Committee on Mutagenicity

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MUT/2023/08

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

Assessment of in 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. The paper provided in Annex A gives an overview of the methodology by which papers on *in vivo* genotoxicity assays, that were assessed in the EFSA opinion, were screened for relevance and reliability. The screening process was split into three tiers, namely tier 1; nanomaterial and generic study design, tier 2; generic genotoxicity study design and tier 3; detailed genotoxicity study design. Papers identified in a recent literature review were also screened for consideration. The spreadsheet used for screening is presented in Annex B.

3. 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

4. Members are asked to consider the paper in Annex A, and in particular 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?

IEH Consulting under contract supporting the UKHSA COC and COM Secretariat
October 2023

Committee on Mutagenicity

MUT/2023/08 – Annex A

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

Assessment of in *in vivo* studies of TiO_2 genotoxicity

Overview of the methodology by which papers on *in vitro* genotoxicity assays that were assessed in the EFSA opinion, were screened for relevance and reliability.

IEH Consulting under contract supporting the UKHSA COC and COM Secretariat
October 2023

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

Review of Titanium Dioxide - 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 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); the generic experimental details of the genotoxicity study including adherence to Organisation for Economic Co-operation and Development (OECD) technical guidelines (tier 2); and finally, detailed experimental details of the genotoxicity study (tier 3). These criteria were assessed by several members of the Committee through an iterative process.

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 data on negative and positive controls and number of animals per group, and hence were given a score of 2 (see bold text in Table 1).

4. Papers with a 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 Assessment criteria for nanomaterial characteristics and generic study design of *in vivo* genotoxicity studies on TiO_2

NM characteristics
• **Test material and crystalline form**
• Particle size
• Particle shape
• Size at the start or at the end of the exposure period
• duration of exposure as well as time-points of observations
Study design
• **Use of negative and positive controls (where required)**
• **Number of animals per group**

Tier 2. Generic genotoxicity study design

5. When assessing papers based on generic genotoxicity study design, papers that scored 7 out of 10 in tier 1 were scored against the criteria outlined in Table 2. As with the assessment of nanomaterial characteristics, some characteristics of the genotoxicity study design were given a higher weighting including use of positive and negative controls and number of animals (see bold text in Table 2). Papers with a score of 12 out of 16 and above went to tier 3 and were further evaluated by assessing the detailed genotoxicity study design.

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

NM characteristics

- Identification of test material
- Source/origin of test material
- Purity (concentration) of test material
- Doses administered or concentration in exposure media
- Test medium or vehicle

Organism characterisation

- Species
- Route of administration
- Frequency and duration of exposure, and timepoints of observations
- **Use of negative controls**
- **Use of positive controls**
- **Numbers of animals**
- Study methods described
- Statistical analysis

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 exclusion criteria listed below, using expert judgement.

Table 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 exclusion criteria.

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

phosphatidylinositol glycan class A gene (Pig-a) mutation assay (OECD TG470) and γ-H2AX assay.

9. Other assays were excluded from further evaluation. 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. A number of criteria such as the absence of appropriate controls, insufficient experimental details and inappropriate sampling times automatically led to these studies being graded as red (RAG rating) and were not further assessed.

12. Overall, from a total of 72 papers that were initially assessed, nine papers were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the *in vitro* genotoxicity assessment of TiO_2 (Figure 1).

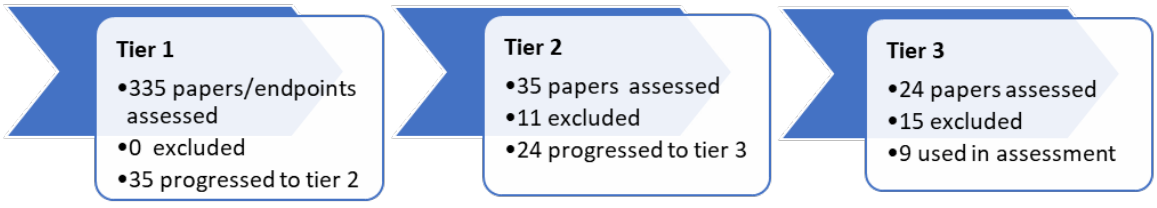

Figure 1 Summary of number of papers assessed in tier 1, 2 and 3

13. The nine papers 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. One of the nine papers were considered as the most robust and were categorised as green and eight papers were categorised as amber. The number and type of assay in each category is shown in Table 4.

