Committee on Mutagenicity

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COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

Assessment of i*n vivo* studies of TiO_2 genotoxicity

1. Following the publication of the opinion on titanium dioxide (TiO_2) by the European Food Safety Authority (EFSA) entitled 'Safety assessment of titanium dioxide (E171) as a food additive' (EFSA, 2021), the Committee on the Mutagenicity (COM) has been asked to provide an opinion on its genotoxicity.

2. For those studies that were considered appropriate, a narrative is presented in the paper, outlining methodology, results, conclusion and COM opinion.

Questions for the Committee

3. Members are asked to consider the following questions:

   i. Do members agree with COM opinions of the *in vivo* papers?

   ii. Do members consider TiO_2 to be genotoxic based on the *in vivo* data?

   iii. Overall, considering *in vitro* and *in vivo* data, do members consider TiO_2 to be genotoxic?

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Committee on the Mutagenicity of Chemicals in Food, Consumer Products and the Environment.

Assessment of *in vivo* studies of TiO_2 genotoxicity

Introduction

1. Following the publication of the opinion on titanium dioxide (TiO_2) by the European Food Safety Authority (EFSA) entitled 'Safety assessment of titanium dioxide (E171) as a food additive' (EFSA, 2021), the Committee on the Mutagenicity (COM) has been asked to provide an opinion on its genotoxicity.

Methodology

Screening and evaluation of papers

2. The *in vivo* studies referenced in the EFSA opinion (EFSA, 2021) were collated. An additional literature search was carried out to identify papers published between 2021-2023 (see Annex I for search methodology). All papers were screened against a series of criteria to assess the characteristics of the nanomaterial used in the study and the generic study design (tier 1), and the generic experimental details of the genotoxicity study including adherence to Organisation for Economic Co-operation and Development (OECD) test guidelines (tier 2). These criteria were assessed by several members of the Committee through an iterative process. Finally, the experimental details of the study were thoroughly evaluated using expert judgement (tier 3).

Tier 1. Nanomaterial and generic study design

3. When assessing papers based on nanomaterial characteristics and generic study design, all papers were scored against the criteria outlined in

Table 1. If sufficient data were available in the paper, a score of 1 was given. For example, a score of 1 was awarded if sufficient data were presented on nanoparticle (NP) size and shape or exposure duration and observation time points. More weight was placed on some criteria, such as availability of data on positive controls and the number of animals per group, and hence these were given a score of 2.

4. Papers with a total score of 7 out of 10 and above proceeded to tier 2 of screening and were further evaluated by assessing the basic genotoxicity study design (see below).

Table 1 Tier 1 Assessment criteria for nanomaterial characteristics and generic study design of *in vivo* genotoxicity studies on TiO_2

NM characteristics

- Test material and crystalline form (score = 2)
- Particle size (score = 1)
- Particle shape (score = 1)
- Size at the start or at the end of the exposure period (score = 1)
- Duration of exposure as well as time-points of observations (score = 1)

Study design

- Use positive controls (where appropriate) (score = 2)
- Number of animals per group (score = 2)

Tier 2. Generic genotoxicity study design

5. When assessing papers based on generic genotoxicity study design, papers were scored against the criteria outlined in Table 2. As with the assessment of nanomaterial characteristics, some characteristics of the genotoxicity study design, including use of positive and negative controls and number of animals, were given a higher weighting. Papers with a total score of 12 out of 16 and above went to tier 3 and were further evaluated by expert

review by COM members, in which the detailed genotoxicity study design was assessed.

Table 2: Tier 2 Assessment criteria for genotoxicity study design of *in vivo* genotoxicity studies on TiO_2

NM characteristics • Identification of test material (score = 1) • Source/origin of test material (score = 1) • Purity (concentration) of test material (score = 1) • Doses administered or concentration in exposure media (score = 1) • Test medium or vehicle (score = 1)
Study Design • Species (score = 1) • Route of administration (score = 1) • Frequency and duration of exposure, and timepoints of observations (score = 1) • Use of negative controls (score = 2) • Use of positive controls (score = 2) • Numbers of animals (score = 2) • Study methods described (score = 1) • Statistical analysis (score = 1)

Tier 3. Detailed genotoxicity study design

6. When assessing papers based on the detailed genotoxicity study design, data on the criteria outlined in Table 3 were collated. The data were assessed using the assessment criteria listed below, using expert judgement.

Table 3: Tier 3 Assessment criteria for genotoxicity study design of *in vivo* genotoxicity studies on TiO_2

Nanomaterial characteristics • Primary and secondary size
Nanomaterial dispersion • Method and surfactant
Test system • Animal model, species, strain and sex • No. animals/group • Exposure route • Dosing regime • Sampling time • Dose range • Number of cells scored • Tissue • Standard test system
Cytotoxicity assessment • Cytotoxicity test used • Extent of cytotoxicity at genotoxic dose
Controls • Negative control (background level) • Positive control • Level of increase over background
Cellular/target tissue uptake
Mechanism of action data
Results
Opinion on study quality and validity of approach

Exclusion criteria

7. Expert judgement was used to assess the quality and interpretation of the genotoxicity studies by noting a number of assessment criteria.

8. Only assays with OECD guidelines were included in the assessment, including the micronucleus (MN) assay (OECD TG474), chromosomal aberration (CA) assay (OECD TG475), Comet assay (OECD TG489),

transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488) and the phosphatidylinositol glycan class A gene (Pig-a) mutation assay (OECD TG470). The γ-H2AX assay and oxidative damage markers were also included for mechanistic information. Other assays were excluded from further evaluation.

9. Other exclusion criteria included the lack of positive controls, inadequate numbers of animals used, inappropriate sampling times, inadequate number of cells scored and high spontaneous levels of damage.

10. The studies were assessed according to the exclusion criteria and, based on the results, were classified as Red, Amber or Green (RAG rating). Green indicates good robust studies without major deficiencies identified; Amber indicates studies considered sufficient for assessment, but with noted deficiencies; and Red indicates studies with significant deficiencies in procedural descriptions or protocols meaning that they were not of sufficient quality for use in the assessment of genotoxicity of TiO_2.

11. Application of the exclusion criteria listed above automatically led to some studies being graded as red (RAG rating) and these were not further assessed.

12. Overall, from a total of 53 papers that were initially assessed, six papers were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the *in vivo* genotoxicity assessment of TiO_2 (Figure 1).

