). • Resolution of the TEM images is insufficient. • No indication of number of cells analysed per sample. • In the single image provided to suggest nuclear uptake, the particles are not sufficiently electron-dense to be TiO2 & has not been confirmed as such by EDX. • Also in this image, the large particle agglomerate appears to be adjacent to the nucleus and is not within the nuclear region.	Particles have potentially entered the cell cytoplasm, but this has not been confirmed by EDX. There is insufficient evidence for nuclear uptake.	Low
Kazimirova et al. (2020)	TEM	NM105 (anatase/ rutile, 15–60 nm) obtained from JRC Nanomaterials Repository.	2 different dispersion protocols: 1) Mixing with 10% FBS in PBS in a glass tube, then probe sonication for 15 min. 2) Suspended in DMEM with 15 mM HEPES buffer (no FBS), then probe sonicated for 10s, aliquoted and stored at −20 °C. Aliquots	V79 cells were treated in the absence of S9 for 24 h at 3, 10 & 30 μg/cm^2	TiO_2 agglomerates were located in the cytoplasm & vesicles. They were also detected in contact with the nucleus. Uptake was not dependent on the dispersion protocol.	• EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • Resolution of the TEM images are insufficient; all images presented were small and vesicles were not clearly visible, thus it is difficult	Particles have potentially entered the cell cytoplasm, but this has not been confirmed by EDX. There is insufficient evidence for nuclear contact and no evidence of nuclear uptake.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			of TiO_2 NP suspensions were thawed, vortexed for 10 s, sonicated again and added to DMEM. DLS showed varying agglomeration for each method: 1) 228 nm at the start of cell treatment and 154 nm after 24 h. 2) 184 nm at the start of cell treatment and 217 nm after 24 h			to assess quality. • Particles are not evidently in the same focal plane as the cells. • Low resolution of images did not provide conclusive evidence of nuclear contact. • No indication of number of cells analysed per sample.		
Stoccoro et al. (2017)	TEM with EDX for elemental analysis; 10 ultrafine slice cells analysed	1. TiO_2 NP nanosuspension (84% anatase, 16% brookite crystal phase) from Colorobbia Italia SpA. The particle size was not stated and was not checked. 2. Citrate and silica coated particles, prepared in the authors' laboratory. 3. P25 - the size was not stated or checked.	Stock solutions were bath-sonicated for 15 min; aliquots were added first to 0.05% BSA in PBS and then to the cultures directly. Suspensions in water or in cell culture medium (Ham's F12 with 10% FBS) were measured by DLS, and, except for P25, showed much higher mean diameters in culture medium than in water. In media uncoated, citrated, silicated & P25 had mean agglomerate size of 1608, 91.3, 563.2, 477 nm respectively.	A549 cells were treated in the absence of S9 with 20 μg/cm^2 of TiO_2 NP for 48 h.	Following 48 h exposure, all particles were found within the cells, P25 mainly as single particles and the other samples as agglomerates. The NPs were mostly located in lysosomes early and late endosomes. No free NP in the cytoplasm were observed.	• Good quality TEM images are presented. • TiO2 uptake is confirmed by EDX elemental analysis. • Analysis of only 10 cells per sample is low.	There is evidence of uptake of TiO_2 NPs into vesicles in the cytoplasm; there were no free NPs in the cytoplasm observed and no evidence of nuclear uptake.	High
Jugan et al. (2012)	TEM	1. Anatase-A12 and Rutile-R20, synthesised by authors. 2. P25 from Degussa. 3. Anatase-A140 and Rutile-R68, purchased from Sigma-Aldrich. Shape & primary particle size measured by TEM. Average diameters were 12 nm (A12), 24 nm (P25), 142 nm (A140), 21 nm (R20), and for R68 length was 68 nm & diameter 9 nm	Dispersed in water by pulse sonication; hydrodynamic diameter and Zeta potential were determined by photon correlation spectroscopy (PCS) and zetametry, but the results are not presented.	A549 cells were treated in the absence of S9 for 4 h with 50 μg/mL suspensions of the NPs	Accumulation of the smallest NPs (A12, A25 and R20) was observed in the cytoplasm; A12 was also observed in the nucleus. For the larger NPs, cytoplasmic accumulation was also observed, but NPs were not observed in the cell nucleus.	• Only two images were presented in the paper that were related to the A12 exposure alone. Representative images for all other TiO_2 exposures were not provided. • EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • Resolution of the TEM images is insufficient. • No indication of number of	Images representing only one particle exposure were presented, so it is not possible to conclude on uptake for all materials presented. For A12, there is no convincing evidence of cellular or nuclear uptake.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						cells analysed per sample. • Particles are not evidently in the same focal plane as the cells in both images presented. Regarding the image showing suspected nuclear uptake, the potential particles (unconfirmed by EDX) appear to be in a different focal plane.		
Uboldi et al. (2016)	ICP-MS analysis	Bulk anatase (BAN) and bulk rutile (BRU) TiO_2, and also 2 nanosized particles (anatase: AN-10 & rutile: RU-10) were synthesised by the authors. Suspensions in deionized water were characterised by DLS and TEM: hydrodynamic diameters were 333.8 nm (BAN), 734.2 nm (BRU), 51.4 nm (AN-10) & 134.4 nm (RU-10).	Suspensions were bath-sonicated (10 min) and then added to cell cultures. Centrifugal liquid sedimentation (CLS) was used to determine agglomerate hydrodynamic diameter in complete cell culture media after 24, 48 & 72 h incubation.	Balb/3T3 cells were treated in the absence of S9 for 72 h with 500 μg/mL suspensions of the NPs	The data indicate all particles were taken up by the cells, and in higher amounts for AN-10 and RU-10, as compared to their bulk counterparts. AN-10 was taken up as efficiently as RU-10 by Balb/3T3 mouse fibroblasts.	• ICP-MS is a quantitative analysis for cell association, accurately identifying the potential presence of the test material. However, ICP-MS does not discriminate between particles attached to the outside of cells vs. internalised. Thus, cell uptake cannot be confirmed by this method (only cell association). • Cellular uptake & sub-cellular location of any particles within the cells cannot be established. • The concentration was much higher and the exposure for much longer than was used for corresponding genotoxicity tests.	Analysis indicates TiO_2 particles are "associated" with the treated cells, but cellular uptake and sub-cellular localisation of the particles cannot be confirmed.	Moderate
Guichard et al. (2012)	TEM	Anatase (A) & rutile (R) nano & micro TiO_2 particles (Sigma-Aldrich), and P25 (Evonik-Degussa). Primary particle sizes checked by TEM: 14 nm (A-nano), 62 × 10 nm (R-nano), 160 nm (A-micro), 530 nm	Particles were suspended in SHE culture medium (DMEM with 20% FBS), sonicated for 20 min. Particle size was determined by DLS and laser diffraction (LD). All particles showed agglomeration in culture medium	Syrian hamster embryo (SHE) cells were treated with 1 μg/cm^2 particles for 24 h in the absence of S9, and protected from direct light exposure.	All particle types were internalised into the cytoplasm in the form of individual particles and agglomerates.	• Only 2 images are presented: one control cell and one P25-treated cell. Representative images for all other TiO2 exposures were not provided. • EDX was not performed on TEM images to confirm the	Images representing only one particle exposure were presented, so it is not possible to conclude on uptake for all materials presented. For P25, there is unconfirmed evidence of uptake into the	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		(R-micro) & 25 nm (P25).	with sizes ranging from 300 to 700 nm.			presence of Ti in the particle-like objects. • Resolution of the TEM images is insufficient. • No indication of number of cells analysed per sample.	cytoplasm and no evidence of nuclear uptake.	
Andreoli et al., (2018)	Flow Cytometry	TiO_2 NPs with different crystalline phases (anatase, rutile, anatase/rutile) & microparticles of anatase & rutile in the micron (MP) size range (Sigma-Aldrich). Primary particle sizes were characterised by TEM.	Particles were dispersed by ultrasonication for 45 min in RPMI medium with 15% FBS. Size distributions were determined by SEM.	Human peripheral blood lymphocytes were treated in the absence of S9 for 24 h with anatase NPs and MPs at 0, 10, 20, 50 and 100 μg/mL, and with rutile TiO_2 NPs & MPs and a mixture of anatase/rutile at 50 μg/mL only.	Both anatase TiO_2 NPs and MPs were predominantly taken up by monocytes. A significant dose-dependent increase in signal intensity was observed in monocytes, irrespective of the TiO_2 size. Similar results were seen with 50 μg/mL rutile & anatase/ rutile particles.	• Flow cytometry-based analysis is not appropriate for evaluating cellular uptake; it can be confounded by optical properties of the particles & only provides information on cell association. • Evaluation of particle interference was assessed & taken into consideration as part of the analysis, strengthening data quality. • Cellular uptake cannot be confirmed and there is no information on sub-cellular localisation.	Flow cytometry data provide some evidence of particles being associated with cells (particularly with monocytes), but cellular internalisation and sub-cellular localisation cannot be determined.	Low
Di Bucchianico et al. (2017)	Flow Cytometry	NM100 (anatase, uncoated, 50–150 nm), NM101 (anatase, coated, 5–8 nm) and NM103 (rutile, coated, 20–28 nm) were obtained from the JRC Nanomaterials Repository.	Particles were suspended in 0.05% BSA and probe sonicated for 15 min before dilution into BEGM culture medium. DLS showed agglomerate size at 0 & 24 h were: 347 and 456 nm (NM100), 531 and 430 nm (NM101), and 238 and 224 nm (NM103).	BEAS-2B cells were treated in the absence of S9 for 48 h with 1, 5, 15 or 30 μg/mL NPs	A linear dose-dependent increase in side scatter suggests cellular uptake of all tested particles. Both anatase TiO_2 NPs increased the side scatter to a higher extent compared to the rutile, suggesting a somewhat higher uptake of NM100 and NM101.	• Flow cytometry-based analysis is not appropriate for evaluating cellular uptake; it can be confounded by optical properties of the particles & only provides information on cell association. • Evaluation of particle interference was not presented and/or controlled for in the analysis. • The authors comment, these data are only "suggestive" of cellular uptake. • Cellular uptake cannot be confirmed and there is no	Flow cytometry data provide some evidence of particles potentially being associated with cells, but cellular internalisation and sub-cellular localisation cannot be determined.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						information on sub-cellular localisation.		