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

Test	Green category	Amber category
MN	1	6
CA		2
Comet		3
Pig-a and γ-H2AX		3

Note: some papers assessed several endpoints

‘Green’ papers – MN assay

Donner et al. (2016)

15. 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.

16. 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.

17. 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. 20,000 RETs per animal were analysed. One pigment grade and one ultrafine material each were evaluated for potential systemic exposure/uptake from the gastrointestinal tract by analysis of TiO_2 into blood and liver.

18. 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 either administered by oral gavage (three studies) or by intraperitoneal (i.p.) injection (three studies). Cytotoxicity was assessed by the %RETs.

19. 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% in males and 0.07-0.1% and 0.05-0.1% in females, at 48- and 72 hours, respectively. The positive controls were 7.9-, 19.7- and 13.4-fold higher than background in males and 7.8-, 15.3- and 14.1-folder in females, with PG-1, PG-2 and PG3, respectively at 48 hours. For UF-1, UF-2 and UF-3, positive controls were 9.3-, 18- and 8.3-fold higher than background in males and 9.3-, 14.7- and 7-fold higher in females, respectively, at 48 hours. No positive controls were used at 72 hours or data were not significantly higher than background.

20. Following treatment, no biologically or toxicologically 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.

21. There were no significant increases in TiO_2 in blood compared to controls (48 or 72 hour) or liver (72 hour) following exposures to 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 observed lack of genotoxic

effects for all six test materials is 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.

22. COM Opinion: This study was deemed as RAG status green[Sb1][JW2]. Whilst considered to be a relatively good study, material preparation and secondary characteristics were not described clearly. Also, the positive control was not significant for some of the 72-hour timepoints, although all 48-hour positive control treatments responded as expected and the vehicle control data were within expected ranges, thus demonstrating laboratory proficiency. 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. The route of exposure was physiologically relevant, but there was a potential lack of adequate bone marrow exposure. Overall, the study was considered to be negative.

'Amber' papers – MN assay

Chakrabarti et al. (2019)

23. 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, according to a previously described method (see other sections for CA and Comet assay).

24. 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 or the method of dispersal, with the authors simply saying NPs were suspended in 500 µl water.

25. 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).

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

27. In the MN test, no clinical signs were observed. The negative control was 0.14% and the positive control was 24-fold higher than background. The PCE:NCE ratio was similar to that of the control group for both the 200 and 500 mg/kg TiO_2 treatment groups.

28. Following treatment, %MNPCE was 4-fold higher than background at 500 mg/kg (11.33±1.21; 0.57%) but remained unaltered at the 200 mg/kg bw dose.

29. COM Opinion: This study was deemed as RAG status amber. The MN assay was considered appropriate, but only 2000 PCE/animal were analysed. There is evidence of hepatic and renal toxicity at 500 mg/kg bw/day that may have confounded interpretation of the test results at 500 mg/kg bw/day, although the authors did not make this connection. Overall, this study was considered to be positive.

El-Bassyouni et al. (2017)

30. El-Bassyouni et al. (2017) tested the genotoxicity of three semi-conducting metal oxides including TiO_2 particles, using the MN and Comet assays in mice. The Comet assay was deemed RAG status red so is not included in the Comet section.

31. Surface characteristics of the particles were analysed by electron microscopy (EM), but no details regarding particle size distribution were provided nor the method of dispersion.

32. Groups of 10 male albino mice were treated with 100, 200 or 400 mg/kg bw of pure TiO_2 powder (anatase form), given as a single i.p. injection once

per week for one month. Five animals from each group were sacrificed at the end of the exposure period. Bone marrow was collected for the MN assay and liver samples for the Comet assay and enzyme activity analyses to assess cytotoxicity.

33. Saline was used as the negative control and CP (40 mg/kg) as the positive control. 3000 PCEs were scored per animal to assess MN formation. Cytotoxicity was assessed by albumin, globulin and the albumin/globulin (A/G) ratio, alanine transferase (ALT) and aspartate aminotransferase (AST) levels.