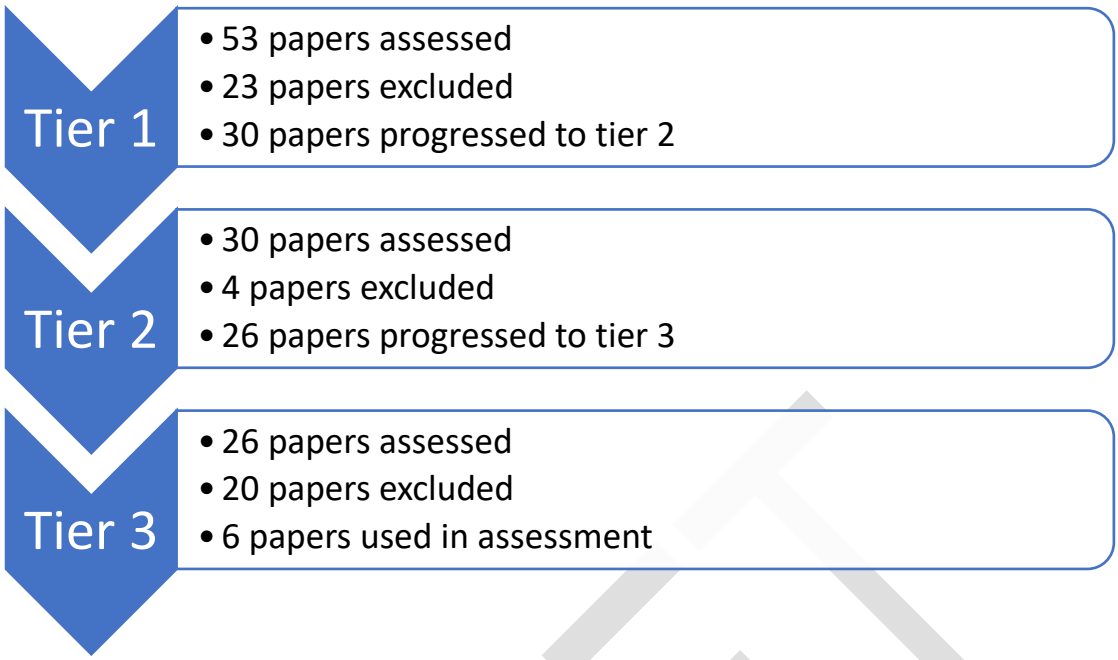

Figure 1 Summary of number of papers assessed in tier 1, 2 and 3

13. The six papers (reporting 12 assays) are summarised below together with a brief summary of the COM opinion for each paper. An overall summary draws a conclusion on the potential *in vivo* genotoxicity of TiO_2.

14. Two of the 12 assays were considered the most robust and were categorised as green, and ten assays were categorised as amber. The number and type of assay in each category is shown in Table 4.

15. Table 4

Table 4 Number and type of genotoxicity study classified as green, amber or red

Test	Green category	Amber category	Red category
MN	2	3	9
CA		1	1
Comet		1	15
hprt			1
Pig-a mutation		3	
gpt mutation		1	
γ-H2AX		1	2

Note: some papers assessed several endpoints

‘Green’ papers – MN assay

Donner et al. (2016)

16. Donner et al. (2016) reported the results of six studies evaluating the potential of three pigment-grade (PG), and three nanoscale (ultrafine (UF)) TiO_2 forms to induce MN by analysis of micronucleated reticulocytes (MN-RETs) in rat peripheral blood cells, according to OECD TG474.

17. The three nanoscale (ultrafine; UF) samples contained anatase and rutile crystal structures (UF-1; 89%/11%; primary size 43 nm), anatase (UF-2; 100%; 42 nm) or rutile (UF-3; 100%; 47 nm). The pigment grade (PG) TiO_2 samples were anatase (PG-1; 100%; primary size 153 nm) and rutile (PG-2; 100%; 195 nm and PG-3; 100%; 213 nm). Secondary sizes were unclear. Particles were dispersed in deionised water by probe sonication.

18. The studies were conducted in two different laboratories on Wistar (UF-3, PG-2 and PG-3) or Sprague Dawley (SD) (UF-1, UF-2 and PG-1) rats. Five animals/sex/dose (seven in the highest dose group) received a single oral dose of 500, 1000 or 2000 mg/kg body weight (bw) of one of the materials. Peripheral blood was collected at 48- and 72-hours post-dosing for MN evaluation and analysis of titanium. In total, 20,000 RETs per animal were analysed. One PG and one UF material each were evaluated for potential systemic exposure/uptake from the gastrointestinal tract by analysis of TiO_2 in blood and liver.

19. The vehicle control (sterile water) was administered as a single dose by oral gavage. The positive control (cyclophosphamide (CP); 10 mg/kg bw/day) was administered either by oral gavage (UF-1, UF-2 and PG-1) or by intraperitoneal (i.p.) injection (UF-3, PG-2 and PG-3). Cytotoxicity was assessed by the %RETs.

20. No clinical signs of toxicity were observed and there was no change in the %RETs. The negative and positive control groups both exhibited responses consistent with historical control data. The negative controls ranged from 0.06-0.08% and 0.07-0.1% RETs in males and 0.07-0.1% and 0.05-0.1% RETs in females, at 48 and 72 hours, respectively. In the PG-1 study, the positive controls showed MN induction levels 7.9--fold higher than background levels in males and 7.8-fold higher in females at 48 hours. For PG-2, positive control levels were 19.7-fold higher than background in males and 15.3-fold higher in females at 48 hours. For PG-3, positive controls were 13.4-fold higher than background in males and 14.1-fold higher in females at 48 hours. At 72 hours, the positive control group was either not evaluated (UF-2, PG-2, PG-3) or the % RETs in this group was not significantly higher than background (UF-1, UF-3, PG-1).

21. Following treatment, no biologically relevant increases in MN-RET frequency was observed in any TiO_2-exposed group, and no biologically relevant decreases in %RETs was seen among total erythrocytes.

22. There were no significant increases in TiO_2 in blood compared to controls (48 or 72 hour) or liver (72 hour) following exposures up to the OECD 474 guideline limit dose of 2000 mg/kg bw TiO_2, indicating that there was little or no absorption of the test material from the GI tract into the blood circulation. The authors noted that the observed lack of genotoxic effects for all six test materials might have been due to a lack of exposure owing to the inability of the test material to migrate from the GI tract into the blood.