Murugadoss et al. (2020)	TEM	NM10202a and NM10200a were obtained from the JRC Nanomaterials Repository.	Two differently agglomerated suspensions of the same TiO_2 NPs were produced by adjusting pH of the solutions & probe sonication. Size characterisation was determined by DLS.	Human bronchial epithelial (HBE) cells were exposed to 50 μg/mL of small and large agglomerates of both particles for 24 h in absence of S9	Cellular internalisation was verified in HBE cells. Some TiO_2 agglomerates were observed close to the nucleus.	• EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • Resolution of the TEM images is insufficient; focus on cells/ particles is also not sufficient to determine if particles are in the same focal plane as the cells. • No indication of number of cells analysed per sample. • Possible drag marks in images suggest particles may have shifted from their original positions during the sectioning process.	There are unconfirmed indications of TiO_2 uptake into the cytoplasm of cells, but no evidence of nuclear uptake.	Low
Hackenberg et al. (2011)	TEM	Anatase NPs <25 nm (Sigma-Aldrich). Particle size was not confirmed.	Dispersion was performed according to Bihari et al. (2008); but several approaches were investigated in this paper & the one selected by Hackenberg et al. was not defined. Morphology, size and size distribution were determined by TEM; but no characterisation in cell culture media.	Human lymphocytes were treated for 24 h at 20, 50, 100 and 200 μg/mL in the absence of S9.	Transfer of NPs into the cytoplasm was low, seen in only 5 out of 100 cells, and mainly as aggregates up to 500 nm diameter. It is claimed that invasion into the nucleus was observed in 1 cell.	• 100 cells analysed per sample, which is a robust approach to the analysis. • EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • No image is provided to demonstrate nuclear uptake. • One low resolution image of cytoplasmic uptake is provided, but suspected particles in cells did not have the same/similar morphology as the stock material.	There is unconfirmed indication of low level TiO_2 uptake into the cytoplasm of cells, but no evidence of nuclear uptake.	Low
Ahlinder et al. (2013)	Raman spectroscopy and TEM	Anatase TiO_2 (Degussa). Primary size characterised by XRD (21 nm).	Dispersed by ultrasonication for 4 min in media before dilution in complete culture media. Characterisation of agglomeration	A549 cells were treated at 10 μg/mL for 4 & 48 h in the absence of S9.	Raman: NPs were internalised into cells, including the nucleus after 4 h exposure. The same number of NPs are located inside the nucleus	• Raman spectroscopy does not convincingly discriminate between internalised particles *versus*	There are unconfirmed indications of TiO_2 uptake into the cytoplasm of cells, but no convincing	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			in media determined by photon cross-correlation spectroscopy.		after 4 h of exposure (42%) as compared with 48 h of exposure. TEM: NPs located inside vacuoles & nuclei. The authors note that the discrepancy is apparently large between TEM & Raman mapping regarding the number of cells with nanoparticles in the cell nucleus.	those on the cell surface. • EDX was not performed on TEM images; thus, it is not possible to determine if suspected particles in nuclei are artefacts, other nanoparticle types also exposed to the cells, or TiO_2. • Resolution of the TEM images is insufficient; focus on cells/ particles is also not sufficient to determine if particles are in the same focal plane as the cells. • Only 30 cells analysed per sample by TEM, which is limited. • Authors claim the Raman technique shows 40% nuclear uptake, and yet they only indicate nuclear uptake occurs in 2 out of 30 cells by TEM (6.7%). • The authors note that damage to the cell samples can arise during the sectioning process, particularly when larger agglomerates are present; this is an important point raised by the authors and demonstrates careful evaluation of sample quality included in the analysis.	evidence of nuclear uptake.	
Louro et al. (2014)	TEM & histological analysis by light microscopy	Anatase 22 nm NM-102 was obtained from the JRC Nanomaterials Repository.	NP powder was prewetted in 0.5% ethanol, 0.05% serum albumin was added & sample was probe sonicated for 16 min on ice. Characterisation of the dispersion	Liver tissue from LacZ plasmid-based transgenic mice; NPs were injected IV via the tail vein at 10 and 15 mg/kg/day for two consecutive days.	TEM revealed electrodense deposits, which the authors state is consistent with the accumulation of NPs in the liver tissue of treated animals, both dispersed within	• Light microscopy is not of sufficient resolution to define NP uptake and/or sub-cellular localisation. • Nuclear uptake observations	There are unconfirmed indications of TiO_2 uptake into the cytoplasm of cells, but no convincing evidence of nuclear uptake.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			was not performed.		the cytoplasm and accumulated in mitochondria & lysosomes. Using light microscopy, the authors claim particles were found inside some of the hepatocytes nuclei in all mice exposed to either dose of NM, but without a clear dose-related effect.	were made by light microscopy, with low resolution (scale bar of 25 μm); thus it is not possible to determine whether particles were "on" rather than "in" the nucleus. It is also not possible to confirm the objects identified as NPs were indeed TiO_2 without high-resolution imaging and elemental analysis. • Whilst the authors suggest nuclear uptake by light microscopy, this was not observed in the TEM imaging. • EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • No indication of number of cells analysed per sample.		
El Yamani et al (2022)	TEM	Anatase NM-101 was obtained from the JRC Nanomaterials Repository.	2 different dispersion protocols: 1) Mixing with 10% FBS in PBS in a glass tube, then probe sonication for 15 min. 2) Nanogenotox protocol (Jensen et al., 2011). Confirmation of size distribution using nanoparticle tracking analysis (NTA) was carried out after NPs dispersion. in cell culture medium (before, during, and after the exposure)	V79 cells were treated in the absence of S9 for 24 h at 3, 10 and 30 μg/cm^2	TiO_2 NPs were taken up by the cells independently of the dispersion protocol used & concentration applied. The NPs were mostly aggregated; observed outside the cells attached to the cell membrane, inside the cell cytoplasm near the membrane (inside cytoplasmic vesicles, vacuoles, endosomes, or lysosome-like structures), or attached to the nuclear membrane, as well as inside the nucleus.	• EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • Resolution of the TEM images is insufficient; focus on cells/ particles is also not sufficient to determine if particles are in the same focal plane as the cells. This is particularly true of the one image presenting nuclear uptake, where the particles that are apparently inside the cells do not have the same density as those in the	There are unconfirmed indications of TiO_2 uptake into the cytoplasm of cells, but no convincing evidence of nuclear uptake.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						adjacent cytoplasmic area. Without EDX, the elemental composition of these articles within the images cannot be confirmed. • No indication of number of cells analysed per sample.		
Vignard et al. (2023)	Confocal microscopy, TEM, npSCOPE analysis	E171 (from a French on-line food supplier). 21 nm TiO_2 (Sigma-Aldrich). NM-102 was obtained from the JRC Nanomaterials Repository. E171 was characterised in a previous paper (Guillard et al., 2020); the 21 nm TiO_2 was not independently characterised by the authors.	For the *in vitro* experiments, TiO_2 samples were sonicated in ultrapure water in an ice bath for 1 min. DLS was used to evaluate size distribution after dispersion in cell culture media.	Human buccal TR146 cells *in vitro* and piglets *in vivo*. TR146 cells were exposed to 50 μg/mL of E171 for 1, 2, 5 and 24 h; or exposed to all test particles for 2 h (5, 50 or 100 μg/mL). 4-week old piglets: exposed to E171 water suspensions (dispersed & non-dispersed simples administered by deposition under the tongue). The dosing procedure was repeated 1, 2 and 3 h later.	Confocal: E171 agglomerates present in the cytoplasm of TR146 cells; numbers of particles increased with duration of exposure; large agglomerates still present after washout. TEM confirmed uptake of E171 particles into cytoplasm of TR146 cells. npSCOPE: single NPs, small and large clusters of electron-dense TiO_2 (E171) embedded in the cytoplasm of TR146 cells after 24 h *In vivo*, E171 particles observed by TEM deep into the buccal tissues in all treated pigs, even 30 min after the 1st sublingual dose. After the 4th dose, electron-dense particles observed in the mucosa of buccal floor as well as in the lumen of blood capillaries. At 1 h after the 4th dose Ti found in 1 isolated particle and in 7 aggregates recovered from tissue sections sampled from the submandibular lymph nodes located underneath the tongue.	• Standard confocal microscopy cannot distinguish between particles that have been internalised *versus* those on the outside surface of the cell. • High quality TEM images are provided. • EDX analysis was used to supplement TEM, to identify the elemental composition of suspected particles in images. • npSCOPE imaging proved to be sensitive, providing detailed information on sub-cellular localisation and elemental composition of the internalised particles.	Convincing evidence is presented demonstrating that E171 is taken up into the cytoplasm of cells cultured *in vitro*, and into the buccal mucosa and submandibular lymph nodes of pigs given sublingual doses *in vivo*. There is no evidence of nuclear uptake.	High