34. No cytotoxicity was seen apart from at the high dose (400 mg/kg bw) as albumin and globulin were significantly lower compared with controls (0.6- and 0.7-fold lower). In addition, AST and ALT were significantly higher at 400 mg/kg bw compared with controls (2- and 1.5-fold, respectively). The negative control was 0.16% and the positive control was 4-fold higher than background.

35. Results of the MN assay showed that high doses of TiO_2 (400 mg/kg bw), but not low (100 mg/kg bw) or medium (200 mg/kg bw) doses, significantly increased the incidence of Micronucleated poly-chromatic erythrocytes (MnPCEs) compared with the negative control group (2.4-fold higher).

36. COM Opinion: The MN assay was deemed as RAG status amber and the Comet assay as red due to the visual scoring (and therefore was not included in this review). The MN assay data were considered acceptable, with the high dose (400mg/kg bw) giving positive results, but toxicity was not measured with PCE/NCE level[Sb3]. The results are in agreement with the finding that TiO_2 inhibits the antioxidant activity of the glutathione peroxidase (GPx) enzyme and that the genetic alterations exerted by TiO_2 involves oxidative stress. No bone marrow exposure data was provided nor information on particle characteristics. Overall, the study was considered to be positive.

El-Ghor et al. (2014)

37. 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.

38. 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.

39. 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 animas treated with 500 and 2000 mg/kg bw TiO_2.

40. 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. 2000 PCEs per animal were scored to determine the number of MNPCEs.

41. 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 7-fold increase over background.

42. 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).

43. 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. A statistically significant decrease was also seen in SOD, CAT, and GPx levels, again in a dose-dependent manner.

44. 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 stress (ROS). Indirect deoxyribonucleic acid (DNA) damage via oxidative stress was confirmed by the reported high p53 mutations, elevated MDA (marker of lipid peroxidation), and decreased antioxidant defence systems.

45. COM Opinion: The study was deemed as RAG status amber. It was unclear what type of TiO_2 sample was used. In the MN assay, the OECD recommend at least two sampling times. Overall, this study was considered to be positive.

Relier et al. (2017)

46. Relier et al. (2017) investigated TiO_2 NP-induced genotoxicity in lung overload and non-overload conditions in SD rats as measured by MN, Comet, Pig-a and γ-H2AX assays.

47. 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.

48. Twelve male SD rats 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. Twenty-four rats (4 x 6 rats) were sacrificed 2 hours after the last treatment to investigate immediate NP-

induced toxicity (data not shown) and the other 24 rats were sacrificed 35 days after last treatment to detect mutations induced in bone marrow erythroblasts. Lung, peripheral blood and liver were collected.

49. NP-induced chromosomal damage was assessed in blood with the MN test. 2000 PCEs were assessed.

50. Cytotoxicity was assessed on bronchoalveolar lavage (BAL) and serum through lactate dehydrogenase (LDH) release. 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) for Comet, γ-H2AX and MN assays, or N-methyl-N-nitrosourea (MNU; 60 mg/kg bw in one i.p. injection 35 days before sacrifice) for the Pig-a mutation assay.

51. Following treatment, LDH levels in serum did not differ from background and no change was seen in the PCE/NCE ratio. The negative control was 0.1% and the positive control was 6-fold higher than background.

52. Following treatment, no changes in MN were seen after 2 hours. After 35 days, small 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). However, such small non-dose related increases may not be considered as biologically relevant.

53. 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.

54. COM Opinion: This study was deemed ‘amber’. The study was considered good, with the only comment being that exposure was by intratracheal instillation. Overall, the study was considered to be negative.

Sadiq et al. (2012)

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

56. The TiO_2-NPs had a narrow size distribution. The particles had a slight ellipsoidal shape, with the minor axes 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.

57. Groups of five male B6C3F1 mice were treated intravenously (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, and %MN-RET frequencies were measured on Day 4 only.

58. 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.

59. The negative control was PBS and the positive control was N-ethyl-N-nitrosourea (ENU; 140 mg/kg bw) administered once via i.p. $2x10^4$ CD71-positive RETS were counted for each animal. No measure of cytotoxicity was given.

60. 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.

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

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

63. The authors concluded that the 10 nm TiO_2-NPs tested were not mutagenic in the Pig-a mutation assay and not clastogenic or aneugenic in the MN assay at the dose levels studied; 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.