23. COM Opinion: Whilst considered to be a good study, with a comprehensive assessment of primary particle characteristics, preparation of test material suspensions and secondary characteristics were not described clearly. Also, the positive control was not significant for some of the 72-hour timepoints, although concurrent positive control data for all timepoints is not mandatory. All 48-hour positive control treatments responded as expected and the vehicle control data were within expected ranges, thus demonstrating laboratory proficiency. Despite such reservations, the study was given a RAG rating of green.

24. The authors claim that lack of absorption from the GI tract (based on terminal toxicokinetic (TK) sampling) was the reason for the lack of genotoxicity, but TK sampling timepoints were not measured robustly enough to be considered definitive. There was a potential lack of adequate bone marrow exposure for hazard identification, however the authors tested up to the maximum recommended dose and the route of exposure was physiologically relevant for dietary exposure of TiO_2 from a risk assessment perspective. Overall, the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

Sadiq et al. (2012)

25. Sadiq et al. (2102) conducted MN and Pig-a mutation assays to evaluate the genotoxicity of TiO_2 anatase NPs in mice.

26. The TiO_2-NPs had a narrow size distribution. The particles had a slight ellipsoidal shape, with the minor axes (smallest diameter) of 12.1 ± 3.2 nm. TiO_2-NPs were prepared in PBS and sonicated. NP agglomerations consisting

of a few hundred NPs had a size distribution of around 130 nm in the treatment solution and around 170 nm in cell culture medium.

27. Groups of five male B6C3F1 mice were treated via intravenous (i.v.) injection for three consecutive days with 0.5, 5.0, and 50 mg/kg bw TiO_2-NPs. Blood was sampled one day before the treatment and on Day 4, and at weeks 1, 2, 4, and 6 after the beginning of the treatment. %MN-RET frequencies were measured on Day 4 only.

28. Additional animals were treated i.v. with three daily doses of 50 mg/kg bw TiO_2-NPs for the measurement of titanium levels in bone marrow, 4, 24, and 48 hours after the last treatment.

29. The negative control was PBS and the positive control was N-ethyl-N-nitrosourea (ENU; 140 mg/kg bw) administered once via i.p. In total, 20,000 CD71-positive RETs were counted for each animal. Cytotoxicity was assessed using %RETs.

30. The titanium levels in bone marrow were significantly increased over the control at all three sampling times, with fold changes ranging from 12.1 to 14.2, suggesting that the TiO_2-NPs reached the bone marrow, the target tissue for the genotoxicity assays.

31. The negative control was approximately 0.3% MN-RETs and the positive control was 8- to 9-fold higher than background.

32. Following treatment, no differences in %MN-RET frequencies were observed between TiO_2-NP-treated and negative control animals.

33. The authors concluded that the 10 nm TiO_2-NPs tested were not clastogenic or aneugenic in the MN assay at the dose levels studied; they were, however, cytotoxic to mouse bone marrow (~35% reduction in RETs at 5mg/kg and 55% reduction at 50 mg/kg TiO_2 on Day 4). Thus, although TiO_2-NPs can reach the mouse bone marrow and are capable of inducing cytotoxicity, they were not demonstrated to be genotoxic.

34. COM Opinion: This study was considered to be of good quality with appropriate dosing and the study was given a RAG rating of green. Whilst the

i.v. route was not physiologically relevant, this did ensure target organ (bone marrow) exposure and overall the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

‘Amber’ papers – MN assay

Chakrabarti et al. (2019)

35. Chakrabarti et al. (2019) evaluated the cytotoxic potential of TiO_2-NPs both *in vitro* and *in vivo*. The *in vivo* genotoxicity tests conducted included MN and CA assays carried out according to OECD TG 474 and 475, respectively, and a Comet assay (see section 63 for CA data). The Comet assay was given a RAG status of red so is not included in this review.

36. Scanning electron microscope (SEM) analysis of TiO_2-NPs (type not given) revealed a spherical, smooth, homogenous, and uniform structure. The average particle diameter was 58.25±8.11 nm. No information on the secondary size was provided, nor on the method of dispersal, with the authors simply saying NPs were suspended in 500 µl water.

37. Groups of 5 male and 5 female Swiss albino mice were orally exposed to either vehicle only (water) or 200 or 500 mg/kg bw/day of TiO_2-NPs suspended in water, for 90 days. Lungs, heart, liver, bone marrow, and kidneys were collected at termination (day 91).

38. Cyclophosphamide (positive control; 40 mg/kg bw/day) was administered as a single dose by i.p. on day 88 and animals were sacrificed 48 hours later. The MN test was conducted on bone marrow, with 2000 polychromatic erythrocytes (PCE) per animal being scored. Polychromatic erythrocyte/ Normochromatic erythrocyte (PCE:NCE) ratio was used to assess cytotoxicity.

39. No clinical signs were observed. However, on gross examination at necropsy, bleeding was observed in the abdominal and pelvic cavities of animals dosed at 500 mg/kg bw/day.

40. The negative control was 0.14% MNPCE and the positive control was 24-fold higher than background. The PCE:NCE ratio was similar in all groups

(the positive and negative control groups and the 200 and 500 mg/kg TiO_2 treatment groups).

41. Following treatment, %MNPCE was 4-fold higher than background at 500 mg/kg (11.33±1.21 MN PCE/2000 PCE, 0.57% MN PCE) but remained unaltered at the 200 mg/kg bw dose. Cell cycle analysis by flow cytometry showed dose-related oxidative damage / apoptosis (measured as accumulation in G2/M) in liver and kidney cells.

42. COM Opinion: The MN assay was considered appropriate, although only 2000 PCE/animal were analysed; also there was no evidence of bone marrow exposure and no confirmation of cellular uptake. Due to these observations, the study was given a RAG rating of amber.

43. Overall, this study was considered to be positive. However, there was evidence of gross toxicity at the highest dose tested and the authors concluded there was evidence of dose-related oxidative damage. This study was cited in the EFSA review (EFSA, 2021).

El-Ghor et al. (2014)

44. El-Ghor et al. (2014) investigated the effects of co-administration of the free radical scavenger chlorophyllin (CHL) on the clastogenicity, genotoxicity, and mutagenicity of TiO_2 in mice as determined by the MN assay and alkaline Comet assay.

45. The primary size of TiO_2 NPs was <100 nm and secondary size was 45.6±12.9 when suspended in water. Small agglomerates were formed in aqueous solution. Transmission electron microscopy (TEM) indicated that most of the TiO_2-NPs had polyhedral morphologies.