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Jalili et al. (2018)	TEM	NM103 (rutile, hydrophobic, 25 nm) and NM104 (rutile, hydrophilic, 25 nm) were obtained from the JRC Nanomaterials Repository.	Nanogenotox protocol (Jensen et al., 2011). Hydrodynamic diameters of the TiO_2 NPs in the stock suspensions were characterised by DLS and NTA, which gave different values. Mean diameters increased over 24 h.	Differentiated Caco-2 and HepaRG cells were treated in the absence of S9 for 24 h with 7.5 and 67.4 μg/cm^2	The two NPs were observed at similar incidences inside both Caco-2 and HepaRG cells even at 7.5 μg/cm^2. Agglomerates of different sizes were seen free or in vesicles, but also in vacuole-like compartments in HepaRG cells, particularly at the highest concentration. No NMs were observed inside the nucleus.	• Good quality images are presented. • EDX was not performed on TEM images to confirm the presence of Ti in the particle-like objects. • No indication of number of cells analysed per sample.	There is unconfirmed indication of TiO_2 uptake into the cytoplasm of cells only. There was no uptake into the cell nuclei.	Moderate
Gea et al. (2019)	3D confocal micro-Raman imaging spectroscopy	Rods, platelets and bipyramids TiO_2-NPs were synthesised by the authors. P25 NPs (Evonik) and food grade NPs (Faravelli Group) were purchased. Particles were characterised by SEM and transmission mode in SEM (T-SEM).	Rods, platelets and bipyramids were dispersed in DMSO 1% in water by ultrasonication for a few hours before the exposure with cells. Particle size distributions were assessed by DLS in this primary suspension, in base RPMI medium, and in RPMI medium containing 10% FBS.	BEAS-2B cells were treated in the absence of S9 for 24 h with 80 μg/mL of each of the particles.	The reconstructed image sections show cell uptake of aggregates of P25, food grade-TiO_2 and platelet NPs, but not bipyramids or rods.	• Method not traditionally used for uptake analysis; uncertainty as to whether or not particle uptake versus those attached to the surface of cells can be robustly determined. • Imaging software was used to develop 3D chemical images both for cells and TiO2 particles. • Limited number of cells analysed (only 5 cells evaluated per sample). • It is not possible to determine whether any intracellular aggregates were located in the cytoplasm, within vacuoles, or in the nucleus.	An unconventional approach has been used to detect cellular uptake; the observations are difficult to interpret and sub-cellular localisation cannot be determined.	Low
Vila et al. (2018)	Laser confocal microscopy; confocal images were processed with the software Huygens Essential 4.4.0p6 and Imaris 7.2.1	NM100 was obtained from the JRC Nanomaterials Repository.	Nanogenotox protocol. Hydrodynamic diameters of the TiO_2 NPs in the stock suspensions were characterised by DLS	Undifferentiated and differentiated Caco-2 cells were treated for 24 h in the absence of S9. The highest concentration (100 μg/mL) was chosen to facilitate the observation of NPs inside cells.	The authors suggest that TiO_2 can be visualised by laser confocal microscopy on the basis of the material's own optical properties. The authors report that undifferentiated Caco-2 cells internalised TiO_2 aggregates in the cytoplasm and	• Authors acknowledge method is qualitative and cannot distinguish between individual NPs and NP aggregates. • No controls or evidence are presented to confirm that the suspected green signal in the	An unconventional & unsubstantiated approach has been used to detect cellular uptake. Evidence for uptake into cells cannot be confirmed.	Low

