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A weight of evidence review of the genotoxicity of titanium dioxide (TiO_2)

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Abstract

Titanium dioxide is a ubiquitous white material found in a diverse range of products from foods to sunscreens, as a pigment and thickener, amongst other uses. Titanium dioxide has been considered no longer safe for use in foods (nano and microparticles of E171) by the European Food Safety Authority (EFSA) due to concerns over genotoxicity. There are however, conflicting opinions regarding the safety of Titanium dioxide. In an attempt to clarify the situation, a comprehensive weight of evidence (WoE) assessment of the genotoxicity of titanium dioxide based on the available data was performed. A total of 192 datasets for endpoints and test systems considered the most relevant for identifying mutagenic and carcinogenic potential were reviewed and discussed for both reliability and relevance (by weight of evidence) and in the context of whether the physico-chemical properties of the particles had been characterised. The view of an independent panel of experts was that, of the 192 datasets identified, only 34 met the reliability and quality criteria for being most relevant in the evaluation of genotoxicity. Of these, 10 were positive (i.e. reported evidence that titanium dioxide

was genotoxic), all of which were from studies of DNA strand breakage (comet assay) or chromosome damage (micronucleus or chromosome aberration assays). All the positive findings were associated with high cytotoxicity, oxidative stress, inflammation, apoptosis, necrosis, or combinations of these. Considering that DNA and chromosome breakage can be secondary to physiological stress, it is highly likely that the observed genotoxic effects of titanium dioxide, including those with nanoparticles, are secondary to physiological stress. Consistent with this finding, there were no positive results from the *in vitro* and *in vivo* gene mutation studies evaluated, although it should be noted that to definitively conclude a lack of mutagenicity, more robust *in vitro* and *in vivo* gene mutation studies would be useful.

Existing evidence does not therefore support a direct DNA damaging mechanism for titanium dioxide (nano and other forms).

Abbreviations:

8-OHdG = 8-hydroxy-deoxyguanosine; ADI = Acceptable Daily Intake; ATCC = American Type Culture Collection; BSA = bovine serum albumin; CA = chromosomal aberrations; DLS = dynamic light scattering; DMSO = dimethyl sulfoxide; DSB = double strand DNA break; ECHA = European Chemicals Agency; EFSA = European Food Safety Authority; FBS = foetal bovine serum; FDA = US Food and Drug Administration; Fpg = formamidopyrimidine-DNA glycosylase; GLP = good laboratory practice; GSH = reduced glutathione; hOGG1 = human 8-oxoguanine glycosylase; HPRT = hypoxanthine-guanine phosphoribosyl transferase; IP = intraperitoneal; IV = intravenous; JRC = Joint Research Centre; MDA = malondialdehyde; MN = micronucleus/micronuclei; MTD = maximum tolerated dose; NCI = National Cancer Institute; nm = nanometres; NP = nanoparticle; NTP = National Toxicology Program; OECD = Organisation for Economic Co-operation and Development; PBS = phosphate buffered saline; PC = physico-chemical; PCE = polychromatic erythrocyte; PDI = polydispersity index; RET = reticulocyte; ROS = reactive oxygen species; RTG = relative total growth; SAR = structure activity relationship; SCE = sister-chromatid exchange; SSB = single strand DNA break; TDMA = Titanium Dioxide Manufacturers Association; TEM = transmission electron microscopy; TG = test guideline; TGR = transgenic rodent mutation assay; TK = thymidine kinase; WoE = weight of evidence

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 sunscreens (Smijs and Pavel 2011), printer inks, medicines, plastics, and even cancer treatments as a sensitising agent in photodynamic therapy (Cesmeli and Avci 2019).

In 2021, TiO_2 pigment production in the US was estimated to be worth $3.2 billion (Mineral Commodity Summaries 2022, US Geological Survey). From the same report, the estimated end-use distribution of TiO_2 pigment consumption was predominantly via paints with 60% total usage.

As a food colourant, the use of TiO_2 (E171) has dramatically increased since the end of the second world war (Oil and Colour Chemists' Association, Australia, 1983) with cheaper mass production techniques and an increased availability of processed foods. It can be found as a whitener in dairy products such as milk and cream, coffee whitener, sweets, chewing gum, sauces and many tablet supplements as well as medicines (Boutillier et al 2021, Weir et al 2012).

In 2016, the European Food Safety Authority (EFSA) re-evaluated the safety of E171 as a food additive (in concordance with EU No 257/2010, as part of the re-evaluation programme for food additives authorised in the EU before 20 January 2009.), and identified several data gaps in the safety profile, notably for reproductive toxicity endpoints. As such, an acceptable daily intake (ADI) could not be calculated (EFSA, 2016) and the no-observed adverse effect level (NOAEL) from a carcinogenicity study was used to establish safe levels of exposure. In 2019 EFSA published a statement based on a review by the French agency for Food, Environmental and Occupational Health and Safety (ANSES), which made similar conclusions around data gaps for reproductive toxicity endpoints and recommended further investigation of *in vivo* genotoxicity endpoints (EFSA, 2019). According to the ANSES opinion, although there were no studies showing direct interaction of TiO_2 (E171) with DNA and/or the mitotic apparatus, a direct effect on genetic material or other molecules interacting with the genetic material could not be excluded.

In 2020 the European Commission requested a review of the safety profile of E171 which EFSA concluded in mid-2021. Since the 2016 and 2019 EFSA opinions, many more studies were conducted, including those published in peer reviewed journals as well as data generated at Contract research labs on behalf of industry or regulatory bodies, leading to a more comprehensive review of the available data by EFSA including studies focussed on new or novel endpoints. In the 2021 EFSA opinion, 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 TiO_2 particles that may be present in E171 could therefore not be ruled out. Several modes of action for the genotoxicity may operate in parallel and the relative contributions of different

molecular mechanisms elicited by TiO_2 particles are not known, and therefore 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 (EFSA, 2021).

Not all countries have agreed with the 2021 EFSA opinion. In the UK the independent Government expert committee, the Committee on Mutagenicity (COM), stated that "Members considered that the lack of quality in the evidence (e.g. mixed particle sizes (micro and nano particles (NP's)) and a wide variety of testing approaches) did not allow definitive conclusions to be drawn and therefore did not agree with the EFSA overall conclusions on the genotoxicity of E171. A review of more reliable and robust datasets may be required before conclusions could be drawn on the mutagenicity of TiO_2 particles." (Committee On Mutagenicity, 2021).

Health Canada have also recently re-evaluated TiO_2 as a food additive (June, 2022) and concluded that "the adverse effects associated with oral exposure to TiO_2 are largely derived from non-standard studies that administered stable, homogenized suspensions of ultrasonically dispersed particles". Health Canada argued that such preparations do not represent TiO_2 as a constituent of food. Whilst they did note that there were uncertainties and gaps in the published data that would benefit from further research, on weight of evidence they concluded that these data gaps were "not significant enough to warrant a more cautionary approach to TiO_2 use in foods at the current time" (Health Canada, 2022). Health Canada alongside many other regulatory bodies globally will continue to monitor the emerging science concerning the safety of TiO_2.

Several reviews on the genotoxicity of TiO_2 have been published, most recently by Wani and Shadab (2020) and Shi et al. (2022). Both publications included extensive data sets, focussing on more recent evidence (predominantly comet and micronucleus studies). However, neither make any qualitative assessment of the data, they both conclude that there are positive and negative genotoxicity studies and recommend that more testing is required to make a clear decision. To date, no published analysis has yet looked at the existing data to determine the robustness of the studies themselves, and relevance of the endpoints studied, before trying to interpret the overall weight of evidence for a genotoxic effect resulting from TiO_2 exposure.

To provide a comprehensive review of the available data, an expert panel was assembled at the request of TDMA to develop a WoE assessment of the genotoxicity of TiO_2 based on the available data identified in the EFSA evaluation, but also including additional studies available since the initial EFSA review including data generated in industrial and contract research laboratories on behalf of TiO_2 producers. None of the panel members are currently employed by companies that manufacture and sell TiO_2. However, it is acknowledged that due to the widespread use of TiO_2, several experts were employed by companies that included TiO_2 in their formulated products. Whilst some experts were funded by TDMA to perform this review, none of the experts were influenced in any way and prepared an entirely independent opinion.

The panel (namely the authors of this paper) included experts in genetic toxicology, general toxicology, bioavailability, carcinogenicity, nanoparticle (NP) characterisation and nanotoxicology.

2. Methods

2.1 Summary of the process

To identify those datasets that were most relevant in terms of predicting genotoxic potential, the following parameters were assessed:

- Relevance of the endpoint and test system investigated in terms of their association with genetic or carcinogenic hazard
- Reliability of the methods, including characterisation of the test substance (in particular for NPs)
- Quality and interpretation of the reported data by weight of evidence using expert judgement.

The processes used in these assessments are described in detail below.

2.2 Data sources

The publications reporting genotoxicity tests on TiO_2 reviewed by EFSA (2021; search criteria described in Appendix A of that publication, EFSA 2021) have been supplemented by additional publications identified by the Engineering Biology Research Consortium (EBRC) using the search criteria detailed in Supplementary data (table S1). In addition, our review included unpublished reports conducted by industry or at contract laboratories (sponsored by industry). The reviews of the various genotoxicity datasets in the publications and reports were tabulated separately (in Data Review Tables) according

to endpoint and test system, *in vitro* or *in vivo,* as detailed in supplementary tables S2-S8 with notes as to whether pigmentary (non nano) or nano-sized TiO_2 was tested (or if it was not clearly stated).

The relevant datasets in the publications and study reports were reviewed by the panellists for reliability using the ToxR Tool (Schneider et al. 2009) which applies modified Klimisch scores (Klimisch, 1997), and is a widely used method for weighting toxicology data based on quality. Each study dataset was assigned a Klimisch reliability score of 1 (reliable without restrictions), 2 (reliable with restrictions) or 3 (unreliable) using the principles of the ToxR Tool (Schneider et al., 2009), together with expert judgement. The standard ToxR Tool template was modified to include NP characterisation as detailed in Card and Magnuson (2010), and a copy of the modified tool is included in the supplementary documentation.

In brief, the ToxR Tool assigns a "0" or "1" to a range of parameters to reflect a "no" or "yes" answer (e.g., "0" would be entered if no details regarding mammalian cell characteristics or culture conditions, or animal husbandry, were included within a paper or by reference, or if a concurrent negative control was not included). The scores for the individual parameters are then totalled and the "Tool" calculates a Klimisch score (1, 2 or 3, as described above), which the reviewer could either confirm or revise (with justification). ToxR Tool parameters and modifications that are relevant to a high reliability score of the TiO_2 genotoxicity review are as follows:

1. Test substance identification (see below for special considerations related to tests on nanomaterial).
2. Test system/organism characterisation: the test system/organism used should be recommended by the relevant OECD guideline. If not, and the test system can be justified, the data may still be reliable.
3. Study design description:
    a. If a nanoform has been administered, it should have been characterised in the biologically relevant experimental medium.
    b. Treatment times of mammalian cells with microparticles and NPs should have been sufficient to allow cellular uptake, or there should have been a clear demonstration of cellular uptake.
    c. Concurrent positive controls should have been included. For those studies/endpoints where this is not required, use of an appropriate positive control measure, e.g., use of "banked or archived" slides (from previous positive control treatments) for bone marrow micronucleus (MN) assessment, or positive control DNA for transgenic rodent mutation

(TGR) assays, was appropriate. If positive controls were not included, justification was needed for still considering the data as reliable (e.g., a clear positive result with the test material, or a concurrent reference or test material was reported).

d. Endpoint scoring should have been adequately coded to protect from analyst bias, unless coding for a particular method was considered unnecessary (e.g., flow cytometric scoring of MN).

e. Assay variation should have been adequately controlled (e.g., timing of animal dosing and tissue sampling, use of a block design for comet slide processing or TGR assay DNA packaging).

4. Study results documentation

a. Acceptability and evaluation criteria should have been defined and compared with OECD TG recommendations. For example, negative control values for gene mutations, MN, CA, and % tail DNA should have been consistent with acceptable normal ranges. Justification was needed if the study did not completely meet OECD TG recommendations but was considered reliable. Where historical ranges were not included in the original report or publication, acceptable values for commonly used cell lines/types were used based on the collective experience of the experts.

b. Laboratory historical control data should have been reported and considered in the evaluation. If not, justification was needed to be provided as in point 5.