46. Groups of 5 male Swiss Webster mice were administered nano-sized TiO_2 (500, 1000, or 2000 mg/kg bw/day) by i.p. for five consecutive days and sacrificed 24 hours after the last treatment. The MN assay was conducted on bone marrow cells. The alkaline Comet assay was performed on bone marrow, liver, and brain tissues. Biochemical evaluation of hepatic 3,4-methylenedioxyamphetamine (MDA) and glutathione (GSH), and superoxide

dismutase (SOD), cationic amino acid transporter (CAT) and GPx activities was done in animals treated with 500 and 2000 mg/kg bw TiO_2.

47. Water was used as the negative control and CP (25 mg/kg bw) as the positive control. Cytotoxicity was assessed by assessing the PCE/NCE ratio per 1000 cells. In total, 2000 PCEs per animal were scored to determine the number of micronucleated poly-chromatic erythrocytes (MNPCEs).

48. A cytotoxic effect of TiO_2 was indicated by a decrease in the PCE/NCE ratio at 500, 1000 and 2000 mg/kg (0.71, 0.57 and 0.44, respectively), compared with the negative controls. The negative control was 0.52% and the positive control was increased 7-fold over background.

49. Following treatment, a significant dose-related increase in MNPCEs was seen at 500, 1000 and 2000 mg/kg bw TiO_2 (7.8-, 9.9- and 12.4-fold increase over controls respectively).

50. Treatment with TiO_2 significantly increased the MDA level in a dose-dependent manner compared with the negative control and induced a significant dose-dependent decrease in the GSH level, both suggestive of oxidative stress. A statistically significant decrease was also seen in SOD, CAT, and GPx levels, again in a dose-dependent manner.

51. The authors concluded that these results demonstrate dose-dependent clastogenicity, genotoxicity, and mutagenicity in the tested organs, with the highest damage in bone marrow cells, and argue that the observed TiO_2-induced genotoxicity could be attributed to the accumulation of reactive oxidative species (ROS). Moreover, the authors considered the indirect deoxyribonucleic acid (DNA) damage via oxidative stress to be confirmed by the reported high p53 mutations, elevated MDA (marker of lipid peroxidation), and decreased antioxidant defence systems.

52. COM Opinion: In this study it was unclear what type of TiO_2 sample was used and only 2000 PCEs per animal were scored. Also, the i.p. route is not recommended for this type of study and the background micronucleus frequencies were relatively high. There was also a relatively high level of

cytotoxicity at the highest dose tested (56% reduction in PCE/NCE ratio relative to control). Due to these observations, the study was given a RAG rating of amber.

53. The level of positive response, and the fact the response was completely ablated by CHL, is unusual. Overall, this study was considered to be positive, which the authors suggested was associated with oxidative damage. This study was cited in the EFSA review (EFSA, 2021).

Relier et al. (2017)

54. Relier et al. (2017) investigated TiO_2 NP-induced genotoxicity in lung overload (in which high levels of exposure cause impairment of particle clearance from the deep lung) and non-overload conditions in Sprague-Dawley (SD) rats, as measured by MN, Comet, Pig-a mutation and *γ*-H2AX assays (see section 68 for Comet, section 91 for Pig-a mutation data and section 104 for *γ*-H2AX data).

55. The test material was TiO_2 NPs (AEROXIDE TiO_2 P25, also named NM-105 in the OECD Nanomaterial Testing Sponsorship Program). The authors confirmed NPs primary size of 25.6±15 nm and a secondary size of 100 nm. Suspensions of TiO_2 NPs were prepared by sonication and diluted in phosphate-buffered saline (PBS). Particle agglomeration was analysed by centrifugal liquid sedimentation and stability of the final suspensions (zeta potential) was measured.

56. Sprague-Dawley (SD) rats (12 per group) were administered three endotracheal instillations at 4-day intervals, so that exposure was spread over 8 days, providing total NP doses of 0.0, 0.5, 2.5 or 10 mg/kg bw. Six animals from each group were sacrificed 2 hours after the last treatment to investigate immediate NP-induced toxicity (data not shown) and the remaining six were sacrificed 35 days after last treatment to detect mutations induced in bone marrow erythroblasts. Lung, peripheral blood and liver were collected.

57. NP-induced chromosomal damage was assessed in blood with the MN test. In total, 2000 PCEs were assessed.

58. The PCE/NCE ratio was also calculated. The negative control was PBS and the positive controls were methyl methanesulphonate (MMS; 150 mg/kg bw final dose administered in three gavages at a one-day interval just before sacrifice).

59. No change was seen in the PCE/NCE ratio. The negative control MN frequency was 0.1% and the positive control induced a 6-fold higher level of MN than background. Tested doses of TiO_2 P25 NPs did not induce GSH changes in lung, blood or liver, indicating lack of oxidative stress at the time points studied.

60. Following TiO_2 treatment, no changes in MN levels were seen after 2 hours. After 35 days, small statistically significant increases in MN were seen at 0.5, 2.5 and 10 mg/kg bw (2-, 1.8- and 1.9-fold higher than control, respectively). The authors state that such small non-dose related increases may not be considered biologically relevant.

61. COM Opinion: The study was considered acceptable, although dosing was by intratracheal instillation which is not a physiologically relevant route of exposure, and there was no evidence of exposure of the bone marrow. Only 2000 PCE were scored. Due to these observations, the study was given a RAG rating of amber.

62. Overall, the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

'Amber' papers – CA assay

Chakrabarti et al. (2019)

63. Chakrabarti et al. (2019) evaluated the cytotoxic potential of TiO_2-NPs both *in vitro* and *in vivo*. The *in vivo* genotoxic endpoints were estimated by means of MN and CA assays carried out according to OECD TG 474 and 475, respectively as well as the Comet assay (see section 35 for the study methodology and for MN data). The Comet assay was given a RAG status of red so is not included in this review.

64. No clinical signs were observed. However, on gross examination at necropsy, bleeding was observed in the abdominal and pelvic cavities of animals dosed at 500mg/kg bw/day. The negative control was 0.76% and the positive control was 12.7-fold higher than background.

65. Following treatment, there was no significant difference in the percentage of CAs observed between the 200 mg/kg bw group and the negative control, but there was a significant difference in the 500 mg/kg bw group (2.5-fold higher; 1.9%).