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Table 3 (*continued*)

Authors	Method used for uptake assessment	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type or tissue/ Concentration or dose	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
					nucleus. However, in differentiated cell monolayers, the particles were only observed in the apical membrane of cells.	confocal images does indeed correlate with the presence of TiO_2. • TEM with EDX was not used to confirm the presence of uptake or sub-cellular localisation of the test material.		

an evaluation of the physio chemical properties of the nano materials being evaluated as well as the robustness and reliability of the studies. 18 toxicologists from the Chinese centre for disease control were trained on how to use the TRAM classification system prior to performing the evaluations. After filtering there were 328 studies that were taken forwards for full text review. Of these, only 12 *in vivo* and 19 *in vitro* studies were considered suitable for drawing conclusions on genotoxicity of TiO_2 nanoparticles.

The review is assessed in detail in Annex 4. In summary the authors did not perform a qualitative evaluation of the studies and instead performed their own analysis of the data extracted from the studies and publications. In doing this, they interpreted the data out of context from the original studies and may be using averages rather than raw data. They do not mention toxicity, histopathology, inclusion of hedgehog cells etc. and as such the conclusion that TiO_2 is genotoxic is naïve without the wider context of additional study specific detail. The authors mention the use of the TRAM evaluation tool but there is no example of the criteria used in the publication. Cao et al. (2023) include a large amount of data from *in vitro* comet studies which Kirkland et al. (2022) did not include in their analysis due to it not having an OECD guideline and its status as an indicator test, having negligible use in a weight of evidence for genotoxicity, although some that were identified from the SCHEER (2023) and SCCS (2024) opinions have now been reviewed in Annex 1.

3.5.1. New or newly identified in vitro studies

Eight new or newly identified publications described *in vitro* studies. As can be seen in Table 4A and Annex 4, only 1 paper (El Yamani et al., 2022) provided reasonably robust results ("moderate" weight) for *Hprt* gene mutations, and the conclusion was negative. All of the other studies measuring comets, phosphorylation of H2AX, or MN had limitations or deficiencies indicating the results (both positive and negative) were not robust. Some were unreliable and uninterpretable. The studies claiming cellular uptake show some evidence of uptake into the cytoplasm but all have limitations and there is no convincing evidence of nuclear uptake.

3.5.2. New or newly identified in vivo studies

Five new or newly identified publications described *in vivo* studies. As can be seen in Table 4B and Annex 4, two of the papers are of poor quality. However, the publications of Akagi et al. (2023), Sun et al. (2023) and Liang et al. (2024) provide reasonably robust negative results for MN in liver, γH2AX foci in liver and bone marrow, comets in liver, and MN and CA in bone marrow following oral gavage dosing.

3.5.3. New or newly identified human studies

Three new or newly identified publications described studies in exposed humans. All 3 publications reported studies of workers in manufacturing plants compared with control subjects working in office areas and, in 2 studies from the same laboratory, also external controls. As can be seen in Table 4C and Annex 4, the studies are difficult to interpret. In most cases the distributions of the frequencies of MN or DNA damage (comets) in exposed and control groups overlapped, and where any increases were seen they were small. In some cases office workers exhibited higher frequencies of genotoxic damage than production workers. Also, oxidative stress and inflammation were observed in the individuals studied, so any differences in genotoxic endpoints may be an indirect or secondary consequence of these processes.

4. Discussion and conclusions

Subsequent to our structured weight of evidence review of the most relevant and robust studies of the genotoxicity of TiO_2, we have now extended this review to include papers describing endpoints and test systems not previously considered relevant (mainly *in vitro* DNA damage studies), mode of action studies, cellular uptake studies, and papers published since the previous review or that were not found at that time.

The *in vitro* DNA damage studies all have limitations which mean firm conclusions are difficult. Reported positive results cannot be confirmed because of the deficiencies, in particular the failure to report on severely damaged cells and whether they were included in or excluded from tail intensity calculations. Some comets may be due to oxidative damage, and the impact of coating of particles cannot be confirmed.

The mode of action (MoA) publications indicate that TiO_2 can bind to naked DNA via intercalation, however there is no convincing evidence of nuclear exposure to TiO_2 particles. Downregulation of genes involved in DNA repair is unlikely to occur due to lack of nuclear exposure, but in any case the study methodology is flawed and changes in gene regulation can also be a consequence of general toxicity or hypoxia, particularly in adherent cell lines. Interaction with chromosome segregation machinery was seen where *in vitro* cell cultures had been grown for an extended period to the point at which they probably suffered spontaneous genetic drift. This study was methodologically flawed and therefore unreliable. At up to 7 weeks of exposure to TiO_2 there were no signs of interference with cell division machinery. The most biologically plausible MoA demonstrated by a large amount of data obtained primarily *in vitro* is generation of ROS. Increased ROS leads to a nonlinear and indirect forms of DNA damage. The generation of ROS from TiO_2 and other large surface NPs is well documented. On careful review of the data, ROS generation could be considered as the only MoA for TiO_2

Table 4A
Recent and missing publications: *in vitro* studies Status March 2024.

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
El Yamani et al. (2022)	Comet assay (±Fpg) *Hprt* gene mutation assay	NM101 (6 nm forming clusters up to 55 nm) obtained from JRC Nanomaterials Repository.	2 different dispersion protocols: • Mixing with 10% FBS in PBS in a glass tube, then probe sonication for 15 min. • The Nanogenotox protocol (Jensen et al., 2011). After dilution in culture medium, DLS showed agglomeration up to mean diameters of 150–170 nm	A549 cells (for comet) and V79 cells (for *Hprt*). Final concs. 0.1–75 μg/cm^2. Treatment 3 & 24 h for comet assay; 24 h for *Hprt* assay. Cellular uptake studied (only in V79 cells) by TEM after 24 h exposure to 3, 10 & 30 μg/cm^2.	Little or no cytotoxicity induced up to 75 μg/cm^2. No significant increase in comet -Fpg, although a trend was observed. No significant response + Fpg. No induction of *Hprt* mutations. TEM showed TiO_2 aggregates attached to the outside of the cell membrane, inside vesicles, vacuoles, and endosomes in the cytoplasm or attached to the nuclear membrane, as well as inside the nucleus.	• Detailed characterisation of suspensions. • Positive and negative controls included. • Concs. tested probably not cytotoxic but some confusion with cytotoxicity data. • Sufficient cells treated, processed and sampled. • Comet results quite heterogeneous. • It does not appear that cells were protected from light during treatment. • It does not appear that slides were coded before comet analysis. • Severaly damaged (hedgehog) cells not reported so not known ehther included in or excluded from % tail DNA calculations. • Higher concs. could have been tested (up to 100 μg/cm^2 according to OECD, 2022). • For cellular uptake the elemental composition of the particles was not confirmed (e.g. by EDX) so may not have been TiO_2, and from the images it is possible particles could have been "on" rather than "in" the cells.	Negative with some limitations for comets and *Hprt* mutations. Unconfirmed evidence of cytoplasmic and nuclear uptake.	Low for comets. Moderate for *Hprt* mutations.
Vieira et al. (2022)	Comet (±Fpg) and MN	NM102, 103 and 105 (22–30 nm) obtained from JRC Nanomaterials Repository. Particles represent different crystalline structures, surface area and coating.	The Nanogenotox protocol (Jensen et al., 2011). Parallel samples were digested. DLS data showed that after digestion NM105 has a lower mean size than the undigested sample. No major differences observed for the other NMs. Sizes of the NMs dispersed in culture medium	Caco-2 and HT29-MTX-E12 cells. 0.14, 1.4 and 14 μg/mL (0.14 μg/mL is considered a realistic conc, reaching the human intestine, based on real-life oral exposure). 24 h exposure for comet assay. For the MN assay, exposures were 72 h for HT29-MTX-E12 cells, and 52 h for Caco-2 cells,	Top conc. of digested NM105 showed some evidence of DNA damage in both cell lines -Fpg. Increased DNA damage + Fpg (evidence of oxidative damage) only seen in HT29-MTX-E12 cells. All 3 NMs after simulated digestion induced MN responses in HT29-MTX-E12 cells, but also some responses with undigested	• Treatments sufficiently long to allow cellular uptake, although this was not confirmed. • Cytochalasin B treatment was delayed in the MN assay to avoid interference with cellular uptake. • Sufficient cells were scored for each endpoint. • Both cell lines have a mutation in the p53 gene which may make them susceptible to misleading positive results since they	Weakly positive, with limitations, for DNA damage and MN induction particularly with digested samples in HT29-MTX-E12 cells. However, the damage may be due to ROS formed during digestion, so the biological relevance is questionable.	Low-Moderate