64. COM Opinion: This study was deemed as RAG status amber. It was considered good but with several weaknesses such as exposure by i.v. injection (rather than oral), but the results were still negative. Only 3 doses were used. In the MN assay, scoring of PCE was low, no cytotoxicity data were presented, and data presentation was generally limited. In addition, the sampling time was unclear. Overall, the study was considered to be negative.

Xu et al. (2013)

65. Xu et al. (2013) investigated the genotoxicity of a single dose of TiO_2-NPs (anatase) in mice, using the MN test.

66. The average size distribution of TiO_2-NPs was 42.3±4.60 nm. TiO_2-NPs were suspended in saline and sonicated.

67. Four male and female Institute for Cancer Research (ICR) mice were administered i.v., with a single dose of TiO_2-NPs at 0, 140, 300, 645, or 1387 mg/kg bw. 14 days after treatment, blood and bone marrow samples were taken from each animal.

68. In the negative control group, mice were given saline only. CP (20 mg/kg; two i.p. injections 24 and 48 hours before sacrifice) was used as the positive control. 1000 PCEs were analysed for MN formation. Blood biochemistry & haematology analysis were used to indicate cytotoxicity.

69. The negative control was 3% and the positive control was 4-fold higher than background.

70. Following treatment, no significant increases in the number of micronucleated cells in the TiO_2-NPs-treated mice were seen compared to the control animals.

71. COM Opinion: This study was deemed as RAG status amber[Sb4]. The study protocol was considered good. Overall, the study was considered to be negative.

‘Amber’ papers – CA assay

Chakrabarti et al. (2019)

72. 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, according to a previously described method (see other sections for MN and Comet assay).

73. SEM analysis of TiO_2-NPs 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 or the method of dispersal, with the authors simply saying NPs were suspended in 500 µl water.

74. 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).

75. Cyclophosphamide (40 mg/kg bw/day) was administered as a single dose by i.p. (positive control). The CA assay was conducted in bone marrow, with 500 metaphases being analysed. No method of assessing cytotoxicity was presented.

76. No clinical signs were observed. The negative control was 0.76% and the positive control was 12.7-fold higher than background.

77. 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%), although it was unclear if the CA frequency includes gaps and no details were provided on the types of aberrations.

78. COM Opinion: This study was deemed as RAG status amber. The CA assay was considered appropriate, but only 2000 PCE/animal were analysed. There is evidence of hepatic and renal toxicity at 500 mg/kg bw/day may have confounded interpretation of the test results at 500 mg/kg bw/day, although

the authors did not make this connection. Overall, this study was considered to be positive.

Manivannan et al. (2020)

79. Manivannan et al. (2020) investigated the genotoxic potential of the rutile form of TiO_2-NP in mice by carrying out a CA and Comet assay. The Comet was assessed as RAG status red so is not included in the section below.

80. The physical characteristics TiO_2-NP according to the supplier was particle size (≤25 nm). EM indicated that the TiO_2NP consisted of both spherical and rod-shaped NPs, with most of the particles occurring singly or in loosely arranged clusters in the size range of 21–31 nm, with mean average size of 25.074±3.593 nm. Suspensions of TiO_2 in double distilled water were dispersed by ultrasonication.

81. Groups of 5 male Swiss albino mice were dosed by oral gavage for 28 consecutive days with vehicle only (double distilled water) or 0.2, 0.4, or 0.8 mg/kg bw of TiO_2-NP dispersed in double distilled water. 16.5 hours after the final administration of TiO_2-NP the animals were injected i.p. with 0.04% colchicine and 1.5 hours later (18 hours after the final dose) the animals were sacrificed and bone marrow cells collected.

82. DNA damage and structural chromosomal aberration and clastogenic effects were evaluated using a CA assay in bone marrow cells.

83. Double distilled water was used as the negative control, administered via oral gavage. In the positive control group, animals received a single i.p. injection of mitomycin-C, 18 hours before sacrifice. A total of 150 metaphase plates were scored (3 slides per animal). The mitotic index (MI) was used as an indicator of cytotoxicity.

84. A decrease in the MI was seen with increasing doses of TiO_2-NP (6.09±0.55, 6.20±0.75, 3.71±0.54, 2.18±0.41 and 3.67±0.70 for negative control, 0.2 mg/kg bw, 0.4 mg/kg bw, 0.8 mg/kg bw and positive control, respectively). The negative control was 0.01±0.01 CA/cell and the positive control was 18-fold over background.