66. COM Opinion: The CA assay was considered appropriate, although suitability of the repeat-dose protocol is questionable (due to potential loss of damaged mitotic cells) and positive results were seen only at the highest dose. Also, there was no reference to concurrent toxicity assessment and there was evidence of gross toxicity at the highest dose tested. It is unclear if CA frequency includes or excludes gaps; no details are provided on the types of aberrations found which makes interpretation problematic. There is also no evidence of bone marrow exposure, and cellular uptake is not confirmed. Due to these observations, the study was given a RAG rating of amber.

67. Overall, this study was considered to be positive, however, cell cycle analysis is suggestive of dose-related oxidative damage and/or apoptosis. This study was cited in the EFSA review (EFSA, 2021).

‘Amber’ papers – Comet assay

Relier et al. (2017)

68. Relier et al. (2017) investigated TiO_2 P25 NP-induced genotoxicity in lung overload and non-overload conditions in SD rats as measured by MN, Comet, Pig-a mutation and γ-H2AX assays (see section 54 for the study methodology and MN data, section 91 for Pig-a mutation data and section 104 for *γ*-H2AX data).

69. NP-induced DNA damage was assessed in lung, peripheral blood, and liver cells using the Comet assay. One hundred and fifty cells per rat were assessed for tail DNA percentages.

70. Cytotoxicity was assessed on BAL and serum through LDH release, inflammatory cytokine markers and haematology parameters. The negative control was PBS and the positive control was MMS (150 mg/kg bw final dose administered in three oral gavages at one-day intervals just before sacrifice). TiO_2 uptake was measured in the lung and liver.

71. Following treatment, increases in LDH levels and macrophage accumulation were observed at 2.5 and 10mg/kg bw. Increases in inflammatory cytokines measured in BAL and plasma, and increases in neutrophil counts, were noted at 10mg/kg bw. The %tail intensity in the negative control was 0.3% and 0.7% in liver and blood, respectively, after 2 hours, and 0.6% and 0.4% after 35 days. The positive control was 57-, 13- and 17-fold higher in lung, liver and blood, respectively. Tested doses of TiO_2 P25 NPs did not induce GSH changes in lung, blood or liver, indicating lack of oxidative stress at the time points studied.

72. Following treatment, the lowest tested doses had no toxicity or genotoxicity effects in the lung. In blood, no lymphocyte DNA damage, could be detected. Following treatment with higher doses, in the lung % tail intensity was increased at 2.5 and 10 mg/kg bw (3.2- and 4.7-increase compared to controls, respectively) after 35 hours. In liver, an increase in DNA damage was seen at both time points at 2.5 and 10 mg/kg bw (2 hours; 3.4- and 3.7-fold increase, respectively; 35 days; 3.8-fold increase at both doses). In blood, no DNA damage was seen after 2 hours but a slight increase was seen after 35 hours at the highest two doses (2.0- and 2.3-fold increase, respectively).

73. TiO_2 was found above the basal level (supplied in diet) in the lung and liver. At 2 days, TiO2 was found in the lung in all dose groups in a dose-dependent manner (0.5, 2.4, and 9.2 mg TiO_2/g lung; almost 100% of administered doses) and in the liver only at the highest dose (10% of the

administered dose). After 3 months, TiO_2 continued to be found in the lung in a dose-dependent manner.

74. COM Opinion: The study was considered acceptable, although dosing was by intratracheal instillation, which is not a physiologically relevant route of exposure, but there was evidence of target organ exposure in the lung and liver. The authors did not include a 24-hour sampling timepoint as recommended by OECD TG 489. Therefore, the study was given a RAG rating of amber.

75. Overall, the study was considered to be positive, however the observed genotoxicity was associated with cytotoxicity and inflammatory changes. This study was cited in the EFSA review (EFSA, 2021).

'Amber' papers – Pig-a mutation assay

Sadiq et al. (2012)

76. Sadiq et al. (2102) conducted MN and Pig-a mutation assays to evaluate the genotoxicity of 10 nm TiO_2 anatase NPs in mice (see section 25 for the study methodology and MN data).

77. Groups of five male B6C3F1 mice were treated by i.v. for three consecutive days with 0.5, 5.0, and 50 mg/kg bw TiO_2-NPs. Blood was sampled one day before the treatment and on Day 4, and at weeks 1, 2, 4, and 6 after the beginning of the treatment. Pig-a mutant frequencies were determined at day −1 and weeks 1, 2, 4 and 6 in $1x10^6$ RBC and $3x10^5$ RET/animal.

78. A reduction in %RETs was observed in TiO_2-NP-treated animals on Day 4, suggesting treatment-related cytotoxicity with an associated rebound erythropoiesis in week 1 and normalisation by Day 28. The negative control was 0 to 1.2×10^{-6} and 0 to 4×10^{-6} in red blood cell (RBC) (CD24−) and RET (CD24−). The positive control was 254-305-fold and 216-868-fold higher in RBC (CD24−) and RET (CD24−), based on a maximum ENU response in week 2 (304.80×10^{-6} mutRBC and 864.00×10^{-6} mutRET).

79. Following treatment, no increase was observed in RBC and RET frequencies in TiO_2-NP-treated animals compared with controls.

80. The authors concluded that the 10 nm TiO_2-NPs tested were not mutagenic in the Pig-a mutation assay; they were, however, cytotoxic to mouse bone marrow. Thus, although TiO_2-NPs can reach the mouse bone marrow and are capable of inducing cytotoxicity, they were not demonstrated to be genotoxic.

81. COM Opinion: This study was considered to be of acceptable quality although only three daily doses were used and only 5 animals per dose were used which is not consistent with OECD test guidelines. Due to these observations, the study was given a RAG rating of amber.

82. Overall, the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

Suzuki et al. (2016)

83. Suzuki et al. (2016) investigated the genotoxicity of TiO_2 NP suspensions in male gpt Delta transgenic C57BL/6J mice using MN, Comet, Pig-a mutation and gpt mutation assays (see section 98 for gpt mutation data). The MN and Comet assays were given a RAG status of red so are not included in this review.

84. Titanium dioxide, Aeroxide P25® (TiO_2-P25; 20% rutile and 80% anatase) had an average particle size of 21 nm, The test material was suspended by sonication in disodium phosphate (DSP) The Z-average diameter of the TiO_2-P25 particles in suspension was about 150 nm.