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Table 4A (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			measured by TEM ranged from 20.4 to 25.7 nm.	with cytochalasin B added at least 24 h after the start of treatment	samples. No effects seen in Caco-2 cells. Small increases in ROS seen with digested samples, but could be due to the digestion process itself.	lack normal apoptosis function. • Cell treatments were not protected from light, which may be a confounding factor. • Comet slides were not coded before scoring so scorer bias cannot be excluded.		
Vignard et al. (2023)	H2AX phosphorylation, induction of 53BP1 foci, comets and MN.	E171 purchased from a French on-line food supplier, Previously characterised by Guillard et al. (2020). NPs (21 nm) purchased from Sigma–Aldrich (not independently characterised). NM102 (115 nm) obtained from the JRC Nanomaterials Repository.	Samples sonicated in ultrapure water in an ice bath for 1 min at 40% amplitude (apparently stable for 15 days at 4 °C). DLS analyses of E171 and NM102 showed a slight increase (<10%) in hydrodynamic diameter after resuspension in Ham's F12 culture medium compared to water. The NP sample exhibited more (>30%) agglomeration in culture medium.	Human buccal TR146 cells, Treated at 5, 50 and 100 μg/mL but only for 2 h. However, confocal microscopy and TEM showed agglomerated particles (apparently) in the cytoplasm even after such short exposures. Not cytotoxic in differentiated TR146 cells, but induced <50% cytotoxicity in proliferating cells.	E171 and NM102 induced DNA damage in the γH2AX and 53BP1 biomarkers, and in the comet assay, but TiO_2-NPs did not. None of the TiO_2 samples clearly induced MN in TR146 cells. Both E171 and NM102 induced ROS, whereas TiO_2-NPs did not.	• TR146 cells have a mutation in the p53 gene which may make them susceptible to misleading positive results since they lack normal apoptosis function. • Treatment time very short but uptake into the cytoplasm appeared to be confirmed. • Cell treatments not protected from light, which may be a confounding factor. • For the comet assay severely damaged (hedgehog) cells not reported so it is not known whether they were included in or excluded from % tail DNA calculations. • Slides not coded before scoring so scorer bias cannot be excluded. • DNA damage may be secondary to the induction of ROS. • Permanent genotoxic effects (MN) not induced but the numbers of cells scored were too few for a robust assessment.	Positive, with limitations, for DNA damage but may be due to ROS. Negative, with serious limitations, for MN.	Low
Kang et al. (2011)	MN	P25 (20 nm) purchased from Degussa; "normal TiO_2" (~99% rutile, primary particle size 1 μm) purchased from Sigma Aldrich. Particle sizes of the received samples were not checked by the authors.	Particles were suspended in sterile PBS (concentration not given) and then dispersed for 30 min by sonication (not specified whether bath or probe) to prevent aggregation. Particle size distributions not	PHA-stimulated lymphocytes treated for 20 h with the particles (0.1 and 5 μg/mL), in the absence or presence of UVA (details of when and for how long not given), and then cytochalasin B	Slight but non-significant increase in MN frequency with P25 -UVA but a significant increase + UVA. No increases in MN frequency with "normal TiO_2" either with or without UVA.	• Cytochalasin B added after 20 h treatment with particles. • Treatment period sufficient to allow cellular uptake but not confirmed. • Slides coded before scoring. • 1000 binucleate cells/	Inconclusive	Low-Moderate

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Table 4A (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			determined either in the PBS stock suspension of after addition to culture medium.	was added for a further 28 h.	P25 induced apoptosis, decreased mitochondrial membrane depolarization, and ROS.	experiment from 3 experiments scored. • Only low levels of cytotoxicity induced. • No positive control included, which is important when concluding negative results. • Higher concs. could have been tested. • Positive responses with P25 +UVA could have been due to ROS.		
Jalili et al. (2018)	Cytokinesis block MN assay. Comet assay ± Fpg and γH2AX foci summarised in Table 1.	NM103 (rutile, hydrophobic, 25 nm) and NM104 (rutile, hydrophilic, 25 nm) obtained from JRC Nanomaterials Repository	Nanogenotox protocol. DLS and NTA showed agglomeration (>135 nm) in stock suspensions but gave different values. After 24 h mean diameters increased by 34–43% by DLS. In culture medium mean diameters were 235–270 nm by DLS, and 111–145 nm by NTA for both particles.	Caco-2 and differentiated HepaRG cells treated for 3 or 24 h with particle concs. ranging from • 1.25–80 μg/cm^2 (top conc. >250 μg/mL)for γH2AX • 28 & 128 μg/mL for comets • 2–67 μg/cm^2 for MN (24 h treatment followed after a delay by cytochalasin B) Uptake into cytoplasmic vesicles & vacuoles observed by TEM but not characterised by EDX. No uptake into nucleus.	Negative for MN. No cytotoxicity induced. No changes in glutathione or ROS levels. Negative for comets both – and + Fpg and no induction of γH2AX foci (see Table 1).	• Positive controls were included. • Sufficient cells scored • Upper conc. limit recommended by OECD (2022) was exceeded. • Delay between end of treatment and addition of cytochalasin B could mean cells with chromosome damage died before harvest. • Not clear if slides were coded. • Errors in data tabulation. • Possible interference of the NPs in scoring MN.	Negative with some limitations for the MN results.	Low - Moderate
Chen et al. (2022)	Comet assay ± Fpg. γH2AX foci. Cellular uptake (but no description of method)	Anatase NPs (25 nm) from Shanghai Macklin Biochemical Co., Ltd. TEM used to confirm particle morphology and size (25.12 ± 5.64 nm). XRD used to confirm crystal form (anatase)	Particles suspended in ultrapure water (100 μg/mL) and culture medium (DMEM). Method of suspension not described but probably sonicated before treatment, but in water, after dilution in DMEM or both is unclear. Particles aggregated in water and to a greater extent in DMEM (mean diameters 609.43 and 878.93 nm respectively). To avoid photocatalytic activity, TiO_2 NPs were shielded from light during storage and use.	BEAS-2B cells treated in serum-free DMEM. Cytotoxicity of >50% was seen after 48 h at 200 μg/mL. Hence for the comet and γH2AX assays cells were treated with 0, 25, 50, 100 μg/mL for 48 h. ,	No induction of comets -Fpg but dose-related increases in comets + Fpg. >2-fold increase in γH2AX foci but not dose-related, and addition of N-acetyl cysteine reversed effect. Dose-dependent increase in particles seen in the cytoplasm but none found in the nucleus. Agglomeration most severe at 100 μg/mL. Elemental nature of particles not determined. Induction of ROS seen by 2 methods.	• Positive control (H_2O_2) included. • Methods for preparing TiO_2 suspensions unclear. • Treatment conditions not clearly explained. • Assumed standard comet assay was pH 13 but not clearly stated. • Not clear whether comet slides were coded. • No method detail for cellular uptake.	Negative with limitations in standard comet assay but induction of oxidative lesions (confirmed by ROS induction). Uninterpretable for γH2AX foci. Probable cytoplasmic uptake, with limitations.	Low
Fayer et al. (2021)	MN	NM01001a, 5–6 nm (formerly known as NM-	1000 μg/mL suspension prepared by Nanogenotox	CHO cells & primary bovine fibroblasts.	Statistically significant increases in MN	• The 3-hr. treatment time was likely too short for	Unreliable and uninterpretable	Low