85. Following treatment, the frequency of aberrant cells and the number of breaks per cell was significantly higher than the control at 0.4 and 0.8 mg/kg bw (14- and 19-fold higher, respectively).

86. The authors concluded that these results indicated that rutile TiO2-NP is genotoxic and clastogenic in Swiss albino male mice, and that long term exposure to low concentrations can induce genotoxicity systemically in organs such as liver, spleen, and thymus.

87. COM Opinion: The overall CA study was deemed as RAG status amber; The CA assay appeared to use a non-standard test system. Additional issues regarding the timing of sacrifice following colchinine exposure were also highlighted. Overall, the study was considered to be positive.

'Amber' papers – Comet assay

Chakrabarti et al. (2019)

88. 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, according to a previously described method (see other sections for CA and MN assay).

89. SEM analysis of TiO_2-NPs 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 or the method of dispersal, with the authors simply saying NPs were suspended in 500 µl water.

90. 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).

91. The Comet assay was conducted on liver and kidney cells. Clinical chemistry markers were used to assess cytotoxicity. CP (40 mg/kg bw/day) was administered as a single dose by i.p. (positive control).

92. In liver and kidney cells, the %DNA in the tail in the negative controls was 0.068±.0.007 and 0.084±0.004, respectively. The positive control was 17.4-higher than background in liver and 8.8-fold higher in kidney. There was a significant increase in clinical chemistry parameters (ALT, AST, alkaline phosphatase (ALP), cholesterol) at 500 mg/kg bw. Decreases in red blood cell (RBC) parameters were also reported and kidney effects included tubule nephrosis with acute tubular necrosis, disrupted glomerulus, and loosely packed epithelial cells from the tubular lumen.

93. Following treatment there was a significant increase in %DNA in the tail at 500 mg/kg bw in both liver (3.5-fold increase) and kidney (3.2-fold increase) compared to the control group, while the mean scores for the 200 mg/kg treated animals were not significantly different.

94. COM Opinion: This study was deemed as RAG status amber. For the Comet assay, no information is provided on the method, analysis system used, or number of cells scored. Evidence of hepatic and renal toxicity at 500 mg/kg/day may have confounded interpretation of the test results at 500mg/kg/day, although the authors did not make this connection. Overall, the study was considered to be positive.

El-Ghor et al. (2014)

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

96. 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. TEM indicated that most of the TiO_2-NPs had polyhedral morphologies.

97. 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 collected from the femur. The alkaline Comet assay was performed on bone marrow, liver, and brain tissues. Biochemical evaluation of hepatic MDA and GSH and SOD, CAT and GPx activities was done in animas treated with 500 and 2000 mg/kg bw TiO_2. Tissue titanium content was also determined.

98. Water was used as the negative control and CP (25 mg/kg bw) as the positive control. It is unclear how cytotoxicity was assessed. One hundred cells were used for Comet analysis.

99. The % tail DNA in negative controls was 15.78±1.04 in bone marrow, 24.33±6.15 in brain and 19.71±5.48 in liver. The positive controls were 2-, 2- and 2.5-fold higher than background, respectively.

100. Following treatment, statistically significant increases in % tail DNA were seen in all organs at all doses. In bone marrow, % tail DNA was 3.3-, 4.0- and 4.2- folder higher than controls at 500, 1000 and 2000 mg/kg bw, respectively. In brain, levels were 2.3-, 2.3- and 2.5-fold increased, and in liver, 2.7-, 2.8- and 2.9-fold increased. Nano- TiO_2-induced DNA damage was higher in bone marrow cells than in liver and brain cells.

101. 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. A statistically significant decrease was also seen in SOD, CAT, and GPx levels, again in a dose-dependent manner.

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

103. COM Opinion: The study was deemed as RAG status amber as was unclear what type of TiO_2 sample was used. For the Comet assay an inappropriate sampling time was used and a high level of % tail DNA was seen in the negative controls. Overall, this study was considered to be positive.

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 and γ-H2AX assay.

105. 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 PBS. Particle agglomeration was analysed by centrifugal liquid sedimentation and stability of the final suspensions (zeta potential) was measured.