5. Plausibility of design and data: Concurrent and historical positive and negative control data should have been consistent with other published data. If not, there was reason to doubt laboratory competence.

Based on the above, the reviewer could decide on a Klimisch score different from that automatically calculated by the ToxR Tool, in which case this was justified by additional text. The evaluator's Klimisch score was then entered into the Data Review Tables.

2.3 Reliability using the Modified ToxR Tool

Not all studies that were reviewed had the same level of characterisation of the PC properties of NPs. Furthermore, over time, expectations of reviewers/journals have increased and so in more recent studies a more comprehensive characterisation of NPs was typically performed.

There are several parameters that have been identified as being important when performing characterisation of NPs (e.g., Warheit et al., 2008; Oberdörster et al., 2005; Luyts et al., 2013;

Mourdikoudis et al., 2018; Bouwmeester et al., 2011; Gubala et al., 2018). The most common techniques used for each PC parameter of interest are outlined in Table 1. One method can however, provide information on more than one PC parameter. For example, Transmission Electron Microscopy (TEM) can be used to visualise particle morphology, quantify particle size and size distribution and assess agglomeration/aggregation status. It should be noted that solubility (dissolution) is also an important parameter but is not included in the modified ToxR Tool form.

The quality of studies in which nano-grade TiO_2 was tested were therefore determined by addressing whether some of the important PC parameters had been characterised as proposed by Card & Magnuson (2010), including agglomeration and/or aggregation, chemical composition, crystal structure, purity, shape, surface area, surface charge, surface chemistry (including composition and reactivity) and whether any characterisation was conducted in relevant culture or formulation media.

A modified version of the ToxR Tool containing an extra tab in which the above 10 parameters could be assessed was prepared for use in this project (a template is provided as Supplementary material). The "nano score" was also then entered into the Data Review Tables.

In order that different panel members addressed these 10 parameters in a consistent way, some specific clarification was required. If a publication or study report stated that TiO_2 NPs were purchased from a recognised supplier who provided information on particular PC characteristics (e.g., a particular size range, surface area, purity, surface chemistry, charge etc.), but the authors did not verify this in their publication, and no further characterisation was reported, this was scored as a "0" against the relevant questions in the nano tab of the ToxR Tool. However, a comment was added that those characteristics were provided by the supplier and not confirmed by the authors. If the authors stated that those characteristics were confirmed in a previous paper, that the paper was quite recent (e.g., within 3 years) and details could be checked, then the relevant characteristics could be scored as "1", but comments that the information was provided in a previous publication were given. If, as discussed above, the NPs were provided as standard reference materials by Ispra (JRC standard NP's) or from NIST in the USA, or BAM in Germany or comparable institutes (KRISS in Korea etc.), these are all well characterized materials with specific documentation containing all important parameters. In that case we did not expect that the authors needed to do the same analysis again. If the supplier was not recognised, or the NPs were synthesised by the authors, and there were no data to confirm the PC characteristics, those categories were scored as "0". Whatever information was provided on characterisation of the NPs as a starting material, characterisation in the vehicle for an *in vivo* study,

or solvent and culture medium for an *in vitro* study, needed to be assessed separately. Some guidance on how these questions were critically assessed is given in Table 2:

2.4 Characterisation of nanomaterials

Many genotoxicity studies were performed using nano-grade TiO_2. In addition to assessing the studies from a hazard identification viewpoint, it was considered critical to identify whether the PC properties of NPs had been characterised as this is important for several reasons (Oberdörster et al., 2005; Warheit et al., 2008; Rasmussen et al., 2018). For example:

1. To identify what PC properties of NPs confer toxicity
2. To determine whether information provided by a supplier on the PC properties of the material was correct
3. To assess whether the PC properties of NPs changed when they were dispersed in the vehicle or media relevant to the test system and study
4. To feed into risk assessments for NPs.

Existing studies have evaluated the genotoxicity of samples of TiO_2 NPs that have been obtained from various sources and which vary with respect to their PC properties (e.g., size, surface area, morphology, agglomeration status, charge, surface chemistry). TiO_2 NPs have frequently been obtained from the JRC Nanomaterial Repository (Totaro et al., 2016) when investigating their genotoxicity. In addition, P25 (Degussa/Evonik) has commonly been used to assess TiO_2 genotoxicity (note that sample NM-105 from the JRC repository is P25). The genotoxicity of food grade TiO_2 (E171) has also been tested with samples obtained from various suppliers. However, such samples have a wide particle size distribution and only contain a small proportion of NPs. Some researchers synthesised their own TiO_2 NPs, but this was less common.

There is evidence that information provided by suppliers on the PC properties of NPs may not always be accurate (Luyts et al., 2013). Therefore, in the studies reviewed in this project, it was expected that some independent characterisation of the PC properties of the NPs was also performed. However, the PC properties of materials from several sources (e.g., JRC, Degussa and Evonik) have been extensively characterised and detailed information on their PC characteristics is available in the published literature (e.g., Rasmussen et al., 2014; OECD, 2016). Thus, for studies using materials from these sources, it was common that no independent characterisation of the properties of these materials in the 'as supplied' (pristine) form was performed. However, it was still expected that studies using these

materials would have summarised what information exists on the PC properties of these NPs, and that relevant literature was cited. By contrast, for NPs received from other suppliers, independent characterisation of their PC properties was considered essential, and it was not sufficient to rely solely upon information provided by the supplier.

It is well known that the PC properties of NPs can change when they are dispersed in biological media (e.g., Warheit et al., 2008) as well as during the dispersion process (Schulze et al., 2008). Therefore, it was expected that researchers characterised the PC properties of the NPs in media relevant to the study and test conditions. Most commonly dynamic light scattering (DLS) measurements are performed on the NP suspensions used in toxicity (including genotoxicity) studies to investigate hydrodynamic diameter (size), zeta potential (indicator of charge) and occasionally polydispersity index (PDI; which provides a measurement of how well the NPs are dispersed). TEM has also been used to characterise NPs suspended in biological media (e.g., to visualise particle morphology and to measure particle size). We therefore recorded whether characterisation in biological media (dosing suspension or culture media for *in vitro* studies, dosing formulation for *in vivo* studies) was performed as part of the nano assessment (nano tab of the ToxR Tool). Interestingly, the concentrations of NPs used for characterisation studies were not always comparable to the concentrations used in the genotoxicity component of the study, or that NP properties were only characterised at one NP concentration. The choice of particle concentration is important as it can influence the PC properties of NPs (e.g., agglomeration status) and therefore their hazard potential (e.g., Gudkov et al., 2020).

There was a lack of harmonisation regarding the methodology employed to prepare NP suspensions (Schultze et al., 2008) as there is a lack of standard methodologies for measuring the PC properties of NPs. It is common to use different strategies to improve the dispersion of NP suspensions and to limit NP agglomeration, but the relevance of this to real-life exposures has been debated. The approach used to disperse NPs is varied and can include the use of sonication (probe and bath), dispersants, solvents, and shaking/stirring/vortexing (Bouwmeester et al., 2011). Importantly, the dispersion protocol can influence the PC properties and toxicity of NPs (e.g., Pradhan et al., 2016). Of relevance is that the German NanoCare project (Schulze et al., 2008) and the EU Nanogenotox project developed protocols for preparation of NP suspensions (Jensen et al., 2011) but this has not been adopted across all nanotoxicology studies. We therefore noted what methodology was used to prepare NP suspensions for hazard studies.

2.5 The weight of evidence (WoE) process

The panel's evidence weighting assumptions for the various genotoxicity endpoints reviewed were based on Brusick et al. (2016). The basic weight descriptors are:

- **Negligible Weight** - The endpoint is not linked to any adverse effect relevant to genetic hazard/ risk (e.g., SCE).
- **Low Weight** - The end point is indicative of primary DNA damage, not directly linked to mechanisms associated with tumorigenicity (e.g., DNA breakage or computer-based SAR results), or the endpoints are evaluated in non-mammalian test systems (other than the Ames test).
- **Moderate Weight** - The endpoint may be: (a) only potentially relevant to tumour initiation, (b) subject to secondary effects (cytotoxicity), (c) subject to threshold dependent mechanisms of induction (aneugens) or (d) the test system exhibits a high rate of false responses with respect to carcinogenicity predictivity (e.g., mammalian cell *in vitro* clastogenicity and gene mutation tests, particularly in p53-deficient cells).
- **High weight** – The endpoint is one that has been demonstrated to play a critical role in the process of tumorigenicity (gene mutation in bacteria (Ames test) or *in vivo*, chromosome aberrations or micronuclei *in vivo*).

By applying the above weight descriptors, the default weights (i.e., for a robust study) for different endpoints studied *in vitro* or *in vivo* as shown in Table 3 are achieved (Brusick et al., 2016). The highest weighting is given to *in vivo* chromosome damage endpoints and *in vivo* gene mutation assays. It should be noted that whilst gene mutations in bacteria (Ames test) is given high weight, the Ames test is not recommended for testing insoluble particles (including nano particles) because they do not readily pass through the bacterial cell wall and prokaryotes do not perform endocytosis (Doak et al., 2012; Elespuru et al., 2018). Therefore, even though the default weight for an Ames test on a soluble chemical would be high, Ames tests on TiO_2 particles (whether micro or nano, irrespective of the bacterial strains tested and the outcome of the study – positive, inconclusive or negative) were given Low-Moderate or Low weighting. Although all the Ames tests reviewed gave negative results, they therefore did not contribute to the overall assessment of genotoxic hazard.

Although we identified 337 datasets within publications or study reports on the genotoxicity of TiO_2 (all listed in the supplementary bibliography), only those endpoints with a default weighting of "moderate" or "high" (according to table 3 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) have not been reviewed, since a "low" or "negligible" default weighted study would not contribute meaningfully to the assessment of genotoxic or carcinogenic hazard. It should

be noted that some publications contained datasets for “moderate” or “high” weighting endpoints that were reviewed in detail, but, within the same publication, also contained datasets for “low” or “negligible” weight endpoints that were not reviewed.

For the WoE process, each dataset was given an initial weighting according to the criteria in Table 3, 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, 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.

There were several specific considerations that were taken into account based on the recommendations from the OECD working party on nanomaterials, including misleading results that can occur if there is simultaneous co-treatment of cells with particles and cytochalasin B (Doak et al 2012). This type of co-treatment is not recommended, therefore studies using the cytokinesis block MN approach could only achieve default "moderate” weight if cells were treated with particles for a sufficient period of time prior to the addition of cytochalasin B. The latest draft recommendations from OECD (OECD, 2021) indicate treatment in the absence of cytochalasin B should be for at least 1 cell cycle, followed by 1.5 cell cycles in the presence of cytochalasin B. Shorter treatment times in the absence of cytochalasin B can be acceptable if there is a clear demonstration that the particles entered the cells. Since uptake into the cells is equally important for *in vitro* CA and gene mutation studies, these same requirements were also applied to these assays in our review process. However, if clear positive results were obtained with TiO_2 following a treatment period of less than 1 cell cycle, it was assumed that intracellular exposure had occurred. Therefore, some *in vitro* MN, CA and gene mutation studies that gave positive or equivocal results with short treatments were considered reliable and retained a "moderate” weight and were considered relevant to the assessment of genotoxic potential. In contrast, studies that gave negative results with short treatments and with no clear demonstration of cellular uptake were considered unreliable and given ”low-moderate” or "low” weights and not considered relevant.

The inclusion of concurrent positive controls in *in vitro* studies, or the inclusion of archived positive control samples in *in vivo* MN and TGR studies, was considered important to demonstrate reliable functioning of the test system and competence of the technicians, particularly when negative results were obtained with the test material. Thus, absence of an acceptable positive control in a study giving negative results with TiO_2 could be considered unreliable and the weighting downgraded. In contrast,

the absence of an acceptable positive control may not have been considered a critical defect in a study giving positive results with TiO_2. Therefore, some *in vitro* MN, CA and gene mutation studies that did not include acceptable positive controls but gave positive results with TiO_2 or other study materials were considered reliable and retained a "moderate" weight and were considered relevant to the assessment of genotoxic potential. In contrast, studies that gave negative results with no acceptable positive control were considered unreliable and given "low-moderate" or "low" weights and not considered relevant. This inevitably will have led to a "bias" towards positive results in the studies that were considered relevant for further assessment, but it was considered important in a rigorous, structured process.