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Table 4A (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
		101), obtained from JRC Nanomaterials Repository.	protocol. After dilution to 0.1, 1, 5 and 10 μg/mL, DLS showed hydrodynamic sizes varied between 500 and 1300 nm in deionized water and DMEM/F12 –FBS, but were much smaller in DMEM/F12 +FBS. (mean 20.8 nm at 0.1 μg/mL up to 444.8 nm at 10 μg/mL).	Treated for 3 h (– and + S9) or 24 h (-S9) with 0.1, 1, 5 and 10 μg/mL. Cytochalasin B was added after the 3-hr. treatment or was present throughout the 24-hr. treatment.	frequencies at the lower concs. in CHO cells, and statistically significant decreases in MN frequencies at the highest conc. in primary bovine fibroblasts. Oxidative stress (by SOD) not induced.	meaningful NP uptake. • The entirety of the 24-hr. treatments was in the presence of CytoB, which is known to interfere with NP uptake. • It is unclear if the MN slides were coded prior to scoring. • The increases in MN frequency in CHO cells were observed only at the lowest concs., were not confirmed to be singificant by independent chi-square analysis, were quite small, within normal published ranges, and therefore of questionable biological significance. • Statistical analysis not performed by the authors on the positive controls, but in the 24-hr. treatment –S9 in CHO cells they did not induce biologically relevant (i.e. not >2-fold) increases in %MN, and not significant by idependent chi-square analysis. • Independent chi-square analysis showed MMS (3 h) and COL (24 h) did induce singificant responses in the bovine fibroblasts, but the data are confusing since data for CP are only given for the 24-hr. -S9 treatment. • The choice and application of the positive controls suggests an unfamiliarity with the *in vitro* MN assay in general. • Historical control data were not reported or used in the assessment (as per OECD TG487; OECD, 2016a). • Treatments were not cytotoxic so higher concs. could have been tested.		

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Table 4A (*continued*)

Authors	Assay	Test substance (nano, pigmented, mixture or not clear)	Dispersion method(s) and characterisation	Cell Type/Concentration	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Fernández-Bertólez et al. (2021)	MN (manual scoring and flow cytometry) Cellular uptake by flow cytometry	TiO_2 NPs (25 nm) obtained from Degussa-Evonik. Characterisation referenced from an earlier paper.	Formulated in deionized water or cell culture medium at 200 μg/mL, sonicated on ice to prevent heating effects. Mean hydrodynamic size and zeta potential were 160.5 nm and −27.8 mV in water, and 228.3 nm and −10.7 mV in culture medium, showing a good dispersion in both media.	SH-SY5Y cell line (derived from human neuroblastoma). Precipitate that would interfere with MN scoring was seen at 200 μg/mL so concs. tested were 10, 50 and 100 μg/mL for 6 or 24 h -S9. Cytochalasin B (CytoB) was included under 3 different protocols – simultaneous, post-treatment and delayed co-treatment. Treatments not toxic (referenced from earlier paper).	Positive at the top 2 concs. by manual scoring with all CytoB regimens (though some quantitative differences). Negative in the absence of CytoB by flow cytometry, but weakly positive at the top 2 concs. with CytoB. CytoB did not interfere with cellular uptake in these cells. However, it was responsible for the differences between manual scoring and flow cytometry results. CytoB could be responsible for the increase in MN in this cell line. Concentration dependent increase in side-scatter indicating cellular uptake.	• Positive control included. • Sufficient cells scored for MN. • Slides were coded before manual scoring. • Negative control MN frequencies were high (10%) by manual scoring. • Cellular uptake methodology is inadequate to determine whether particulates are intracellular or adhere to the cell surface and the treatment regimens are mostly inconsistent and incomparable. • The cell line used (SH-SY5Y) whilst being p53 competent is not commonly used in genotoxicity studies and its sensitivity for this purpose is untested. • Historical ranges for this cell line are not available in the literature and not mentioned or used in this study. • There was no mention of protecting treated cultures from light. • Conclusions on effect of CytoB are confusing and contradict previous publications.	Uninterpretable	Low

Table 4B
Recent and missing publications: *in vivo* studies Status March 2024.

Authors	Endpoint(s) tested	Test substance (nano, pigmented, mixture or not clear)	Dispersion method (s) and characterisation	Species/Doses	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Akagi et al. (2023)	MN in liver. γH2AX foci in liver and bone marrow.	AMT-100 (6 nm) obtained from Takya, Japan. Not independently characterised.	Dispersed in ultrapure water with 0.2% Na_2HPO_4 to form a uniform emulsion-like suspension. Prepared daily and used within 2 h. DLS showed median secondary particle size was 206.3 ± 44.2 nm. A small fraction with particles <100 nm in diameter (4.9 ± 11.1%) was occasionally observed	Male and female F344/DuCrlCrlj rats. Oral gavage at 10, 100, and 1000 mg/kg/day (5/sex/group) for 28 days, and at 100, 300, and 1000 mg/kg/day (10/sex/group) for 90 days.	In the 28-day study no increases in MN-hepatocytes in either sex. No increases in γ-H2AX foci in the liver or bone marrow. In the 90-day study no γ-H2AX induction in liver or bone marrow, or at sites of yellowish-brown material deposited in the nasal cavity, BALT, trachea, Peyer's patches, or cervical and mediastinal lymph nodes.	• Animals exposed primarily to larger agglomerated particles (nanosized secondary particles constituted only a small fraction of the dosing suspension). • Fewer hepatocytes were scored for MN than required by the current OECD test guideline 474 for MN in bone marrow (OECD, 2016b). • No concurrent positive control for the MN or γ-H2AX endpoints. • No confirmation of liver exposure to TiO_2 NPs or of cellular uptake in any tissue analysed for MN or γ-H2AX, although the high-dose animals showed a statistically significant increase in Ti levels in liver after 28 days. • It does not appear that MN or γ-H2AX slides were coded before scoring so scorer bias cannot be excluded.	Negative with some limitations	Moderate
Hashem et al. (2020)	Comet assay in spleen	E171 purchased from Sigma. No independent characterisation of sample supplied.	Suspensions in 0.5 % HPMC prepared daily and kept at room temperature until used. No other details regarding preparation, No characterisation of the dose formulations was reported.	Male Wistar rats. Oral gavage, 20 and 40 mg/kg/day (5/sex/group) daily for 90 days.	Dose-related increase in all comet parameters in spleen. Significant histological changes in spleen and bone marrow, in splenic index and some haematological, immunological and cytotoxicity parameters. Evidence of inflammation.	• No characterisation of the test material or dose formulations. • Dosing formulations, tissue samples and slides do not appear to have been protected from light. • The study did not meet the OECD TG 489 (OECD, 2016c) for the comet assay and there were no details provided regarding the methodology or scoring. • There was no concurrent positive control. • Not known how many slides were prepared or how many cells scored. • It does not appear that the slides were coded before scoring so scorer	Positive with serious limitations.	Low

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Table 4B (*continued*)

Authors	Endpoint(s) tested	Test substance (nano, pigmented, mixture or not clear)	Dispersion method (s) and characterisation	Species/Doses	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						bias cannot be excluded. • Severely damaged (hedgehog) cells were not reported so it is not known whether they were included in or excluded from calculation of tail length, % tail DNA or Olive tail moment. • The comet and some of the tissue images provided are of extremely poor quality. • There was no confirmation of splenic exposure to TiO_2. • There was clear evidence of inflammation and tissue toxicity.		
Sun et al. (2023)	Bone marrow MN and CA	TiO_2 NPs obtained from Nanostructured & amorphous materials. Characterisation of crystal structure, surface area & chemical composition determined by XRD, BET and FT-IR. TEM used to determine size (38 × 31 nm) and morphology (ellipsoid or approx. spherical).	NPs dispersed in 0.8% Tween 80 solution, sonicated on an ice bath, followed by addition of FBS to 3% (v/v) and further sonicated. Samples stored at 4 °C and used within 1 week, shaking well before each use. Dispersibility & stability of 1 mg/mL and 25 mg/mL solutions (the latter, for both fresh & stored preparations, diluted to 1 mg/mL) determined by DLS. Mean diameters were 206–210 nm.	Male and female Kunming mice. Oral gavage of 50, 150 and 500 mg/kg/day (5/sex/group) for 15 days.	No significant increases in %MN or %CA.	• Positive controls included. • Sufficient cells scored. • It does not appear that the slides were coded before scoring so scorer bias cannot be excluded. • MN frequencies in vehicle controls normal. • There was no concurrent measure of bone marrow toxicity (i.e., mitotic indices) in the CA assay, and there was no bone marrow toxicity according to %PCE. • Prolonged dosing for the CA assay is not recommended because chromosomally damaged mitotic cells may be lost at toxic doses. • CA frequencies in vehicle controls seem high. • Historical control data was not reported or used for comparison. • There was no confirmation of target tissue exposure to TiO_2.	Negative with some limitations for MN. Negative with more limitations for CA.	Moderate for MN. Low-Moderate for CA.
	Comet assay in liver			Male Sprague Dawley rats. Oral gavage of 50, 150 and 500 mg/	No significant increases in % tail DNA.	• Positive controls included. • Sufficient cells scored.	Negative with limitations	Low-Moderate