106. Twelve rats per group of male SD rats 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 rats per group were sacrificed 2 hours after the last treatment to investigate immediate NP-induced toxicity (data not shown) and the other 24 rats were sacrificed 35 days after last treatment to detect mutations induced in bone marrow erythroblasts. Lung, peripheral blood and liver were collected.

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

108. Cytotoxicity was assessed on BAL and serum through LDH release. The PCE/NCE ration was also calculated. The negative control was PBS and the positive controls were MMS (150 mg/kg bw final dose administered in three gavages at a one-day interval just before sacrifice) for Comet, γ-H2AX and

MN assays, or MNU (60 mg/kg bw) in one i.p. injection 35 days before sacrifice for the Pig-a mutation assay.

109. In serum, LDH levels following treatment did not differ from background and no change was seen in the PCE/NCE ratio. 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.

110. Following treatment, in the lung, DNA damage was significantly increased after 2 hours at all doses of TiO_2 (but within historical control levels). After 35 hours, % tail intensity was increased at 2.5 and 10 mg/kg bw (3.2- and 4.7-increase compared to controls, respectively). 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).

111. The lowest tested doses had no toxicity or genotoxicity effects in the lung. In blood, no lymphocyte DNA damage, erythrocytes chromosomal damage or gene mutation could be detected. 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.

112. COM Opinion: This study was deemed ‘amber’. The study was considered good, with the only comment being that exposure was by intratracheal instillation. Overall, the study was considered to be positive.

‘Amber’ papers – PigA assay and γ-H2AX

Sadiq et al. (2012)

113. Sadiq et al. (2102) conducted MN and Pig-a mutation assays to evaluate the genotoxicity of 10 nm TiO_2 anatase NPs in mice.

114. The TiO_2-NPs had a narrow size distribution. The particles had a slight ellipsoidal shape, with the minor axes 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.

115. 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, and %MN-RET frequencies were measured on Day 4 only.

116. 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.

117. The negative control was PBS and the positive control was ENU (140 mg/kg bw) administered once via i.p. %RETs were calculated as a measure of cytotoxicity.

118. A reduction in %RETs was observed in TiO_2-NP-treated animals on Day 4, suggesting treatment-related cytotoxicity. The negative control was 0 to 1.2 × 10^{-6} and 0 to 4 × 10^{-6} in 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).

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

120. The authors concluded that the 10 nm TiO_2-NPs tested were not mutagenic in the Pig-a mutation assay and not clastogenic or aneugenic in the MN assay at the dose levels studied; 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.

121. COM Opinion: This study was deemed as RAG status amber. It was considered good but with several weaknesses; exposure was by i.v. injection (rather than oral), but the results were still negative. Only 3 doses were used. The Pig-a mutation assay should have used 6 animals as a minimum per group for 28 days. Overall, the study was considered to be negative.

Suzuki et al. (2016)

122. Suzuki et al. (2016) investigated the genotoxicity of TiO_2 NP suspensions in male gpt Delta transgenic C57BL/6J mice using a MN, Comet and Pig-a mutation assay. MN and Comet assays were deemed RAG status red so have not been included in the sections above.

123. 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.

124. 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.

125. DSP was used as a negative control and ENU (70 mg/kg) as the positive control (single i.p. dose sampled 30 days after dosing). 1,000,000 TER-119-positive cells were analysed to determine the frequency of CD24-negative RBCs. Assessment of cytotoxicity was not specified but %RETs was used in a previous publication.

126. No data on cytotoxicity were presented. The negative control was $0.4\pm0.55 \times 10^{-6}$ mutRBC (CD24-/CD71-/TER-119+ cells). The positive control was 127.5-fold higher than background.

127. 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.

128. COM Opinion: This study was deemed as RAG status amber. The study protocol is generally good, although group size is lower than recommended and there is no mention of cytotoxicity measurements. The vehicle and positive controls gave expected results, and despite the i.v. route the results are clearly negative.

Relier et al. (2017)

129. 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 and γ-H2AX assay.

130. 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 PBS. Particle agglomeration was analysed by centrifugal liquid sedimentation and stability of the final suspensions (zeta potential) was measured.