Thus, an initial "moderate" weighting may have been down-graded to "low-moderate", or a "high" weighting may have been down-graded to "moderate-high" (or even lower) if the quality of the study design and/or results were questionable. This approach is the same as used for the review of acetaminophen (Kirkland et al., 2021).

Since multiple experts were working across several different endpoints, consistency was addressed by having 2 or more experts assess the reliability and WoE. Any assessments that appeared "out of line" with the majority of review comments for a given endpoint and test system were discussed either directly with the assigned individual reviewer or more widely by the panel. 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

Details of the 192 datasets with default "moderate" or "high" weights from publications and study reports that were reviewed in detail are given in Supplementary Tables S2-8 (for Ames tests, *in vitro* mammalian cell gene mutation tests, *in vitro* MN & CA tests, *in vivo* gene mutation tests, *in vivo* MN & CA tests, *in vivo* comet assays and *in vivo* 8-OHdG assays). The remaining 145 datasets (with default "low" or "negligible" weightings) that were not reviewed are listed in Supplementary Table 9.

The ToxR Tool was used to assess the reliability of the methods reported for the datasets reviewed in all publications and study reports. Whilst the details required for a robust reliability assessment were lacking in many publications, leading to Klimisch scores of 3, this was not used as a primary criterion to exclude a study from further evaluation; conclusions based on overall WoE assessment were used as the primary selection criteria for studies that should be considered most relevant for evaluation of genotoxic potential. More recent publications and reports of studies conducted by industry or at

contract research laboratories tended to contain more detail on methodology as well as including raw, unprocessed data, and included the necessary design components to lead to Klimisch scores of 1 or 2. Nonetheless, it was clear that the quality of available genotoxicity studies with TiO_2 is variable, and therefore the structured reliability and WoE assessment approach carried out in this project was considered important.

Tables 4 and 5 show summary data from those *in vitro* and *in vivo* studies (respectively) which, after review, achieved "moderate", "moderate-high" or "high" weighting, and were therefore considered most relevant from which to draw conclusions on genotoxicity.

3.1 Characterisation of physico-chemical properties

Supplementary Tables S2-8 document the nano score taken from the modified ToxR Tool, and reflect the level of information provided on the characterisation of PC properties of TiO_2 NPs in published studies. More specifically, we have considered what information was provided by a supplier, whether independent characterisation was performed, and whether characterisation was performed in the vehicle and/or media relevant to the genotoxicity studies. Where detailed characterisation data was available then high nano scores were obtained, but in several cases very limited characterisation was performed and the nano scores were low, sometimes even zero. In addition, details of the approaches used to suspend NPs is provided (e.g., media used, sonication approach and time) as this was varied across existing studies and can influence the PC properties and toxicity of particles. Comments on characterisation of NPs in the most relevant studies are given in summary Tables 4 and 5.

In some cases, we observed that the (geno)toxicity of the same material had been reported across several publications e.g., assessing different endpoints, using different biological models etc. Accordingly, the characteristics of the NPs were commonly reported in the first publication, and subsequent publications then cited the first publication for characterisation information. However, there is evidence that different batches of NPs may vary with respect to their PC properties (e.g., Mülhopt et al., 2018) and the approach used to prepare NPs may vary between studies and influence their PC properties. Thus, it was considered important that authors clarified the relevance of existing characterisation information.

Whilst some studies did report characterisation of NPs in biological media, many did not. We observed that published studies do not always provide a sufficient level of detail on the methodology that was employed to perform the characterisation of the NPs. For example, studies often neglected to include details of the concentrations of NPs used, and the approach used to disperse NPs (e.g., vehicle or media used to suspend NPs, and whether sonication was used and the time of sonication, when used).

466 This made it challenging to identify whether characterisation relevant to the hazard studies had been
467 performed.
468
469 The NTP genotoxicity studies (for example see Tennant et al., 1987; Ivett et al., 1989; Myhr & Caspary,
470 1991; Shelby & Witt, 1995; Shelby et al., 1993; in Supplementary Tables S2-8) apparently used the
471 same grade of TiO_2 (Unitane 220) as was used in the NCI carcinogenicity study (see Tennant et al.,
472 1987). Following an FDA request, an analytical comparison was made between 2 samples of Unitane
473 220 that had been retained by TDMA members (it is no longer manufactured) and food grade TiO_2
474 (E171). It was concluded that Unitane 220 is very similar in all PC characteristics to the current E171
475 grades and lies within the draft E171 specification. Therefore, it can be concluded that the NTP
476 genotoxicity studies effectively tested samples of TiO_2 that were comparable to food grade E171.

477 **3.2 Genotoxicity findings**

478 Of the 192 datasets reviewed, only 34 achieved a final weighting of “moderate” or higher and were
479 therefore considered relevant for the assessment of genotoxic hazard. The numbers of datasets in the
480 different categories are given in Table 6.

481 More details on the 34 datasets that achieved "moderate”, "moderate-high” or ”high” weighting after
482 review are summarised in Table 4 (for *in vitro* studies) and Table 5 (for *in vivo* studies) and most of
483 these achieved a Klimisch score of 1 or 2 within the Tox” Tool reliability assessment. As discussed, the
484 Ames test is not recommended for testing insoluble particles, so no Ames tests are included in Table
485 4.

486 As can be seen from Tables 4 and 5, many of the tests were performed on NPs of TiO_2. Some comments
487 on the characterisation of the NPs are provided in Tables 4 and 5, and also in Supplementary Tables
488 S2-8. Whilst some studies included quite extensive characterisation (nano scores of 8-10), others did
489 not (nano scores of 1-3), and this variability in characterisation was seen for datasets giving both
490 negative and positive results.

491 **3.3 *In vitro* studies**

492 Table 4 shows a summary of the expert evaluated scores for *in vitro* studies with “moderate”,
493 “moderate-high” or “high” weight. A total of 14 data sets comprising 9 MN, 3 CA, a single HPRT and a
494 single TK gene mutation data set with 10 out of the 14 data sets being with nano TiO_2.

495 There was no evidence of induction of gene mutations *in vitro*, although only 2 mammalian cell gene
496 mutation studies achieved a final weight of “moderate”. Most *in vitro* tests for MN and CA were
497 negative. Only 2 *in vitro* MN studies in Table 4 were positive or weakly positive, and the concentrations

498 at which these effects were seen induced oxidative damage, apoptosis and necrosis. However, these
499 changes were also seen in negative studies. Therefore, it is highly likely that the increases in MN were
500 secondary to oxidative stress and cytotoxicity.

501 The pattern of *in vitro* results from "moderate" or higher weighted studies is illustrated in Fig 1.

502 It should be noted that there was much variability across the different datasets in terms of the particle
503 concentrations tested in mammalian cells *in vitro*. This may be due to different forms of TiO_2 being
504 tested, cell type, method of formulation, etc., but it makes comparison of any effects between studies
505 very challenging.

506 As described previously, failure to expose mammalian cells for at least 1 cell cycle, or, for shorter
507 exposures, failure to clearly demonstrate that the particles entered the cells, was not considered
508 acceptable when negative results were obtained. Therefore, some *in vitro* MN, CA and gene mutation
509 studies that gave positive or equivocal results with short treatments suggested there must have been
510 intracellular exposure, so were considered reliable and retained a "moderate" weight (so were
511 considered relevant to the assessment of genotoxic potential and included in Table 4). On the other
512 hand, studies that gave negative results with short treatments, and with no clear demonstration of
513 cellular uptake, were considered unreliable and given "low-moderate" or "low" weights and not
514 considered relevant (and were excluded from Table 4). There could therefore be a "bias" towards
515 positive results in the datasets that are included in Table 4, that were considered relevant for overall
516 evaluation of genotoxic potential. Nonetheless, 10 *in vitro* MN/CA and 2 *in vitro* mammalian cell gene
517 mutation studies that were negative did include sufficiently long exposures (prior to cytochalasin B
518 treatment in the MN studies) to provide robust negative results.

519 **3.4 *In vivo* studies**

520 Table 5 shows a summary of the expert evaluated scores for *in vivo* studies with "moderate",
521 "moderate-high" or "high" weight. A total of 20 data sets comprising 11 MN (bone marrow and
522 peripheral blood), 2 CA, 2 transgenic rodent mutation studies (*gpt* and *Spi* mutants), 3 comet assays
523 (2 in liver and lung and a single study in liver) and two 8-OHdG adduct studies in the lung. Sixteen out
524 of the 20 data sets were nano TiO_2.

525 There was no evidence of induction of gene mutations *in vivo* from the 2 TGR studies in Table 5,
526 although neither study fully complied with OECD guideline recommendations. Similarly, none of the
527 *in vivo* Pig-a mutation studies reviewed in Supplementary Table 5 (S5) met recent best practices
528 recommendations (Dertinger et al., 2021) or the just approved OECD TG (OECD, 2022) and were
529 therefore not sufficiently robust to achieve "moderate" or higher weight.

Of the 13 *in vivo* MN/CA studies in Table 5, 7 were considered positive. However:

- 1 was probably an indirect consequence of high bone marrow toxicity since increased CA frequencies only increased at >40% mitotic inhibition (Manivannan *et al*, 2020)
- 3 showed only weak (approximately 2-fold) increases in MN and therefore of questionable biological relevance (Shelby & Witt, 1995, Shakula *et al*. 2014, Relier *et al*., 2017).
- 1 was positive for MN in rat bone marrow which was stained with Giemsa, but negative in bone marrow reticulocytes (stained with acridine orange) in the same animals (Dobezynska *et al.*, 2014). Giemsa is not a recommended stain for rat bone marrow since mast cell granules can stain and look like MN (Pascoe & Gatehouse, 1986), so the bone marrow response with the Giemsa stain could be an artefact and the negative result with acridine orange could be more reliable
- All positive responses other than those listed above were associated with inflammation, oxidative stress and/or apoptosis.

In addition to the above, 2 of these 7 datasets scored a Klimisch 3 in the ToxR Tool and as such are considered unreliable. Therefore, there are reasons to question whether any of these positive *in vivo* MN/CA responses are biologically relevant and indicative of a direct DNA-damaging effect of TiO_2.

It is notable that different dosing routes, dose levels and dosing periods were used in these 7 positive studies. Dose levels and dosing period were variable even by the same route of administration:

- 4 oral gavage studies
  - 1 study on nano TiO_2 (rutile, 25 nm) using doses up to 0.8 mg/kg/day for 28 days,
  - another study on nano TiO_2 (anatase, 5-10 nm) using doses up to 200 mg/kg/day for 60 days,
  - a 3rd study on nano TiO_2 (58 nm) using doses up to 500 mg/kg/day for 90 days,
  - a 4th study on micro TiO_2 using doses up to 1000 mg/kg/day for 7 days.
- 1 drinking water study on nano TiO_2 P25 using doses calculated up to 500 mg/kg over 5 days
- 1 IP study on pigmentary TiO_2 using doses up to 1500 mg/kg/day for 3 days
- 1 IV study on nano TiO_2 NM-105 using a single dose of 5 mg/kg.

This variability in the form of TiO_2 tested, dose levels, dosing routes and dosing periods makes it extremely challenging to draw any conclusions on what form(s) of TiO_2 and/or exposure routes might be associated with a genotoxic hazard.

Five of the seven positive MN/CA studies used oral gavage or drinking water administration, and yet absorption via the oral route has been shown to be very low. In an oral bioavailability study in rats,

562 only 0.0006% of a single 1000 mg/kg oral dose of E171-E was found in the total blood compartment,
563 thus covering any dissolved titanium as well as any TiO_2 NPs that may have crossed the intestinal
564 barrier (Provivo Biosciences & Fraunhofer Institute, 2022). Other grades of TiO_2 (G6-3, G2-5)
565 administered at the same dose, were below the limit of detection in blood, so the percentages
566 absorbed were even lower. With such low oral bioavailability, bone marrow exposure would be
567 negligible, and therefore the plausibility of these positive MN/CA results is questionable. By contrast,
568 3 of the 4 studies that used IV dosing, where exposure of the bone marrow would be assured, were
569 negative.

570 There are 3 *in vivo* comet studies in rats in Table 5. Two of these were negative (one in lung after
571 intratracheal instillation, the other in liver and lung after oral dosing). The third study was positive in
572 lung and liver after endotracheal instillation, but the responses were associated with inflammation
573 and oxidative stress. Again, this route is different from those leading to increased MN or CA, and so
574 comparing effects across different *in vivo* studies is challenging. Thus, again, there are reasons to
575 question whether this positive *in vivo* comet response is a biologically relevant indicator of a direct
576 DNA-damaging effect.