85. Four male gpt Delta transgenic C57BL/6J mice were administered TiO_2-P25 (2, 10 or 50 mg/kg bw per week), via i.v., for 4 consecutive weeks. Mice were sacrificed 9 days after the final injection and blood collected.

86. Disodium phosphate was used as a negative control and ENU (70 mg/kg) as the positive control (single i.p. dose sampled 30 days after dosing). A total of 1,000,000 TER-119-positive cells were analysed to determine the frequency of CD24-negative RBCs.

87. No data on cytotoxicity was presented. The RBC mutation frequency in the negative control group was 0.4±0.55 $x10^{-6}$ (CD24-/CD71-/TER-119+ cells). The mutation frequency in the positive control group was 127.5 times higher than background.

88. Following treatment, the Pig-a mutant frequency was not significantly different in TiO_2-treated groups at any dose compared with the DSP-treated control group.

89. COM Opinion: The study protocol was generally acceptable, although group size was lower than recommended in OECD test guidelines, weekly dosing is not optimal, and there is no mention of mutant cell enrichment in the methodology (albeit the minimum number of cells as recommended in OECD TG 470 were analysed), and no cytotoxicity measurements were described. Due to these observations, the study was given a RAG rating of amber.

90. The vehicle and positive controls gave expected results, and despite the i.v. route the results are clearly negative. This study was cited in the EFSA review (EFSA, 2021).

Relier et al. (2017)

91. Relier et al. (2017) investigated TiO_2 P25 NP-induced genotoxicity in lung overload and non-overload conditions in SD rats as measured by MN, Comet, Pig-a mutation and γ-H2AX assays (see section 54 for the study methodology and MN data, section 68 for Comet data and section 104 for *γ*-H2AX data).

92. NP-induced Pig-a mutation was assessed in the blood of rats sacrificed only at 35 days (three rats per dose group). The number of mutants was recorded relative to a cell population of over 10^8 total RBC and 10^6 RETs.

93. Cytotoxicity was assessed using %RETs. The negative control was PBS and the positive control was N-methyl-N-nitrosourea (MNU; 60 mg/kg bw), given in one i.p. injection 35 days before sacrifice.

94. Following treatment, no change in cytotoxicity was observed at any dose tested. The negative control produced 0.5 $x10^{-6}$ mutant RBCs and 1.3 $x10^{-6}$

mutant RETs. The positive control showed a 52-fold increase in mutant RBCs and a 12.3-fold increase in mutant RETs. Tested doses of TiO_2 P25 NPs did not induce GSH changes in lung, blood or liver, indicating lack of oxidative stress at the time points studied.

95. Following treatment, no increase in the frequency of mutant RBC and RETs was observed.

96. COM Opinion: The study was considered acceptable, although dosing was by intratracheal instillation which is not a physiologically relevant route of exposure, and there was no evidence of bone marrow exposure. The intermittent dosing regimen used in the study is not optimal. Due to these observations, the study was given a RAG rating of amber.

97. Overall, the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

'Amber' papers – *gpt* and *Spi-* mutation assay

Suzuki et al. (2016)

98. Suzuki et al. (2016) investigated the genotoxicity of TiO_2 NP suspensions in male gpt Delta transgenic C57BL/6J mice using MN, Comet, Pig-a mutation and gpt mutation assays (see section 98 for the study methodology and for Pig-a mutation data). The MN and Comet assays were given a RAG status of red so are not included in this review.

99. Four male gpt Delta transgenic C57BL/6J mice were administered once weekly i.v. injections of TiO_2-P25 (2, 10 or 50 mg/kg bw per week) for 4 consecutive weeks. Mice were euthanized on day 9 after the final injection of TiO2-P25; portions of the middle lobe of the liver were removed and stored at −80◦C until genomic DNA isolation. High-molecular-weight genomic DNA was extracted from the liver by the standard method.

100. The negative control produced background mutation frequencies of $0.8x10^{-6}$ for *gpt* and $7.86x10^{-6}$ for *Spi-*. The positive control induced an 11.2-fold increase in *gpt* mutants and a 41.5-fold increase in *Spi-* mutants.

101. Compared with the DSP-treated controls, no significant increase in liver gpt mutation frequency (point mutations) or *Spi-* mutant frequency (deletion mutations) was observed in mice administered TiO2-P25 at any dose, suggesting that TiO2-P25 had no genotoxic effect.

102. COM Opinion: The study protocol was generally acceptable, although group size was lower than recommended in OECD test guidelines, weekly dosing is not optimal, and there is no mention of cytotoxicity measurements. Due to these observations, the study was given a RAG rating of amber.

103. The vehicle and positive controls gave expected results, and despite the i.v. exposure route the results are clearly negative. This study was cited in the EFSA review (EFSA, 2021).

'Amber' papers – *γ*-H2AX assay

Relier et al. (2017)

104. Relier et al. (2017) investigated TiO_2 P25 NP-induced genotoxicity in lung overload and non-overload conditions in SD rats as measured by MN, Comet, Pig-a mutation and *γ*-H2AX assays (see section 54 for the study methodology and MN data, section 68 for Comet data and section 91 for Pig-a mutation data).

105. NP-induced DNA double-strand breaks (DSBs) were assessed in lung, blood lymphocytes, and liver cells with c-H2AX immunostaining. The number of foci per nucleus among 100 cells was counted by fluorescent microscopy and cells were grouped into four categories: 0 foci, 1–4 foci, and 4–10, >10 for analysis. Mean number of foci per cell per rat were also calculated.

106. Cytotoxicity was assessed on BAL and serum through LDH release, inflammatory cytokine markers and haematology parameters. The negative control was PBS and the positive control was MMS (150 mg/kg bw final dose administered in three gavages at a one-day interval just before sacrifice). TiO_2 uptake was measured in the lung and liver.

107. Following treatment, increases in LDH levels and macrophage accumulation were observed at 2.5 and 10mg/kg bw. Increases in inflammatory cytokines measured in BAL and plasma, and increases in neutrophil counts, were noted at 10mg/kg bw. Tested doses of TiO_2 P25 NPs did not induce GSH changes in lung, blood or liver, indicating lack of oxidative stress at the time points studied. The negative controls showed 2-3 mean foci per cell per rat in lung, blood & liver after 2 hours and 35 days. The positive control values were 2-fold higher than background.