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Table 4B (*continued*)

Authors	Endpoint(s) tested	Test substance (nano, pigmented, mixture or not clear)	Dispersion method (s) and characterisation	Species/Doses	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
				kg/day (5/sex/ group) for 15 days.		• It does not appear that the slides were coded before scoring so scorer bias cannot be excluded. • The sample images provided for the comet are of poor quality. • Hedgehogs were excluded from comet scoring but not quantified/ reported. • Historical control data was not reported or used for comparison. • There was no confirmation of target tissue exposure to TiO_2 although histological changes in liver were seen.		
Bakare et al. (2016)	MN in bone marrow. Sperm morphology.	Anatase TiO_2 NPs (<25 nm) obtained from Sigma Aldrich. Confirmatory characterisation of the received particles described in a previous characterisation several years earlier (Shukla et al., 2011).	TiO_2 NPs suspended and ultrasonicated in double distilled water at a stock concentration to achieve a dose of 150 mg/kg but actual concentration not stated. The authors refer to Liu et al. (2009) but the formulation is not described there. Particle distributions in the suspensions not characterised.	Male Swiss Albino mice (4/group) dosed by intraperitoneal (IP) injection daily for 5 or 10 days at 9.38, 18.75, 37.50, 75 and 150 mg/kg/ day. Top dose considered LD_{50} according to Liu et al. (2009).	MN frequencies and sperm abnormalities increased significantly in treated mice.	• Positive control included. • No characterisation of particles on receipt or in suspension. • Intraperitoneal dosing is not considered physiologically relevant and is not recommended in OECD guidelines. • Slides for MN analysis not coded. • Too few cells scored for MN. • Staining of MN different from staining of normal nuclei. • Unclear whether control MN frequencies were acceptable due to inadequate presentation of data. • No measure of bone marrow toxicity.	Uninterpretable	Low
Liang et al. (2024)	MN and CA in bone marrow.	Food grade TiO_2 obtained from Jiangsu Hushen Titanium White Technology Co., Ltd. TEM and X-ray diffraction showed the sample met Chinese standards	TiO_2 was dispersed in water and shaken, but no ethanol, BSA or sonication was used. Dispersibility and stability (after storage at 4 °C for 1 week) of 1 mg/mL and 50 mg/mL solutions (the latter, for both fresh &	Kunming mice dosed daily by gavage at 250, 500 and 1000 mg/ kg/day for 15 days. The top dose is the limit dose for >14 days dosing. For MN mice sacrificed 6 h after last dose. For CA mice	No significant increases in MN-PCE. No significant increases in CA frequencies	• Positive controls included. • Sufficient cells scored. • Slides were coded before analysis of MN and CA. • Analysing CA after 15 days of repeated dosing is not recommended when toxic doses are used (damaged	Negative for MN and CA with some limitations.	Moderate

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Table 4B (*continued*)

Authors	Endpoint(s) tested	Test substance (nano, pigmented, mixture or not clear)	Dispersion method (s) and characterisation	Species/Doses	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
			stored preparations, diluted to 1 mg/mL) determined by DLS. Mean diameters were consistent with those of the primary particles, so they were considered to be well dispersed.	sacrificed 16 h after last dose.		mitotic cells may have died and been lost from the population). However it is acceptable when the limit dose is used and is not toxic, as was the case here. • Although mitotic index was not measured in the CA assay, there was no bone marrow toxicity according to PCE/RBC ratios. • Systemic exposure not determined within this study. Authors rely on data in mice from Talamini et al. (2019) which does not provide convincing data.		
	Comet assay in liver.			Male SD rats dosed daily by gavage at 250, 500 and 1000 mg/kg/day for 15 days. The top dose of 1000 mg/kg/day is the limit dose for >14 days dosing. Liver samples were taken 6 h after the last dose.	No significant increases in % tail DNA.	• Positive controls included. • Sufficient cells scored. • Slides were coded before analysis. • "Hedgehog" cells were counted but not included in % tail DNA calculations. • Systemic exposure not determined within this study. Authors rely on Kreyling et al. (2017) and although there were some differences it did demonstrate that the liver would have been exposed.	Negative.	Moderate

exposure that is supported by robust data.

The cellular uptake publications indicate some evidence of cytoplasmic uptake, particularly in cells treated *in vitro*, but most studies did not confirm the particles they saw were actually TiO_2. Importantly, there is no convincing evidence of nuclear uptake.

The results from the most recent studies (published after the Kirkland et al., 2022 review), and newly identified studies, report both positive and negative results for induction of DNA strand breaks and MN both *in vitro* and *in vivo*. Many of the studies have limitations and deficiencies although some of the negative studies are reasonably robust. For example, the recent *in vivo* study of Akagi et al. (2023) adds further weight to the lack of genotoxic effects in rats after oral administration, even when TiO_2 particles are found in some tissues. Where positive results are seen, most notably in *in vitro* studies, there is evidence these are due to ROS. There is no evidence of induction of gene mutations *in vitro*. Whilst there is unconfirmed evidence of uptake into the cytoplasm of cells there is no evidence of nuclear penetration.

Thus, whilst the publications reviewed here show again that the published data on the genotoxicity of TiO_2 are inconsistent, often of poor quality, and in some cases difficult to interpret, the conclusions of Kirkland et al. (2022) and Shi et al. (2022) that existing evidence does not support a direct DNA damaging mechanism for titanium dioxide (including nano forms), and that the main mechanism leading to TiO_2 genotoxicity is most likely indirect damage to DNA through ROS generation, are still valid. As noted in our previous review, carefully designed studies of apical endpoints (gene mutation, MN and/or CA), following OECD recommended methods, performed with well characterised preparations of TiO_2, would allow firmer conclusions on mutagenicity to be reached.

CRediT authorship contribution statement

David Kirkland: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Arne Burzlaff:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Andreas Czich:** Writing – review & editing,

Table 4C
Recent and missing publications: human studies Status March 2024.