577 There are two *in vivo* 8-OHdG studies in Table 5. Both used a single intratracheal instillation of doses
578 up to 1.0 and 1.2 mg, and one study also used long-term whole-body inhalation. The outcomes of both
579 studies were negative.

580 The pattern of *in vivo* results from moderate or higher weighted studies is illustrated in Fig 2.

581

582 **4. Discussion**

583 We have used a structured approach to assess reliability and weight of evidence (WoE) in reviewing
584 192 datasets from publications and study reports on the genotoxicity of TiO_2 focusing on endpoints
585 considered relevant to genetic or carcinogenic risk. Using this approach, only 34 datasets met the
586 criteria for reliability and quality of data and were considered relevant (i.e., “moderate” or higher
587 weight based on WoE evaluations) for assessment of genotoxic risk. A further 145 datasets covering
588 endpoints that could, at best, have contributed only “low” or “negligible” weight to the overall
589 assessment of genetic or carcinogenic risk, were not reviewed. Therefore, considering the full 337
590 datasets with available genotoxicity data on TiO_2, only 10.1% finally provided relevant data, and
591 although this may seem low, it is higher than the 3.88% of published mutagenicity studies that were
592 considered suitable for inclusion in the GUIDEnano hazard assessment approach of Fernandez-Cruz et
593 al. (2018). There are many studies in which, according to our assessments, the endpoint evaluated has
594 lower weight, the study designs and/or the data are not reliable, or the results are questionable for

various reasons, and are too poor to support a robust assessment. Thus, if all datasets had been considered to contribute relevant results to an assessment of genotoxicity, as was the case in the EFSA (2021) opinion, different conclusions would likely be reached than if a structured reliability and WoE approach, as reported here, had been used.

Indeed, comparisons between the EFSA and Expert Panel approaches highlight differences in terms of the types of studies and endpoints that were included or excluded in the respective assessments, how reliability was scored, and how different aspects of test design were assessed. Table 7 highlights some of these differences, particularly in terms of the relevance (or weight) of certain genotoxicity endpoints *in vitro* and *in vivo*. The main differences in approach can be summarised as follows:

- The reliability of genotoxicity studies was assessed by EFSA using criteria published by Klimisch et al. (1997) whereas the Expert Panel used Klimisch scores derived from the modified ToxR Tool (Schneider et al., 2009).
- EFSA assessed relevance based on reliability (standard Klimisch score), some general aspects (e.g., genetic endpoint, route of administration and status of validation), and nano score (NSC), whereas the Expert Panel used the structured WoE approach described above.
- EFSA attributed relevance into 3 categories but only studies achieving ”High” or “Limited” relevance were considered in the overall assessment, whereas the Expert Panel initially attributed relevance into 4 main categories, and only studies achieving "moderate”, "moderate-high” or "high” weight after WoE reviews were considered in the final assessment.
- EFSA did not independently review the genotoxicity data in the relevant datasets, and the conclusions of the authors were accepted as published, whereas the Expert Panel re-evaluated the data in each of the 192 datasets with default ”moderate” or "high” weights using current standards (including OECD recommendations on testing NPs) and, on some occasions, did not confirm the authors’ conclusions.

As can be seen in Table 8, these differences in approach resulted in EFSA considering many more studies to be “relevant” than the Expert Panel. Many of the additional studies included by EFSA (>50% of those achieving ”high” or “limited” relevance) were *in vitro* comet assays, of which 71.8% were positive. These *in vitro* comet assays were excluded by the Expert Panel on the basis of being only indicator tests (OECD, 2015a) of DNA damage and not necessarily indicative of an ability to induce stable genetic changes (as also described in the OECD guidance document, OECD, 2015a). EFSA also included *in vitro* DNA binding, 8-OHdG adducts and γH2AX foci studies which were excluded by the Expert Panel on similar grounds.

The Expert Panel included more *in vivo* studies than EFSA, mainly due to inclusion of routes of administration not considered relevant for TiO_2 in food (i.e., i.v, i.p., or instillation, which could potentially have led to higher exposures than via the oral route considered by EFSA), but concluded many fewer studies were positive (in particular *in vivo* comet assays).

We noted that the more recent studies tended to contain more detail on methodology, test item characterisation and inclusion of unprocessed data, and were more robust than older studies. It was therefore considered useful to compare the EFSA and Expert Panel assessments of the more recent studies. In Tables 9 and 10 a comparison is made of only the “new” studies reviewed by EFSA (Appendices J and K, EFSA., 2021), and it can be seen that EFSA assessed some studies as ”high” relevance whereas the Expert Panel assessed them as contributing only "low” or "low-moderate” weight. There were very few datasets where the reverse was the case, i.e., where EFSA gave a lower relevance evaluation than the Expert Panel. As a result, EFSA included more study types and datasets as being relevant than the Expert Panel. Again, even with the more recent datasets, EFSA included multiple *in vitro* comet assays as ”high” relevance, many of which were positive, and DNA binding studies, which were also positive, whereas the Expert Panel WoE approach considered these to be “low” weight indicator tests (as also described in the OECD guidance document, OECD, 2015a). It is therefore not surprising that in the EFSA (2021) opinion, different conclusions were reached than in the structured reliability and WoE approach, as reported here.

Within the 34 datasets that were included in the WoE assessment, there was little evidence of reproducible effects for the same endpoint. This made comparison of effects very challenging due to different non-standardised protocols e.g., forms of TiO_2 tested, varied characterisation of the preparations tested, different concentrations or doses, different dispersion protocols, different exposure routes, different cell types showing differences in endocytosis, and the fact that study designs in many cases differed markedly from, and often fell short of, the recommended approaches in OECD test guidelines.

Of the 34 relevant datasets, only 10 (29.4%) were positive for genotoxicity. All were from studies of DNA strand breakage (*in vivo* comet assay) or chromosome damage (*in vitro* and *in vivo* MN or CA assays), and it is accepted within many regulatory guidelines that DNA and chromosome breakage can be secondary to physiological stress (for example see Kirkland et al., 2007 and note 9 ICHS2R1 (ICH 2013). Since all of the positive findings were associated with high cytotoxicity, oxidative stress, inflammation, apoptosis, necrosis, or combinations of these, it is highly likely that the observed genotoxic effects of TiO_2, including those with NPs, are secondary to physiological stress, as has been described recently in a comparable review (Krug, 2022). There were no positive results from the *in*

vitro and *in vivo* gene mutation studies evaluated, which is consistent with DNA/chromosomal damage being secondary to physiological stress, although it should be noted that to definitively conclude a lack of mutagenicity more robust *in vivo* gene mutation studies would be useful. As shown in Table 11, the profile of genotoxicity results from the most robust studies with TiO_2 does not fit the pattern expected for a genotoxic carcinogen.

As the data analysed contained a number of different sizes of TiO_2 from macro to nanoscale particles, there was the opportunity to determine whether particle size was related to genotoxicity outcome. However, we found no pattern of genotoxicity responses consistent with different sizes of TiO_2. Nano forms of TiO_2 under 100 nm, particles between 100 and 1000 nm and those above 1 µm did not correlate with any specific genotoxicity response.

The lack of correlation with particle size is consistent with the data from the German NanoInVivo project (The Federal Institute for Occupational Safety and Health, the German Environment Agency (UBA) and the Federal Institute for Risk Assessment (BfR)) that is looking at the long-term effects of nanomaterials on the lungs and other organs. Using inhaled Cerium Oxide in rat models (from 0.1 – 3 mg/m^3) they found that at a low load, the lungs showed a dose-related inflammatory response alongside tissue changes, and the higher the CeO_2 particle concentration in the lung, the stronger the inflammatory response was. Despite inflammation in the lungs, no tumour development was observed (Reihlen and Zimmermann 2018). TiO_2 showed analogous responses to those reported here, namely negative genotoxicity outcomes unless under conditions associated with generation of reactive oxygen or tissue overload, i.e., not directly DNA damaging.

5. Conclusions

The 34 robust datasets reviewed here, do not support a direct DNA-damaging mechanism for TiO_2 in either the nano or micro form.

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.

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CRediT authorship contribution statement

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Declaration of Competing Interest

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. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Approaches used to characterise the PC properties of NPs. The most commonly employed approaches to characterise different NP PC properties are identified.

Property	**Approach**
Size and size distribution	Transmission electron microscopy (TEM) Scanning electron microscopy (SEM) Dynamic Light Scattering (DLS) X Ray Diffraction (XRD) Nanoparticle tracking analysis (NTA)
Agglomeration/Aggregation	TEM SEM DLS NTA
Shape (Morphology)	TEM SEM
Surface Area	Brunauer, Emmett and Teller (BET) **only applicable to powders*
Surface Chemistry (composition and reactivity)	X-ray photoelectron spectroscopy (XPS) Nuclear magnetic resonance (NMR) spectroscopy
Charge	DLS (zeta potential)

Crystal Structure	**XRD**
Composition & Purity	Inductively coupled plasma mass spectrometry *(*ICP-MS) (ICP-MS) Fourier Transform Infrared Spectroscopy (FTIR)

Table 2: guidance for individual experts filling in the nano section of the modified ToxR tool

Category	**Comments / Explanation / Justification**
Agglomeration and/or aggregation	May be measured or not, not so important for in vitro tox
Chemical composition	A must to know if it is pure or coated or a mixture
Crystal structure/crystallinity	For TiO2 this is important and should be analysed by authors
Particle size/particle distribution	A must and should be measured by authors
Purity	Important, thus a "1" only if analysed by authors
Shape	Important, should be measured by authors using TEM
Surface area	Important, but may be calculated from size distribution. But if a value has been mentioned it should be measured by the authors
Surface charge	Should be measured by the authors
Surface chemistry (including composition & reactivity)	Coating etc. should be stated and analysed by authors
Whether any characterization was conducted in the relevant experimental media	It would be helpful if agglomeration, size distribution and surface characteristics could be provided in the culture media, dose formulation, to judge the effects in a more relevant way
Total score	

Table 3 – Default weighting of genotoxicity studies by endpoint

Endpoint*	Negligible Weight	Low Weight	Moderate Weight	High Weight
DNA binding (adduct formation) *in vitro*		■		
DNA binding (adduct formation) *in vivo*		■		
SSB/DSB *in vitro (including comet)*		■		
SSB/DSB *in vivo (including comet)*			■	
Sister Chromatid Exchanges (SCE) *in vitro*	■			
Sister Chromatid Exchanges (SCE) *in vivo*	■			
Oxidative DNA Damage *in vitro*		■		
Oxidative DNA Damage *in vivo* (detection of 8-OHdG adducts)			■	
DNA repair effects *in vitro*		■		
DNA repair effects *in vivo*			■	
Micronuclei (MN) *in vitro*			■	
Micronuclei (MN) *in vivo*				■
Chromosomal aberrations (CA) *in vitro*			■	
Chromosomal aberrations (CA) *in vivo*				■

Gene mutation in bacteria (Ames Test)				
Gene mutation in mammalian cells *in vitro*				
Gene mutation *in vivo*				

* SSB, single strand breaks; DSB, double strand breaks; SCE, sister chromatid exchange

Principles of WoE are consistent with endpoint specific guidance document of the European Chemicals Agency (ECHA, 2015), and the "Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines" (OECD, 2015a).