108. The number of cells in each of the four categories of foci per nucleus was not different from the vehicle exposed control group, with the exception of the lung in which an increase of DSB was found immediately after exposure to the highest dose. This result was confirmed through analysis of mean foci per cell, which was 2 foci/cell for vehicle, low and mid dose, and 4 foci/cell for the high dose. These results suggest that a high dose of TiO_2 NPs may induce DNA DSBs in the lung.

109. TiO_2 was found above the basal level (supplied in diet) in the lung and liver. At 2 days, TiO2 was found in the lung in all dose groups in a dose-dependent manner (0.5, 2.4, and 9.2 mg TiO_2/g lung; almost 100% of administered doses) and in the liver only at the highest dose (10% of the administered dose). After 3 months, TiO_2 continued to be found in the lung in a dose-dependent manner.

110. COM Opinion: The study was considered acceptable, although dosing was by intratracheal instillation, which is not a physiologically relevant route, but there was evidence of target organ exposure in the lung and liver. The positive control was only 2-fold higher than background. Due to these observations, the study was given a RAG rating of amber.

111. Overall, the study was considered to be positive in the lung 2h after dosing at the highest dose, which was also associated with inflammation and particle overload. This study was cited in the EFSA review (EFSA, 2021).

Summary

112. Six papers were identified, following screening of papers cited in the EFSA opinion (EFSA, 2021) as described in the methodology section and an assessment of the newer literature (2021 – 2023; Annex 1), to be of sufficient quality to warrant further assessment.

113. Regarding the *in vivo* genotoxicity of TiO_2, a total of eleven studies covering five genotoxicity assays, namely the MN (green=2; amber=3), CA (amber=1), Comet (amber=1), Pig-a (amber=3) and *gpt* and *Spi*- mutation (amber=1) assays, all of which are recognised by the OECD and other international regulatory bodies, were considered to be of sufficient quality. Data from the γ-H2AX assay (amber=1) were also included to give mechanistic information.

114. Studies were assessed as red, amber or green based on study design, using criteria outlined in Table 3. An overall summary of the data are presented in

115. Table 4 and results of each study are summarised in **Error! Reference source not found.** to **Error! Reference source not found.**.

116. Several of the papers also outlined non-regulatory experiments on the role of oxidative stress and DNA interactions which may aid insight into mechanisms of action.

117. Exposure to the test material in the studies was via the oral route, by i.v. injection, i.p. injection, or by endotracheal instillation.

118. Positive results for *in vivo* genotoxicity were obtained in the MN assay by both oral and i.p. exposure, in the CA assay after oral exposure, and in the comet assay following endotracheal instillation. All three i.v. studies were negative, but limitations in the study designs were noted.

119. Only two studies were deemed to have used robust methodology. In one of these, utilised the MN assay to detect MN-RET in peripheral blood cells following oral dosing. No biologically relevant increases in MN-RET frequency were observed in any TiO_2 exposed group and no biologically

relevant decreases in %RETs was seen. The observed lack of genotoxic effects was attributed by the authors to a lack of exposure due to the inability of the test material to migrate from the GI tract into the blood.

120. In the other 'green' study (Sadiq et al., 2021), no differences in %MN-RET frequencies were observed between TiO_2-NP-treated and negative control animals following i.v. injection. The authors concluded that the 10 nm TiO_2-NPs tested were not clastogenic or aneugenic in the MN assay at the dose levels studied; they were, however, cytotoxic to mouse bone marrow. Thus, although the TiO_2-NPs were demonstrated to reach the mouse bone marrow and be capable of inducing cytotoxicity, they were not shown to be genotoxic.

121. Results from the three oral studies are considered the most physiologically relevant for dietary exposure to TiO_2. These utilised the MN assay (two studies) and the CA assay (one study). Positive results were obtained in the CA assay, but only at the highest dose, and in one MN assay, again only at the highest dose. These positive changes were associated with cytotoxicity, oxidative damage and/or inflammation. As noted above, the only robust oral study (which utilised the MN assay) yielded negative results.

Table 5 Summary of the 'Green' MN results

Test material	**Primary size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
PG and UF TiO_2	PG 153-213 nm UF 42-47	500-1000	5 male and females Wistar or CD rats	Oral (no further details) Single dose	MN	Neg	Donner et al. (2016)
TiO_2 NPs (anatase)	Ellipsoidal, with minor axes 12.1 ± 3.2 nm. Agglomerations had size distribution of c.130-170 nm	0.5-50	5 male B6C3F1 mice	I.V. injection Three consecutive daily doses	MN-RET	Neg	Sadiq et al. (2012)

Table 6 Summary of the 'Amber' MN results

Test material	**Primary size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
TiO_2-NPs (no further info)	58.25±8.11 nm	200-500	5 male and female Swiss albino mice	Oral (no further details) 90 days	MN	Pos (associated with gross toxicity and oxidative damage)	Chakrabarti et al. (2019)
TiO_2 NPs	<100 nm	500-2000	5 male Swiss Webster mice	i.p. 5 days	MN	Pos (associated with oxidative damage and a relatively high level of cytotoxicity)	El-Ghor et al. (2014)
AEROXIDE TiO_2 P25 (NM-105)	25.6±15 nm	0.5-10	12 male SD rats	Endotracheal instillation 3 instillations over 8 days	MN	Neg	Relier et al. (2017)

Table 7 Summary of the 'Amber' CA results

Test material	**Primary size**	**Dose** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
TiO_2-NPs (no further info)	58.25±8.11 nm	200-500	5 male and female Swiss albino mice	Oral (no further details) 90 days	CA	Pos (associated with gross toxicity and oxidative damage)	Chakrabarti et al. (2019)

Table 8 Summary of the 'Amber' Comet assay results

Test material	**Primary size**	**Dose** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
AEROXIDE TiO_2 P25 (NM-105)	25.6±15 nm	0.5-10	12 male SD rats	Endotracheal instillation 3 instillations over 8 days	Comet	Pos (associated with cytotoxicity and inflammatory changes)	Relier et al. (2017)

Table 9 Summary of the 'Amber' Pig-a mutation, *gpt* mutation and γ-H2AX assay results