Authors	Endpoints evaluated	Test substance (nano, pigmented, mixture or not clear)	Exposure conditions/groups	Tissues/cells sampled	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
Bonetta et al. (2024)	MN Comet (±Fpg)	TiO_2-containing powders used in production were analysed previously and reported to be 100% rutile.	15 TiO_2-exposed individuals working in production areas, and 20 non-exposed individuals working in administration, design and marketing roles at the same paint manufacturing company. No significant differences between exposed and unexposed subjects with respect to height, weight, body mass index, age, working exposure years or smoking habits. Highest levels of respirable dusts and Ti were observed in the production areas (0.137 mg/m^3 and 0.114 μg/m^3, respectively), while the lowest levels were in the administrative areas (0.033 mg/m^3 and 0.013 μg/m^3, respectively).	Exfoliated buccal epithelial cells for the MN assay. Salivary leukocytes for the comet assay.	Statistically significant increase in MN frequency in buccal cells, and statistically significant increases in direct and oxidative DNA damage in salivary leukocytes. Statistically significant associations between direct DNA damage and oxidative stress (i.e., oxidative DNA damage and MDA) and inflammation (i.e. TNF-α, IL-1β and IL-10). Also, oxidative DNA damage associated with oxidative stress (i.e. MDA and 8-iso-prostane) and inflammation (i.e., IL-1β and surfactant protein D). Direct and oxidative DNA damage correlated with urinary Ti levels. No correlation between MN frequency and any biomarkers evaluated.	• Unclear whether subjects were checked for potential confounding effects/exposures shortly before tissue isolation. • Unclear whether the health status was checked immediately before tissue isolation, e.g. for infections in the oral cavity. • Authors failed to check the workplace atmosphere for other potentially genotoxic substances. • Slides were coded for MN analysis but unclear if they were coded for comet analysis. • Increase in MN frequency was extremely small (<15% over control values), the distributions of the two groups essentially overlapped, so the "increase" was of questionable biological relevance. • Buccal cells exhibited no differences in other DNA damage, cell proliferation, or cell death/apoptosis. • OECD guidelines recommend scoring 4000 cells/animal *in vivo* or 2000 cells/concentration *in vitro* for MN, but only 1000 cells/subject were scored. • OECD TG489 recommends scoring 150 cells/animal but only 100 cells/subject were scored here. • Severely damaged cells (hedgehogs) not reported so not known whether they were included in or excluded from calculations of % Tail DNA. • It appears the comet assay results were based upon mean % Tail DNA rather than the mean of medians from replicate slides or wells. • No historical positive or negative	MN results so small as to not be biologically relevant. Comet results probably due to oxidative stress.	Moderate

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Table 4C (*continued*)

Authors	Endpoints evaluated	Test substance (nano, pigmented, mixture or not clear)	Exposure conditions/groups	Tissues/cells sampled	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						control data was presented (other comet studies have utilized ex vivo treatments to confirm acceptable assay performance). • Cellular uptake was not investigated or confirmed. • Oxidative stress and inflammation were confirmed, and any genotoxic responses observed may be secondary to those processes.		
Ursini et al. (2021) But see also Cavallo et al. (2023)	MN, nuclear buds (NB), and broken eggs (BE). Cytokinesis defect or arrest (binucleated cells, BIN). Necrosis and apoptosis (karyolytic cells, KL). Condensed chromatin (CC).	The final manufactured TiO_2 product is 280–310 nm rutile TiO_2 with different metal oxide coatings but the workforce is presumably exposed to other forms of TiO_2 during the manufacturing process.	40 individuals working in various production areas of a TiO_2 production facility, 5 non-exposed individuals working in administrative office area of the same facility, and 18 external non-occupationally exposed healthy individuals living in the same area as the workers. No significant differences between control subjects and exposed workers for smoking, age, job seniority, dietary habits, scan tests, drug intake. A slight difference was found for respiratory disease between workers producing TiO_2 and the other groups (office workers and controls).	Exfoliated buccal epithelial cells.	Significant increases in MN (compared to external controls (0.05 ‰MN) for maintainers (3.57‰), cleaners (2.89 ‰) and technicians (2.79‰). MN were also higher, but not statistically significant, in mobile operators (1.53‰), bagging operators (0.72‰), and office workers (1.81‰). Mean MN frequencies of all the groups except the bagging operators was higher than the 1.5 ‰MN cut-off value chosen to define a positive response. BE + NB‰ was significantly increased only for maintainers. BIN frequency significantly increased relative to the external controls but the increase was extremely small and the distributions overlapped. The frequency of cells with CC were higher in the maintainer group	• Unclear whether subjects were checked for potential confounding effects/ exposures shortly before tissue isolation. • Unclear whether the health status was checked immediately before tissue isolation, e.g. for infections in the oral cavity. • Authors failed to check the workplace atmosphere for other potentially genotoxic substances. • The distributions of the endpoints in many of the groups overlapped, indicating very high "within-group" variability and thus any "increases" were of questionable biological relevance. • Even office workers exhibited a MN frequency that exceeded the 1.5 ‰ cut off. • OECD guideline 474 (OECD, 2016b) recommends scoring 4000 cells/animal (*in vivo*) but only 2000 cells/subject were scored here for MN. • It is unclear if the slides were coded prior to scoring. • Cellular uptake was not investigated or confirmed. • Oxidative stress and inflammation were not investigated in this study but were seen in the same individuals in Cavallo et al. (2023), so any genotoxic responses observed may be	Uninterpretable	Low-moderate

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Table 4C (*continued*)

Authors	Endpoints evaluated	Test substance (nano, pigmented, mixture or not clear)	Exposure conditions/groups	Tissues/cells sampled	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						secondary to those processes.		
Cavallo et al. (2023). But see also Ursini et al. (2021)	Comet (±Fpg) Apoptotic cells characterised by nuclear condensation and/or fragmented chromatin Inflammatory markers by TNFα, IL-6, and IL-8 cytokine release	Same as Ursini et al. (2021)	Same as Ursini et al. (2021)	Separated lymphocytes from whole blood samples.	No statistically significant differences in % Tail DNA, tail moment, tail length, % apoptotic cells, oxidative DNA damage, or oxidative positive subjects (with >4 cutoff) between the three groups (production workers, office workers, and external controls). However, a significant increase in the mean value of % comets was reported for the combined TiO_2 production group as compared to the external control group. Slight but significant IL-8 increases were found in technicians, and to a lower extent in the bagging operators. Technicians and bagging operators also exhibited slight (but not significant) increases in oxidative DNA damage, in terms of Fpg sites.	• OECD guideline 489 (OECD, 2016c) recommends scoring 150 cells/animal but only 100 cells/subject (apparently from a single slide) were scored in the direct DNA damage version of the comet assay. • It is unclear why significantly more cells were scored -Fpg comet (1000 cells/subject; and apparently from a single slide) for determination of % comets. • It is unclear if the comet assay slides were protected from light, which can influence the results. • It is unclear if the comet assay slides were coded prior to scoring to exclude scorer bias. • It appears the comet assay results were based upon mean values from a single slide per subject, rather than the mean of the median % Tail DNA from replicate slides or wells (as recommended by OECD guideline 489; OECD, 2016c). • The magnitude of any reported differences in any of the comet or cytokine release parameters were quite small, and the distributions of the groups largely overlapped, indicating very high within-group variability and thus any changes were of questionable biological relevance. • No historical positive or negative control data was presented for the comet assay (while a positive control and historical data could be problematic for a human study, other comet studies have utilized ex vivo treatments to confirm acceptable assay	Uninterpretable	Low-moderate

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Table 4C (*continued*)

Authors	Endpoints evaluated	Test substance (nano, pigmented, mixture or not clear)	Exposure conditions/groups	Tissues/cells sampled	Response (according to authors)	WoE Considerations	Reviewer's conclusion (based on reliability and WoE considerations)	Weight
						performance). • Oxidative stress and pro-inflammatory responses were confirmed, and any genotoxic responses observed here (or in the previous MN evaluation in buccal cells) may be secondary to those processes.		

Writing – original draft, Conceptualization. **Shareen H. Doak:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Paul Fowler:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Stefan Pfuhler:** Writing – review & editing, Writing – original draft, Conceptualization. **Leon F. Stankowski:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andreas Czich is a Sanofi employee and may hold shares and or stock options in the company. Stefan Pfuhler is an employee of the Procter and Gamble company who market consumer products that may contain titanium dioxide. Paul Fowler, David Kirkland, Arne Burzlaff, Leon F. Stankowski and Shareen H. Doak report that financial support was provided by Titanium Dioxide Manufacturers Association.

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Appendix A. Supplementary data

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Data availability

No data was used for the research described in the article.

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