Table 4 – Summary of moderate, moderate-high or high weight *in vitro* studies.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
Kazimirova et al. (2020)	Nano, P25 anatase/rutile (NM-105), average size 21 nm. When dispersed in PBS and sonicated, mean size distribution increased to 112 nm (with FBS) and 296 nm (without FBS). *NanoTEST dispersion protocol used for hazard studies. NPs suspended in 10% FBS (in PBS) at a concentration of 5 mg/ml probe sonicated for 15 min. and diluted in cell culture medium.	Nano score 10. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	HPRT mutations	V79-4 cells	3-75 µg/cm^2 for 24 hours.	Negative	Top concentration equivalent to 585 µg/mL. Only slight cytotoxicity. ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Du et al. (2019)	Nano (40 nm). *Lack of detail provided about NP preparation for genotoxicity studies. Stock concentration of NPs suspended in deionised water.	Nano score 1. * No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. No characterisation performed in relevant biological medium.	TK mutations	L5178Y cells;	4 hours treatment – and + S9, 24 hours treatment -S9; 312-2000 µg/mL in each case.	Negative	Top concentration induced ~50-60% reduction in RTG. Followed OECD guideline 490 (2016). ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Donner (2006); unpublished study report published in Warheit et al. (2007)	Ultrafine (called uf-C in Warheit et al., 2007; 140 nm median size).	Not done – not relevant	CA	CHO-K1	4+16 hours - S9 at 750, 1250 & 2500 µg/mL; 4 + 16 hours +S9 at 62.5, 125 & 250 µg/mL; 20+0 hours - S9 at 25, 50 & 100 µg/mL.	Negative	GLP study, complied with OECD guideline 473 (1998). >60% mitotic inhibition at top concentration in all parts of study. ROS/oxidative stress not investigated.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
							ToxR Klimisch score 1.
Riley (1999)	Nano/bulk not specified but for T 805. *Stock concentration of NPs prepared in ethanol for genotoxicity studies.	Nano score 3. *Limited independent characterisation performed – reliant on information provided by the supplier. No characterisation performed in relevant biological medium.	CA	CHO cells	88.72; 209.7 and 800 µg/mL (-S9 20 hour treatment); 167.8; 640; 800 ug/mL (+S9 3 hour treatment).	Negative	-S9 3 hour treatment performed in separate study. 800 µg/mL is approximately10 mM. GLP study, complied with OECD guideline 473 (1998). ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Glover (2011)	Assumed to be pigmentary since nano is not mentioned.	Not done – not relevant	CA	CHO-K1 cells	4+16 hours -S9 (25, 50, 100 µg/mL), 4+16 hours +S9 (25, 50, 75, 100, 150 µg/mL), or 20+0 hours -S9 (25, 50, 75 µg/mL).	Negative	Little or no mitotic inhibition but >50% growth inhibition at top concentrations scored. GLP study complied with OECD guideline 473 (1998). ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Zijno et al. (2015)	Nano; anatase <25 nm (Sigma Aldrich). *NPs suspended in serum free culture medium (0.1 mg/ml) and probe sonicated for 20 minutes on ice for genotoxicity studies.	Nano score 7. * Information on NP characteristics obtained from the supplier provided. Some independent characterisation performed. Some characterisation	MN	Caco-2 cells (from ATCC)	1, 2, 3.5, 5, 10 and 20 µg/cm^2 (corresponding to 6.4–128.0 µg/ml) in culture medium (without FCS.); 6 and 24 hours then adding cytochalasin B for 24 hours.	Negative	6 hours treatment without cytochalasin B may not be long enough for nanos, but 24+24 hours is robust. ROS/oxidative stress not investigated in this study but previously shown ROS induced at these concentrations.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
		performed in relevant biological medium.					ToxR Klimisch score 2.
Landsiedel et al. (2010)	T-Lite™ SF (TiO_2 for Sunscreens), 10 x 50 nm, Rutile, coated with aluminium hydroxide and dimethicone/methicone copolymer. *For the MN assay NPs were suspended in cell culture medium for genotoxicity studies.	Nano score 8. * Some information on NP characteristics obtained from the supplier provided. Independent characterisation also performed. Characterisation performed in relevant biological medium.	MN	V79 cells	75 to 300 µg/ml for 4 hours; 18.8 to 75 µg/ml for 24 hours.	Negative	The authors clearly identified that NP can be seen on the slides at 2.5 µg/ml and above. ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Armand (2016)	Nano; AEROXIDE P25, (NM105 manufactured by Evonik for JRC Ispra); 24 nm, 86% anatase/14% rutile. *NPs were suspended in ultrapure sterile water (10 mg/ml) and probe sonicated (in pulsed mode) for 30 min. Suspensions were vortexed and diluted in cell culture medium for genotoxicity studies.	Nano score 8. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	MN	A549 cells	1 – 50 µg/ml over 2 months with 2 medium changes (containing nano particles) per week. MN measured at 24 hours, 1 week, 2 weeks, 1 month and 2 months.	Negative	No cytotoxic effect even after 2 months of treatment with 50 µg/ml. ROS increased and oxidative DNA damage (measured with Fpg modified comet) has been shown. ToxR Klimisch score 1.
Vales et al. (2015))	Nano; NM-102 (JRC, Ispra) 21 nm. *Nanogenotox dispersion protocol used: NPs were pre-wetted in 0.5% ethanol and then suspended in 0.05% BSA in MilliQ water (2.56 mg/ml) and probe sonicated for 16 min. on ice. Stock suspension diluted in cell culture medium for genotoxicity studies.	Nano score 9. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-102) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	MN	BEAS 2B cells	1, 10 and 20 µg/ml for acute (24 hours) and chronic treatment (1 to 3 weeks); sequential treatment with NPs and cytochalasin B.	Negative	Cytotoxicity not assessed. Oxidative stress investigated but no positive effect for TiO_2. ToxR Klimisch score 1.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
Di Bucchianico et al. (2017)	Nano; NM-100 (anatase, 50–150 nm, non-coated), NM-101 (anatase, 5–8 nm, coated) and NM-103 (rutile, 20–28 nm, coated). *NANOoREG dispersion protocol used for hazard studies: NPs were suspended in 0.05% BSA in MilliQ water (2.56 mg/ml) and probe sonicated for 15 min. on ice. Stock suspensions were then diluted in 0.05% BSA to a concentration of 0.1 mg/ml and then diluted in cell culture medium.	Nano score 10. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-101 and NM-103) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	MN	BEAS-2B cells	1–30 µg/mL, 3, 24 or 48 hours treatments under serum free conditions. MN scored with flow cytometry and manually by the CBMN cytochalasin B assay (added after 20 hours).	Weak positive (<2-fold and inverse dose-response) for NM-103	Authors noted induction of oxidative DNA damage for all three materials & increased necrotic cells particularly for NM-103. ToxR Klimisch score 1.
Stoccoro et al. (2016)	Commercial TiO_2 (84% anatase, 16% brookite crystal phase composition, 8), NP as nanopowder and as colloidal nanosuspension (nanosol). Pristine (uncoated), citrate-coated and silica-coated TiO_2 were tested with Aeroxide® P25 as benchmark material. *No information on NP preparation for genotoxicity studies provided.	Nano score 6. * Information on NP characteristics obtained from the supplier provided. Some independent characterisation also performed. Some characterisation performed in relevant biological medium.	MN	BALB/3T3 cells	10, 20 and 40 µg/cm^2, (corresponding to 32, 64, and 128 ug/mL); 48 hour treatment.	Positive for citrate-coated TiO_2 and P25 (only at lowest concentration), others weakly positive.	Oxidised purines & pyrimidines induced by all particles tested. Significant apoptotic & necrotic cells induced by citrate-coated & P25. ToxR Klimisch score 1.
Andreoli et al. (2018)	Nano: anatase 20-60 nm; Rutile 30 x 100 nm rods; Mixture anatase and rutile 45 – 262 nm; Anatase 50 – 270 nm; Rutile 50 – 3000 nm (Sigma-Aldrich, USA).	Nano score 4. * Reliant on information provided by the supplier. Limited independent characterisation performed. Some characterisation	MN	Human peripheral blood lymphocytes from 2 healthy male donors (<40 years old)	50, 100 and 200 µg/ml, 20 hours.	Negative for all particle types	Authors used 2 protocols: (1) sequential treatment (20 hours NP and then cytochalasin B was added for the next 28 hours); (2) co-treatment (30 min NP alone and then together

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
	*NPs were suspended in cell culture medium without serum and bath sonicated for 45 min.	performed in relevant biological medium.					with cytochalasin B for 28 hours). The results did not differ. Treatments carried out in the dark. Oxidative DNA damage suggested, 8-OHdG induced at highest concs. 100 and 200 µg/ml. ToxR Klimisch score 1.
Brandao et al. (2020)	AEROXIDE_ P25 (Degussa-Evonik); 25 nm, 80% anatase/20% rutile. *NPs were suspended in cell culture medium and probe sonicated for 5 min. on ice (1.5 min. on and 1 min. off twice, and 2 min. on) for genotoxicity studies.	Nano score 3 *Reliant on information provided by the supplier. Limited characterisation performed in relevant media. *Whilst limited information on NP characteristics was provided in the manuscript P25 has been extensively characterised in the published literature.	MN	A549, A172, HepG2 & SH-SY5Y cells	10, 50, 100 and 200 µg/ml, 3 and 24 hours treatments.	Negative	Uptake of TiO_2 was clearly shown for all cell lines. ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Pittol et al. (2018)	Commercial rutile (TiPure R-103). *NPs were suspended in cell culture medium for genotoxicity studies.	Nano score 6. *No information on NP characteristics obtained from the supplier provided. Some independent characterisation performed. No characterisation in	MN	L-929 mouse fibroblasts	15, 30 and 60 ppm, 6- and 24-hour exposures without S9, cytochalasin B then added until harvest at 72 hours. Data given for 24-hour exposures only.	Negative	Agglomeration of nanos in culture medium. ROS/oxidative stress not investigated. ToxR Klimisch score 1.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Cell type	Concentrations tested	WoE conclusion	Comments
		relevant biological medium.					

Table 5 - Summary of moderate, moderate high or high weight *in vivo* studies

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
Suzuki et al. (2016)	Nano (P25). DLS showed particle sizes of 145-147 nm in dosing vehicle (disodium phosphate). *NPs sterilised by heating (180°C for 1 hour), suspended in 2 mg/ml disodium phosphate (DSP) at a concentration of 10mg/ml and bath sonicated for 30 min. Suspensions then diluted in DSP for genotoxicity studies.	Nano score 3. * Information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium. *NB Whilst there was a reliance placed on presenting information obtained from the suppliers on the characteristics of the NPs, P25 has been extensively characterised in the published literature.	*Gpt* and *Spi* mutations	*Gpt* delta mice	Intravenous; 2, 10 & 50 mg/kg, once per week for 4 weeks; liver sampled 9 days after last administration.	Negative with restrictions. Unusual dosing schedule may not support negative outcome, although TiO_2 shown to be localised in liver by TEM.	ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Suzuki et al. (2020)	Nano (P25). DLS showed particle sizes of 145-147 nm in dosing vehicle (disodium phosphate). *NPs sterilised by heating (180°C for 1 hour), suspended in 2 mg/ml disodium phosphate (DSP) at a concentration of 10 mg/ml and bath sonicated for 30 min. Suspensions then diluted in DSP for genotoxicity studies.	Nano score 3. * Information on NP characteristics provided by supplier. Limited independent characterisation performed. Some characterisation in relevant biological medium. *NB Whilst there was a reliance placed on presenting information obtained from the suppliers on the characteristics of the	*Gpt* and *Spi* mutations NB. Methods described in Suzuki et al. (2016)	*Gpt* delta mice	Intravenous; 2, 10 & 50 mg/kg, once per week for 4 weeks; liver sampled 90 days after last administration.	Negative with restrictions. Unusual dosing schedule may not support negative outcome, although TiO_2 shown to be localised in liver by TEM.	ROS/oxidative stress not investigated. ToxR Klimisch score 1.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
		NPs, P25 has been extensively characterised in the published literature.					
Shelby & Witt (1995)	Unitane 220 (comparable to food grade E-171) pigmentary with a nano tail.	Not relevant. Pigmentary grade tested.	Bone marrow CA	Mice	Single IP dose of 625, 1250 & 2500 mg/kg; bone marrow sampled 17 & 36 hours later.	Negative with some limitations.	Only 50 cells/animal scored for CA. Not clear whether slides coded. No direct measure of bone marrow toxicity, but %PCE reduced in MN study in same paper. IP route not considered physiologically relevant. ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Manivannan et al. (2020)	Nano – rutile form (25 nm, forming agglomerates of >300 nm after dispersion in water). *NPs suspended in distilled water and bath sonicated for 30 min for genotoxicity studies.	Nano score 8. * Some information on NP characteristics obtained by supplier provided. Independent characterisation of NPs also performed. Some characterisation in relevant biological medium.	Bone marrow CA	Mice	Oral gavage dosing of 0.2, 0.4 & 0.8 mg/kg/day for 28 days.	Positive, but chromatid and chromosome breaks may be indirect consequence of high bone marrow toxicity.	>40% reduction in mitotic index at top 2 doses where increased CA frequencies seen. ROS/oxidative stress not investigated. ToxR Klimisch score 3, unreliable.
Shelby & Witt, (1995) & Shelby et al. (1993)	Unitane 220 (comparable to food grade E-171) pigmentary with a nano tail.	Not relevant. Pigmentary grade tested.	Bone marrow and peripheral blood MN	Mice	3 IP studies. 3 daily doses, #1: 250, 500 & 1000 mg/kg/day, bone marrow 24 hours; #2: "DRF" 500, 1000 &1500 mg/kg/day,	Positive, with reproducible, weak increase at 1000 mg/kg/day in bone marrow, but at lowest dose in blood so no significant trend.	IP route not considered physiologically relevant. Only 2000 PCE/animal scored for MN. Peripheral blood 52% toxicity seen; minimal bone marrow toxicity.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
					Peripheral blood 48 hours; #3: 500, 1000, 1500 mg/kg, bone marrow 24 hours		ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Trouiller et al. (2009)	Nano (Aeroxide P25). * NP suspended in drinking water and bath sonicated for 15 minutes.	Nano score 6. * Information on NP characteristics obtained from supplier provided and this information is summarised. Limited independent characterisation performed but P25 has been extensively characterised and citations are provided to relevant literature. Some characterisation in relevant biological medium.	Peripheral blood MN	Mice	Drinking water, 50, 100, 250 500 mg/kg total from 5 days dosing. Water consumption ranged 3-7 mL/mouse/day. Average of 5 mL/day for 30g avg. weight mouse was used to calculate total dose.	Positive, 2.1x increase at top dose, but error bars for control and treated measurements overlap, so may not be biologically relevant.	Not clear whether NCE or PCE were scored. Difficult to verify exposure doses from the descriptions, and whether settling out of particles in drinking water was controlled. Oxidative stress indicated since 8-OHdG increased, and evidence of pro-inflammatory response. ToxR Klimisch score 1.
Sadiq et al. (2012)	Nano, 10 nm anatase. *NPs suspended in PBS (5 mg/ml) and vigorously mixed and sonicated for genotoxicity studies.	Nano score 7. * NPs synthesised by the researchers. Characterisation of NPs performed. Some characterisation performed in relevant biological medium.	Peripheral blood reticulocytes MN	Mice	IV dosing at 0.5, 5.0, and 50 mg/kg/day for 3 days. Blood sampled on day 4.	Negative	Target tissue exposure assessed by measuring titanium in bone marrow. ROS/oxidative stress not investigated. ToxR Klimisch score 1.
Dobrzynska et al. (2014)	Nano, NM-105 (20 nm). *NPs suspended in deionised water containing DMSO and probe sonicated for 5 min. on ice and diluted in PBS	Nano score 7. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and some of	Bone marrow PCE and reticulocytes MN	Rats	Single IV dose of 5 mg/kg. Bone marrow sampled 24 hours, 1 and 4 weeks after dosing.	Positive in bone marrow PCE (with limitations) at 24 hours but negative at 1 and 4 weeks and negative in reticulocytes.	Method incompletely described. PCE stained with Giemsa which can produce artefacts (mast cell granules) but