Test material	**Primary size**	**Dose** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
TiO_2 NPs (anatase)	12.1 ± 3.2 nm	0.5-50	5 male B6C3F1 mice	i.v. 3 days	Pig-a mutation	Neg	Sadiq et al. (2102)
Aeroxide P25® (TiO_2-P25; 20% rutile and 80% anatase)	21 nm	2-50	4 male gpt Delta transgenic C57BL/6J mice	i.v. 4 weeks	Pig-a mutation	Neg	Suzuki et al. (2016)
AEROXIDE TiO_2 P25 (NM-105)	25.6±15 nm	0.5-10	12 male SD rats	Endotracheal instillation 3 instillations over 8 days	Pig-a mutation	Neg	Relier et al. (2017)
Aeroxide P25® (TiO_2-P25;	21 nm	2-50	4 male gpt Delta transgenic C57BL/6J mice	i.v. 4 weeks	*gpt* mutation	Neg	Suzuki et al. (2016)

Test material	**Primary size**	**Dose** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**Reference**
20% rutile and 80% anatase)							
AEROXIDE TiO_2 P25 (NM-105)	25.6±15 nm	0.5-10	12 male SD rats	Endotracheal instillation 3 instillations over 8 days	γ-H2AX	Pos lung (associated with cytotoxicity and inflammatory changes)	Relier et al. (2017)

COM opinion

122. After reviewing the in vivo genotoxicity studies performed to date on TiO_2, we note the following points:

i. The highest quality *in vivo* studies labelled here as "green" (n=2), both show negative results for the micronucleus endpoint (Donner et al., 2016; Sadiq et al., 2012). There were no "green" studies for other endpoints.

ii. Only Donner et al., (2016) used pigment grade TiO_2 (including micro-sized anatase that was most similar to E171) and therefore was most relevant to the concern for human health in this case. This study showed no micronucleus induction.

iii. The Donner et al., (2016) paper also used a physiologically relevant oral route, which is most appropriate for the assessment of dietary exposure of food grade TiO_2.The authors acknowledge that absorption from the GI tract is low, meaning poor bone marrow exposure. This is important for risk assessment purposes where the oral bioavailability of E171 in humans is very low (≤0.0013% - refer to COT opinion XXXX)

iv. The Sadiq et al., (2012) study, that used an i.v. route (a route that is most likely to achieve bone marrow exposure), also showed a negative micronucleus response and confirmed bone marrow exposure to titanium.

v. The studies labelled as "amber" (i.e., contained some suboptimal aspects) showed a mixture of positive (4/9) and negative (5/9) results for the genotoxicity endpoints studied.

vi. The positive studies included chromosomal and DNA damage endpoints and were all associated with cytotoxicity and/or indirect mechanisms of genotoxicity, such as oxidative damage and inflammation. There was no evidence of gene mutations, however no definitive conclusion can be made due to the deficiencies in the study designs and limited number of available studies.

vii. The route of administration of nano-sized TiO_2 in these "amber" studies was often not via the most relevant oral route (only 2/9 studies) when considering the use of E171 as a food grade material. The less relevant endotracheal route was employed in 3/9 studies and the i.v. route and i.p. route were employed in 3/9 and 1/9 studies, respectively. Often the dosing regimens employed in these studies were suboptimal and did not follow the recommendations of the OECD test guidelines, which also makes interpretation difficult.

viii. All these "amber" studies used a nano-sized TiO_2 material which is less relevant to the E171 material.

Overall, we conclude that there is little evidence in the literature to suggest that there is a health concern related to genotoxicity induction by TiO_2, particularly via the oral route and especially the micro sized TiO_2 fraction (most studies used the nano-sized material).

Currently a definitive assessment of the safety of food grade E171 is difficult when there are no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. We also note that there is a dearth of low-quality data sets that are not OECD compliant and this has led to a lot of conflicting data and uncertainty in the risk assessment for TiO2.

Abbreviations

BAL	Bronchoalveolar lavage
CA	Chromosomal aberrations
CAT	Cationic amino acid transporter
CHL	Chlorophyllin
COM	Committee on the Mutagenicity
CP	Cyclophosphamide
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
DSP	Disodium phosphate
EFSA	European Food Safety Authority
ENU	N-ethyl-N-nitrosourea
GSH	Glutathione
i.p.	Intraperitoneal
i.v.	Intravenous
LDH	Lactate Dehydrogenase
MDA	3,4-Methylenedioxyamphetamine
MMS	Methyl methanesulphonate
MN	Micronucleus
MNPCEs	Micronucleated poly-chromatic erythrocytes
MN-RETs	Micronucleated reticulocytes
MNU	N-methyl-N-nitrosourea
NCE	Normochromatic erythrocyte
NP	Nanoparticle
OECD	Organisation for Economic Co-operation and Development

PBS	Phosphate-buffered saline
PCE	Polychromatic erythrocytes
PCE/NCE	Polychromatic erythrocytes/Normochromatic erythrocyte
Pig-a	Phosphatidylinositol glycan class A gene
RAG	Red, Amber, Green
RBC	Red blood cell
RET	Reticulocyte
ROS	Reactive oxygen species
SD	Sprague Dawley (rats)
SEM	Scanning electron microscope
SOD	Superoxide dismutase
TEM	Transmission Electron Microscopy
TiO_2	Titanium dioxide (E171)
TG	Test guideline
TK	Toxicokinetic

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ANNEX 1

Literature Search Strategy

The principal assessment of literature was based on the references used in the EFSA review 'Safety assessment of titanium dioxide (E171) as a food additive' (EFSA, 2021). This literature search was made by ANS in 2016 and the methodology used for this was detailed in Appendices A and B of their review. This search was subsequently update to 2021 using methodology outlined in Appendices J and L (EFSA, 2021).

For this review of genotoxicity, the literature was again updated using the following methodology.

Scopus:

("titanium dioxide" AND nanoparticle AND genotox* AND "in vivo") AND PUBYEAR > 2020 AND PUBYEAR > 2020: 29

PubMed:

"titanium dioxide"[Title/Abstract] AND nanoparticle[Title/Abstract] AND genotox*[Title/Abstract] AND "in vivo"[Title/Abstract]: 1

Both 2021-2023 and only English language.

Exclusion criteria applied by EFSA were also used following criteria for exclusion were applied:

• Non-biological, toxicological or genotoxicity studies (e.g., synthesis, photocatalytic performance, soil analysis)

• Studies on non-mammal species (e.g., fish, *Drosophila*, bees) or plants

• *In vivo* studies that have used a non-relevant route of administration (e.g., dermal, dental and bone implants).

• Studies performed only with coated TiO2

• Studies performed only with TiO2 nanofibres, nanocomposites or nanotubes

• Reviews, editorials, letters to the editors, etc

Terms like derma* OR dental OR "bone implant*" OR soil OR plant OR fish were also excluded.