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
	(containing BSA) for genotoxicity studies.	this information is summarised. Additional characterisation performed in relevant biological medium.					reticulocytes stained with acridine orange. ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Louro et al. (2014) & Fessard (2013)	Nano, anatase average diameter 22 nm (NM-102). *Nanogenotox dispersion protocol used: NPs were pre-wetted in 0.5% ethanol and then suspended in 0.05% BSA in MilliQ water (2.56 mg/ml) and probe sonicated for 16 min. on ice and diluted in PBS.	Nano score 10. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-102) which have been extensively characterised and some of this information is summarised. Additional characterisation performed in relevant biological medium.	Peripheral blood MN	C57Bl/c mice with lacZ reporter gene.	IV doses of 10 and 15 mg/kg on 2 consecutive days. Blood sampled 42 hours after last dose.	Negative	15 mg/kg maximum feasible dose based on stability of stock dispersion. Tissue exposure at this dose level described in a different study. ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Suzuki et al. (2016)	Nano (P25). DLS showed particle sizes of 145-147 nm in dosing vehicle (disodium phosphate) *NPs sterilised by heating (180°C for 1 hour), suspended in 2 mg/ml disodium phosphate (DSP) at a concentration of 10 mg/ml and bath sonicated for 30 min. Suspensions then diluted in DSP for genotoxicity studies.	Nano score 3. * Information on NP characteristics obtained from supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium. *NB Whilst there was a reliance placed on presenting information obtained from the suppliers on the characteristics of the NPs, P25 has been	Peripheral blood MN	*Gpt* delta mice	Intravenous; 2, 10 & 50 mg/kg, once per week for 4 weeks; blood sampled 2 & 9 days after last administration.	Negative	MN measured by flow cytometry using Microflow PLUS kit. No reduction in % RETs. ROS/oxidative stress not investigated. ToxR Klimisch score 1.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
		extensively characterised in the published literature.					
Grissa et al. (2015)	Nano, anatase 5-10 nm, suspension in water sonicated. *NPs suspended in distilled water and bath sonicated for 30 min., then mechanically vibrated for 5 min. for genotoxicity studies.	Nano score 4. * No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. No characterisation in relevant biological medium.	Bone marrow MN	Rats	Oral dosing at 50, 100, 200 mg/kg daily for 60 days; unclear when bone marrow was sampled	Positive at 100 & 200 mg/kg/day	Not clear whether MN frequencies were %, per 1000 or per 2000 – Methods says %, in which case control levels are high. Slight bone marrow toxicity at top dose. Haematological changes and inflammation in many tissues. ROS/oxidative stress not investigated. ToxR Klimisch score 2.
Shukla et al. (2014)	Nano, anatase, particle size 20-50 nm, purity 99,7 %. *NPs suspended in MilliQ water (8 mg/ml) and probe sonicated for 20 min. for genotoxicity studies.	Nano score 5. * Some information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium.	Bone marrow MN	Mice	Oral dosing at 10, 50 and 100 mg/kg/day for 14 days. Bone marrow sampled 24 hours after last dose.	Borderline positive (<3-fold increase)	Oxidative stress (increased MDA & ROS at 50 & 100 mg/kg, decreased GSH at 100 mg/kg). ToxR Klimisch score 2.
Relier et al. (2017))	Nano, P25. *NPs suspended in ultrapure water (15 mg/ml) and probe sonicated for 3 min. (1 min.	Nano score 7. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-105) which	Peripheral blood MN	Rats	Endotracheal instillation to lung 3 times 4 days apart; 0.5, 2.5 & 10 mg/kg total doses; blood	Equivocal (significant response after 35 days but not 2 hours after 3rd dose	MN frequencies in treated groups almost identical at 2 hours & 35 days, but ≤2-fold increase at 35 days

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
	on/1 min. off) and then diluted in PBS for genotoxicity studies.	have been extensively characterised and this information is summarised. Some independent characterisation also performed. Some characterisation performed in relevant biological medium.			sampled 2 hours & 35 days later.	(on day 8) seems not plausible).	only statistically significant because vehicle control MN frequency was lower. A decrease in glutathione was observed immediately after exposure at the highest dose in lung cells and 35 days after exposure at the mid dose in liver cells but was not statistically significant due to a large variability. ToxR Klimisch score 2.
Chakrabarti et al. (2019)	Nano, avg. diameter 58 nm. *Method used for NP dispersion not clear.	Nano score 3. *No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium.	Bone marrow MN	Mice	Oral dosing at 200 & 500 mg/kg/day for 90 days. Not clear when bone marrow was sampled.	Positive – significant ~4-fold increase at top dose.	Very long dosing period for assessment of MN in bone marrow. Dose-related increases in oxidative stress & apoptosis. Although oxidative stress was not measured directly, the dose-related accumulation of cells in G2/M suggested this was due to oxidative stress which led to DNA damage.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
							ToxR Klimisch score 2.
Sycheva et al. (2011)	Micro (TDM) and nano simethicone (TDN). *NPs suspended in distilled water forgenotoxicity studies.	Nano score 2. *No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. No characterisation in relevant biological medium.	Bone marrow, forestomach, colon & testis MN	Mice	Oral dosing at 40, 200 & 1000 mg/kg/day for 7 days. Bone marrow and testis sampled 24 hours after last dose.	TDM induced 2X increase in MN in bone marrow; TDN simethicone was negative. TDM and TDN negative in forestomach, colon & testis.	TDM and TDN induced apoptosis in testis and cytotoxicity in forestomach & colon. Authors conclude genotoxic effects are secondary to inflammation and/or oxidative stress. ToxR Klimisch score 3, unreliable.
Naya et al. (2012)	Nano, anatase (ST-01), 5 nm. *NPs suspended in 2 mg/ml disodium phosphate followed by agitation in a bead mill with 15 µm zirconium oxide beads for 2 hours, centrifuged and the supernatant used for genotoxicity studies.	Nano score 5. *Limited information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium.	Comet in lung	Rats	Intratracheal instillation; 1 & 5 mg/kg single dose, 0.2 & 1 mg/kg once per week for 5 weeks.	Negative	Slides not coded. Inflammatory response at 1 & 5 mg/kg. Inflammation induced, oxidative stress discussed, but no DNA damage. ToxR Klimisch score 2.
Relier et al. (2017)	Nano, P25 *NPs suspended in ultrapure water (15 mg/ml) and probe sonicated for 3 min. (1 min. on/1 min. off) and then diluted in PBS for genotoxicity studies.	Nano score 8. *TiO_2 NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and this information is summarised. Some independent characterisation also performed. Some	Comet in lung & liver	Rats	Endotracheal instillation to lung 3 times 4 days apart; 0.5, 2.5 & 10 mg/kg total doses; tissues sampled 2 hours & 35 days later.	Positive in lung (35 days) and liver (both sampling times).	Inflammation and oxidative stress. ToxR Klimisch score 2.

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
		characterisation performed in relevant biological medium.					
Jensen et al. (2019)	E171 purchased from Bolsjehuset (DK). 99.8% anatase, 0.2% rutile. *NPs suspended in 2% FBS in water for genotoxicity studies.	Nano score 3. *No information on NP characteristics obtained from the supplier provided. Some independent characterisation performed in previous studies which are cited and data summarised. Characterisation performed in biological medium not relevant to this study.	Comet in lung & liver	Rats	Oral dosing of 50 & 500 mg/kg/week, once per week for 10 weeks. Tissues sampled 24 hours after last dose.	Negative	Positive control only via in vitro slides. Study done with and without Fpg and hOGG1. No changes to oxidatively damaged DNA in liver and lung. ToxR Klimisch score 1.
Rehn et al. (2003)	P-25 and T805 (trimethoxyoctylsilane-coated). *NPs suspended in saline with 0.25% lecithin and sonicated for 5 min. for genotoxicity studies.	Nano score 7. *Some information on NP characteristics obtained from the supplier provided. Some independent characterisation also performed. Characterisation performed in relevant biological medium. *NB The characteristics of the NPs (P25) have been extensively characterised in the published literature.	8-OHdG adducts in lung cells	Rats	Single intratracheal instillation of 0.15, 0.3, 0.6 & 1.2 mg. Tissues sampled 90 days later.	Negative	Although 30 rats/group were treated, unclear how many were sampled. No oxidative damage found. ToxR Klimisch score 1.
Li et al. (2018)	Nano (rutile, MT-150AW, from Teyka Co. Ltd., Osaka, Japan); 44.9 nm *NPs suspended in distilled water for genotoxicity studies.	Nano score 5 *No information on NP characteristics obtained from the supplier provided. Some independent characterisation performed in previous	8-OHdG adducts in lung cells	Rats	Single intratracheal instillation of 0.2 and 1.0 mg, and whole-body inhalation of 0.50 ± 0.26 mg/m^3 and 1.84 ± 0.74 mg/m^3 for 6 hours/day	Negative	For intratracheal instillation, tissues frozen at −80°C, obtained in previous studies were analysed. Was top dose for

Reference	Type of TiO_2 tested	Nanoparticle characterisation	Endpoint tested	Species	Doses tested & dosing/sampling regimen	WoE conclusion	Comments
		studies which are cited and data summarised. Characterisation performed in relevant biological medium.			and 5 days/week for 4 weeks.		inhalation study high enough? No oxidative damage found. ToxR Klimisch score 2.

Table 6: Datasets reviewed by study type/endpoint and those achieving moderate or higher weight.

Study type	**Nº. of datasets reviewed**	**Nº. achieving moderate or higher weight after WoE assessment**
In vitro		
Bacterial reverse mutation (Ames test)	15	0
Mammalian cell gene mutation	16	2
MN or CA	62	12
In vivo		
Gene mutation	9	2
MN or CA	35	13
Comet	51	3
8-OHdG adducts	4	2
Totals	**192**	**34**

Table 7: Comparison of EFSA and Expert Panel approaches to evaluation of the genotoxicity of TiO_2 (shaded rows show discrepancies)

Parameter	EFSA approach	Expert Panel approach
Non-biological studies	Excluded (at TiAb stage)	Excluded (only studies with a conventional genotoxic endpoint were reviewed)
Studies on non-mammal species (e.g., fish, *Drosophila*, bees) and plants	Excluded (at TiAb stage)	Excluded
In vivo studies with a non-relevant route of administration (e.g., dermal, dental, bone implants)	Excluded (at TiAb stage)	None found
Studies performed only with coated TiO_2	Excluded (at TiAb stage)	Included (if endpoint and test system had default "moderate" or "high" weight)
Studies performed only with TiO_2 nanofibres, nanocomposites or nanotubes	Excluded (at TiAb stage)	Included (if endpoint and test system had default "moderate" or "high" weight)
Reviews, editorials, letters to the editor etc.	Excluded (at TiAb stage)	Excluded (but if original data included in a review paper was found, this was included and both references cited)
Abstract only	Excluded (at TiAb stage), unless there was sufficient information provided	Included (if endpoint and test system had default "moderate" or "high" weight)
In vitro and *in vivo* studies	Included	Included
Gut microbiota studies	Included	Excluded
Toxicokinetic studies	Included	Included (if genotoxicity data in the same publication)
Genotoxicity studies	Included	Included
Local effects (e.g., inflammation, proliferation)	Included	Included (if genotoxicity data in the same publication)
Apical effects, general toxicity	Included	Included (if genotoxicity data in the same publication)

Mechanisms of action (e.g., oxidative stress)	Included	Included (if genotoxicity data in the same publication)
Test/measured endpoints	Included	Only those endpoints and test systems with default "moderate" or "high" weight were included
Information on study design (e.g., type of cells/animal species, doses tested, duration of studies etc.)	Included	Included
Scoring for reliability	Klimisch (1997) giving 5 categories	ToxR Tool (Schneider et al., 2009) giving 3 Klimisch categories
Relevance categories for endpoints	2	4
Gene mutations *in vivo* and the Ames test	High relevance	High default weight
Gene mutations in mammalian cells *in vitro*	High relevance	Moderate default weight
Structural and numerical chromosomal aberrations *in vivo*	High relevance	High default weight
Structural and numerical chromosomal aberrations *in vitro*	High relevance	Moderate default weight
In vivo comet assay	High relevance	Moderate default weight
Other genetic endpoints (presumably SCE, UDS etc., but not clear whether this includes *in vitro* comet assay)	Lower relevance (but included)	Low or negligible default weight (and therefore excluded)
Exposure of cells *in vitro*	More weight was given to study designs including observations confirming that cells were exposed to the nanoparticles. Negative results from studies where the cell uptake was not	Negative results in mammalian cells were accepted, even if cellular exposure was not demonstrated, as long as treatment was for at least 1 cell cycle. Relevance

	demonstrated were considered as inconclusive (to which only low relevance was assigned)	(weight) of the study was then determined by other design and quality factors.
Concentrations tested *in vitro*	A low weight was given to studies performed using only excessively high concentrations i.e. higher than 100 µg/ml (because of aggregation/agglomeration and precipitation of the tested nanoparticles at high concentration).	The relevance (weight) of the study was not changed just because high concentrations were tested, but agglomeration/aggregation was noted if it was measured and reported. Several studies with testing to concentrations >100 µg/mL retained moderate weight.
Cytotoxicity evaluation *in vitro*	A low weight was given to studies in which no parallel toxicity evaluation was performed or an inappropriate toxicity test had been used.	Both negative and positive studies in which there was no concurrent measure of cytotoxicity, or an inappropriate measure of cytotoxicity was used, were considered unreliable and weight was downgraded.
Ames test	Bacterial reverse mutation (Ames) assay is not considered suitable for investigation of gene mutations (due to limitations in the penetration of particles through the bacterial cell wall and the lack of internalisation in bacteria), and therefore assigned low relevance. Hence a higher weight was given to mammalian cell models.	All Ames studies reviewed were given only Low or Low-moderate weight for the reasons given, whereas mammalian cell studies could retain moderate weight if otherwise well-conducted.
In vitro micronucleus test	Higher weight was given to studies with an extended treatment, covering at least one cell cycle. A low weight was given to studies in which cytochalasin B and nanoparticles were simultaneously added (cytochalasin B needs to be added after the nanoparticles, since cytochalasin B might inhibit the cellular uptake of nanoparticles).	Studies with an extended treatment, covering at least one cell cycle (either without cytochalasin B or before cytochalasin B was added) were more likely to retain Moderate weight.
In vitro micronucleus test	A higher weight was given to studies in which the uptake capability of the selected cell lines was demonstrated.	The uptake capability of the cells was not considered since there are few comparative data to make such judgements. The final weight was assessed on multiple design and quality factors.
In vitro micronucleus test	A low weight was given to studies based on cell lines with high background micronuclei frequency (higher than 2%).	The weight of a study was not influenced by whether the background MN frequency was high, but on whether the control MN frequencies were within pre-agreed normal ranges. The same approach was applied to *in vitro* CA and gene mutation studies (not discussed by EFSA).

In vitro comet assay	Evaluation of the relevance of the test design included identification of possible interferences (e.g. interaction of nanoparticles with dye and lysis condition) within the comet assay at the applied test conditions.	*In vitro* comet assays were not reviewed (not included) because, as indicator tests (as specified in OECD guidance document; OECD, 2015a), they are less relevant in terms of genotoxic or carcinogenic risk.
In vivo studies	Because TiO_2 needs to be assessed as a food additive, administration by non-oral routes of exposure was considered of limited or low relevance, depending on the reliability of the study and other aspects such as information on the level of dispersion.	Of the non-oral routes, IP dosing was considered less physiologically relevant. However, IV studies were considered particularly relevant since exposure of the target tissue (e.g., bone marrow, liver) was more likely than by oral dosing.

TiAb = title and abstract (initial stage of screening literature)

Table 8: Comparison of EFSA and Expert Panel studies considered appropriate for review and included in the final assessments

	EFSA approach		Expert Panel approach	
Study type	**No. of studies available for evaluation**	**No. of studies achieving High or Limited relevance (No. positive)**	**No. of datasets reviewed**	**No. achieving Moderate or higher weight after WoE assessment (No. positive)**
In vitro				
Bacterial reverse mutation (Ames test)	8	0	15	0
Mammalian cell gene mutation	14	7 (3 positive)	16	2 (0 positive)
MN or CA	56	43 containing 67 datasets (26 datasets positive)	62	12 (2 positive)
Comet assay	142	106 containing 142 datasets (102 datasets positive)	0	0
DNA binding	5	5 (unclear whether these considered positive)	0	0
8-OHdG adducts	5	5 (4 positive)	0	0
γH2AX foci	4	4 (2 positive)	0	0
ToxTracker	1	1 (0 positive)	0	0
Sub-totals	**235**	**231 datasets (137 positive)**	**93**	**14 (2 positive)**
In vivo				
Gene mutation	6	6 (1 positive)	9	2 (0 positive)
MN or CA	26	15 (8 positive)	35	13 (7 positive)
Comet	44	18 containing 19 datasets (12 datasets positive)	51	3 (1 positive)
DNA binding	2	2 (unclear whether these considered positive)	0	0
8-OHdG adducts	2	1 (1 positive)	4	2 (0 positive)
γH2AX foci	2	2 (2 positive)	0	0
Sub-totals	**82**	**45 (24 positive)**	**99**	**20 (8 positive)**
Totals	**317**	**276 (161 positive)**	**192**	**34 (10 positive)**

Note: Studies measuring formation of reactive oxygen species, epigenetic DNA methylation and cell transformation were discussed in the EFSA opinion, but not included in the table above since they appear to be taken as supporting information rather than direct evidence of genotoxic effects.

Table 9: Comparison of specific *in vitro* study datasets from Appendix J of EFSA (2021): Shaded rows show differences in relevance and/or conclusion

Publication and dataset	EFSA assessment*	Expert Panel assessment**
Kazimirova et al. (2020); mammalian cell Hprt gene mutation test	**High relevance**; Reliability score 1; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 2; Nano score 10; **Negative**
Du et al. (2019); mouse lymphoma Tk mutation test	**Low relevance**; Reliability score 3; Nano score 3; **Inconclusive** (based on cellular uptake not demonstrated although cytotoxicity induced)	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 1; **Negative** (based on 24-hour exposure and induction of cytotoxicity)
Andreoli et al. (2018); *in vitro* MN study on human lymphocytes with 5 forms of TiO_2	**High relevance**; Reliability score 1; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 4; **Negative**
Li et al. (2017a); *in vitro* MN study on TK6 cells; microscopy & flow cytometry methods	**High relevance**; Reliability score 1 (for microscopy results); Nano score 2; **Positive** for microscopy; **Inconclusive** for flow cytometry	**Low weight (excluded)**; ToxR Klimisch score 2; Nano score 7; **Negative (<2-fold increase) with limitations** for microscopy; **Uninterpretable** for flow cytometry
Zijno et al. (2015); *in vitro* MN study in Caco-2 cells	**High relevance**; Reliability score 1; Nano score 2; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 2; Nano score 7; **Negative**
Stoccoro et al. (2017); *in vitro* MN study in A549 cells	**High relevance**; Reliability score 1; Nano score 1; **Positive**	**Low-moderate weight (excluded)**; ToxR Klimisch score 2; Nano score 3; **Inconclusive** (test materials not well characterised)
Kurzawa-Zegota et al. (2017); *in vitro* MN study on human lymphocytes	**High relevance**; Reliability score 1; Nano score 2; **Positive**	**Low weight (excluded)**; ToxR Klimisch score 3; Nano score 2; **Uninterpretable** (abstract only, very few details)
Kazimirova et al. (2019); *in vitro* MN study in human lymphocytes & TK6 cells	**High relevance in TK6 cells**; Reliability score 2; **Low relevance in human lymphocytes**; Reliability score 3; Nano score 1; **Negative** in both cell types	**Low-moderate weight (excluded)**; ToxR Klimisch score 2; Nano score 10; **Negative with limitations** (treatment time prior to cytochalasin B too short)
Demir et al (2015); *in vitro* MN study on HEK293 human embryonic kidney cells and NIH/3T3 mouse fibroblasts	**High relevance**; Reliability score 1; Nano score 1; **Positive** in both cell types	**Low weight (excluded)**; ToxR Klimisch score 2; Nano score 3; **Positive** with limitations (unusual cells for MN studies, negative control MN frequencies not established, slides not coded)
Di Bucchianico et al. (2017); *in vitro* MN study on BEAS-2B cells with NM-100, NM101 & NM-103	**High relevance**; Reliability score 1; Nano score 1; **Negative for NM-101** but results for other forms not mentioned	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 10; **Negative for NM-100 & NM101, weak positive for NM-103**

Vales et al. (2014); *in vitro* MN study BEAS-2B cells	**High relevance**; Reliability score 1; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 9; **Negative**
Pittol et al. (2018); *in vitro* MN study on L-929 mouse fibroblasts	**Limited relevance**; Reliability score 2; Nano score 3; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 6; **Negative**
Brandao et al. (2020); *in vitro* MN study on 4 cell lines	**High relevance**; Reliability score 1; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 3; **Negative**
Zijno et al. (2020); *in vitro* MN study on BEAS-2B cells	**High relevance**; Reliability score 1; Nano score 1; **Negative**	**Low-moderate (excluded)** based on too short exposure time before cytochalasin B; ToxR Klimisch score 2; Nano score 7; **Negative**
In vitro comet studies	**High relevance for 20 studies; 16 Positive**	**Low weight; all *in vitro* comet assays excluded**

* Reliability score range 1-5; Nano score range 1 (highest) to 4 (lowest)

** ToxR Klimisch score range 1-3; Nano score range 0 (lowest) to 10 (highest)

"Limited relevance" in the EFSA scheme is considered similar to "Moderate weight" in the Expert Panel scheme, since both were considered suitable for further evaluation.

Table 10: Comparison of specific *in vivo* study datasets from Appendix K of EFSA (2021): Shaded rows show differences in relevance and/or conclusion

Publication and dataset	EFSA assessment*	Expert Panel assessment**
Suzuki et al. (2020); *in vivo* gpt & spi mutation studies in transgenic mice, 1x/week IV dosing for 4 weeks	**Limited relevance**; Reliability score 2; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 3; **Negative with restrictions** (based on only 1x/week dosing)
Chakrabarti et al. (2019); *in vivo* MN & CA studies in mouse bone marrow, 90 daily oral doses	**Limited relevance**; Reliability score 2; Nano score 4; **Positive for both MN & CA**	**Low-moderate weight for CA study (excluded); Moderate-high weight for MN study (included);** ToxR Klimisch score 2; Nano score 3; **CA data uninterpretable; MN data Positive with limitations** (MN in bone marrow after 90 days dosing unusual; evidence of oxidative stress)
Grissa et al. (2015); *in vivo* MN study in rat bone marrow, 60 daily oral doses	**Limited relevance**; Reliability score 2; Nano score 2; **Positive**	**Moderate weight (included)**; ToxR Klimisch score 2; Nano score 4; **Positive** (associated with haematological changes & inflammation)
Suzuki et al. (2016); *in vivo* MN study in mouse reticulocytes, 1x/week IV dosing for 4 weeks	**Limited relevance** (based on IV route not being relevant); Reliability score 2; Nano score 1; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 3; **Negative**
Manivannan et al. (2020); *in vivo* CA study in mouse bone marrow, 28 daily oral doses	**Limited relevance**; Reliability score 2; Nano score 2; **Positive**	**Moderate-high weight (included)**; ToxR Klimisch score 3 (unreliable); Nano score 8; **Positive (**at high bone marrow toxicity)
Shukla et al. (2014); *in vivo* MN study in mouse bone marrow, 14 daily oral doses	**High relevance**; Reliability score 1; Nano score 1; **Positive**	**Moderate-high weight (included)**; ToxR Klimisch score 2; nano score 6; **Borderline positive (<3-fold), associated with oxidative stress**
Relier et al. (2017); *in vivo* MN study in rat peripheral blood, endotracheal instillation to lung 3 times 4 days apart	**Low relevance**; Reliability score 3; Nano score 1; **Equivocal**	**Moderate weight (included)**; ToxR Klimisch score 2; Nano score 8; **Equivocal**
Jensen et al. (2019); *in vivo* comet assay in lung and liver, oral dosing once/week for 10 weeks	**High relevance**; Reliability score 1; Nano score 2; **Negative**	**Moderate weight (included)**; ToxR Klimisch score 1; Nano score 3; **Negative**
Shukla et al. (2014); *in vivo* comet assay in mouse liver, 14 daily oral doses	**High relevance**; Reliability score 1; Nano score 1; **Positive without and with Fpg**	**Low weight (excluded)**; ToxR Klimisch score 3 (unreliable); Nano score 5; **Positive with limitations** (inadequate description of method,

		no early sample times, high control %tail intensity, increases in ROS and liver injury)
Relier et al. (2017); *in vivo* comet assay in rat lung, blood and liver, endotracheal instillation to lung 3 times 4 days apart	**Limited relevance**; Reliability score 2; Nano score 1; **Positive in all 3 tissues**	**Moderate weight (included)**; ToxR Klimisch score 2; Nano score 8; **Positive in lung and liver** (associated with inflammation and oxidative stress)
Jin et al. (2013); *in vivo* DNA binding assay in rat liver, 45 daily intranasal administrations	**High relevance**; Reliability score 1; Nano score 1; **Positive** for NPs anatase and anatase/rutile mixture	**Low weight (excluded)**; not reviewed since adducts are only an indicator of genotoxic potential, not an apical endpoint.
Li et al. (2010); *in vivo* DNA binding assay in mouse liver, 14 daily IP injections	**High relevance**; Reliability score 1; Nano score 1; **Positive**	**Low weight (excluded)**; not reviewed since adducts are only an indicator of genotoxic potential, not an apical endpoint.

* Reliability score range 1-5; Nano score range 1 (highest) to 4 (lowest)

** ToxR Klimisch score range 1-3; Nano score range 0 (lowest) to 10 (highest)

“Limited relevance” in the EFSA scheme is considered similar to “Moderate weight” in the Expert Panel scheme, since both were considered suitable for further evaluation.

Table 11 Comparison of test response profiles from TiO_2 to the profile characteristics of confirmed genotoxic carcinogens (adapted from Brusick et al. (2016); based on Bolt et al. (2004) and Petkov et al. (2015)).

Characteristic	**Carcinogens with a proven genotoxic mode of action**	**TiO_2**
Profile of Test Responses in Genetic Assays	Positive effects across multiple key predictive endpoints (i.e. high weight studies such as gene mutation in bacteria or *in vivo*, chromosomal aberrations or micronuclei *in vivo).*	No valid evidence for gene mutation in mammalian cells or *in vivo*; chromosomal damage in rodents only at doses inducing cytotoxicity, inflammation, oxidative stress.
Structure Activity Relationships	Positive for structural alerts associated with genetic activity.	Not done
DNA binding	Agent or breakdown product are typically electrophilic and exhibit direct DNA binding.	No evidence of DNA binding, and no evidence of 8-OHdG adducts in robust *in vivo* studies
Consistency	Positive test results are highly reproducible both *in vitro* and *in vivo.*	Conflicting and/or non-reproducible responses in the same test or test category both *in vitro* and *in vivo.*
Response Kinetics	Responses are dose dependent over a wide range of exposure levels.	Dose responses in robust, reliable test systems generally not observed.
Susceptibility to Confounding Factors (e.g. Cytotoxicity)	Responses are typically found at non-toxic exposure levels.	Positive responses in robust, reliable test systems typically associated with evidence of apoptosis, necrosis, inflammation and oxidative stress.

Fig 1: Profile of results for *in vitro* studies

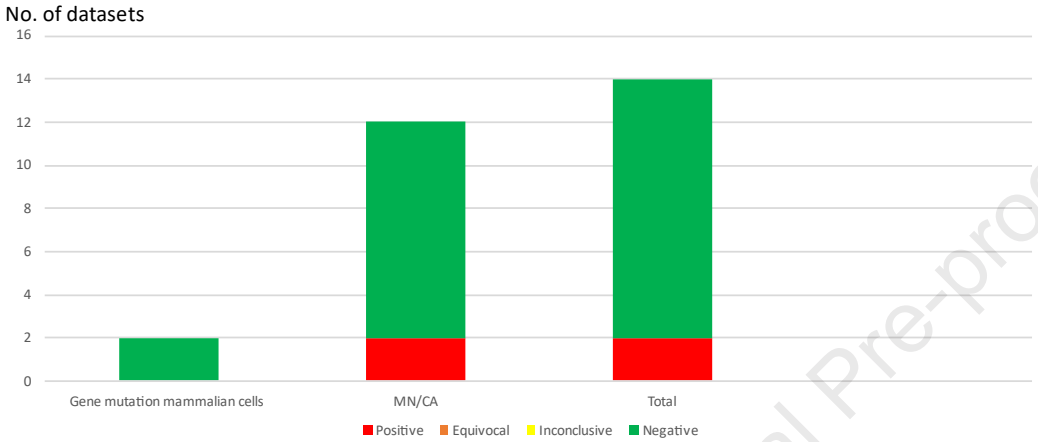


Fig 2: Profile of results for *in vivo* studies

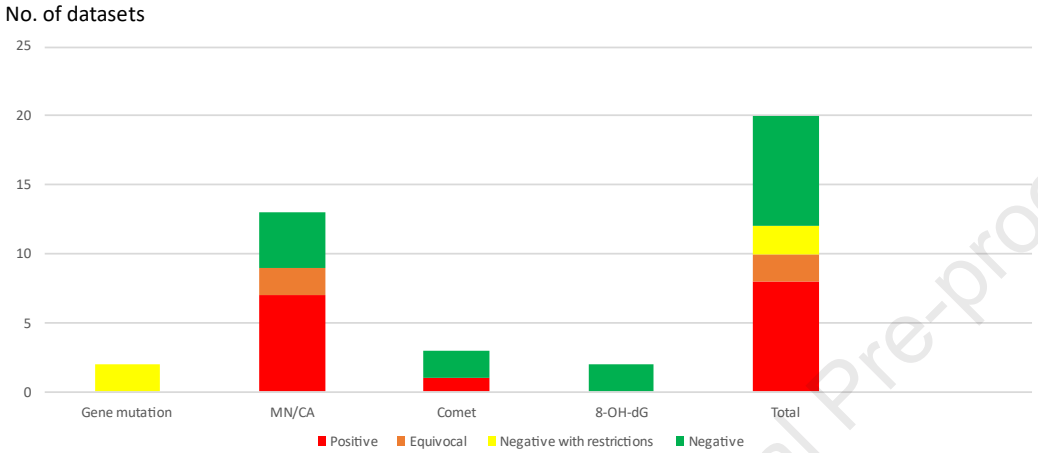


Fig 1: Profile of results for *in vitro* studies

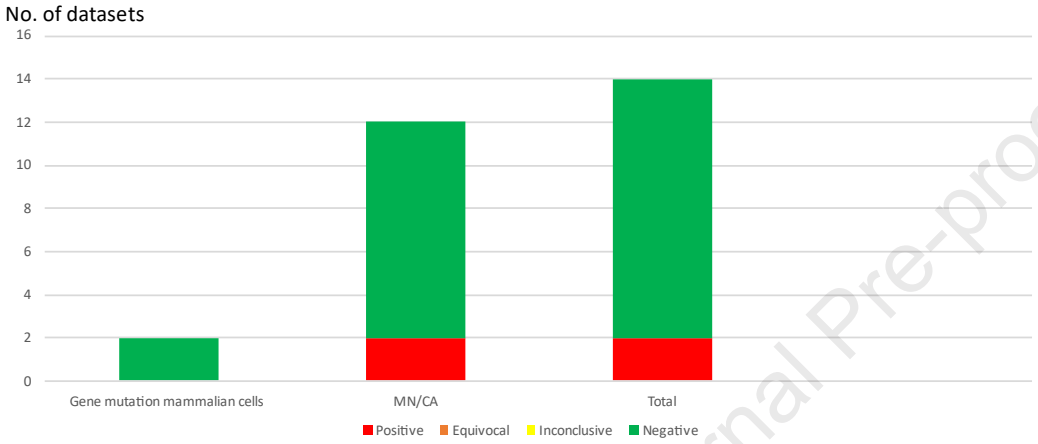
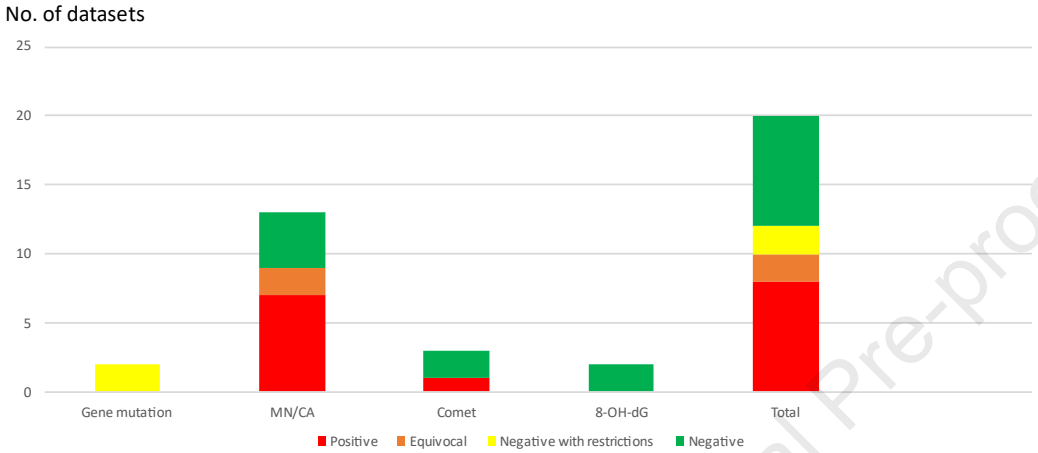


Fig 2: Profile of results for *in vivo* studies



Highlights

- EFSA have recently banned titanium dioxide in foods due to concerns over genotoxicity
- A tiered weight of evidence analysis was performed on genotoxicity data for TiO_2, according to relevance and reliability.
- TiO_2 was positive for chromosome damage mainly at levels where reactive oxygen or other cellular toxicity were prevalent.
- TiO_2 was negative for point mutations *in vivo*, the panel noted more data would be required to make definitive conclusions.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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