131. Twelve rats per group of male SD rats 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 rats per group were sacrificed 2 hours after the last treatment to investigate immediate NP-induced toxicity (data not shown) and the other 24 rats were sacrificed 35 days after last treatment to detect mutations induced in bone marrow erythroblasts. Lung, peripheral blood and liver were collected.

132. NP-induced DNA double-strand breaks (DSBs) were assessed in lung, blood lymphocytes, and liver cells with γ-H2AX immunostaining. The number of foci per nucleus among 100 cells was assessed.

133. 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 RET.

134. Cytotoxicity was assessed on BAL and serum through LDH release. The PCE/NCE ration was also calculated. The negative control was PBS and the

positive controls were MMS (150 mg/kg bw final dose administered in three gavages at a one-day interval just before sacrifice) for Comet, γ-H2AX and MN assays, or MNU (60 mg/kg bw) in one i.p. injection 35 days before sacrifice for the Pig-a mutation assay.

135. In serum, LDH levels following treatment did not differ from background and no change was seen in the PCE/NCE ratio.

136. In the H2AX assay, the negative control showed 2-3 mean foci in lung, blood and liver after 2 hours and 35 days. The positive control was 2-fold higher than background. No differences were seen in the number of foci in the liver and blood, following exposure to TiO_2 NPs. However, an increase in the number of foci in the lung after 2 hours was reported at 10 mg/kg bw, indicating that a high dose of TiO_2 NPs may induce DNA DSBs.

137. In the Pig A assay, the negative control 0.5 Mutant RBCs x 10^{-6} and 1.3 Mutant RETs x 10^{-6}. The positive control showed a 52-fold increase in Mutant RBCs x 10^{-6} and a 12.3-fold increase in Mutant RETs x 10^{-6}.

138. Following treatment, no increase in the frequency of mutant RBC and reticulocytes (RETs) was observed.

139. 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.

140. COM Opinion: This study was deemed ‘amber’. The study was considered good, with the only comment being that exposure was by intratracheal instillation. Overall, the study was considered to be negative.

Summary

141. Nine 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 regarding the *in vivo* genotoxicity of TiO_2. Studies included CA assay, Comet assay, MN assay Pig-a mutation assay and the γ-H2AX assay. Studies were assessed as red, amber or green b assessing the genotoxicity study design, using criteria outlined in Table 3.

An overall summary of the data are presented in Table 5 and the results of each study are summarised in Table 6, Table 7, Table 8, Table 9 and Table 10.

Table 5 Summary of test results[Sb5]

	Comet	MN	CA	Pig-a
Exposure route				
Oral	(+)*	— (+)*	+ (+)*	
i.v.	—	— — —		— —
i.t.	+	—		—
i.p.	+	+ (+)*		

* At highest dose only

Table 6 Summary of the 'Green' MN results[Sb6]

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**Reference**
PG and UF	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	G	Donner et al. (2016)

Table 7 Summary of the 'Amber' MN results

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**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	A	Chakrabarti et al. (2019)

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**Reference**
TiO_2-NPs (no further info)	No info	100-400	10 male albino mice	i.p. Single dose	MN	Pos	A	El-Bassyouni et al. (2017)
	<100 nm	500-2000	5 male Swiss Webster mice	i.p. 5 days	MN	Pos	A	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	A	Relier et al. (2017)
Anatase	12.1 ± 3.2 nm	0.5-50	5 male B6C3F1 mice	i.v. 3 days	MN	Neg	A	Sadiq et al. (2102)
Anatase	42.3±4.60 nm	140-1387	4 male and female ICR mice	i.v. Single dose	MN	Neg	A	Xu et al. (2013)

Table 8 Summary of the 'Amber' CA results

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**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	A	**Chakrabarti et al. (2019)**
Rutile	≤25 nm	0.2-0.8	5 male Swiss albino mice	Gavage 28 days	CA	Pos	A	Manivannan et al. (2020)

Table 9 Summary of the 'Amber' Comet assay results

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**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	Comet	Pos	A	Chakrabarti et al. (2019)
TiO_2-NPs (no further info)	<100 nm	500-2000	5 male Swiss Webster mice	i.p. 5 days	Comet	Pos	A	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	Comet	Pos	A	Relier et al. (2017)

Table 10 Summary of the 'Amber' Pig-A and γ-H2AX assay

Test material	**Size**	**Conc.** mg/kg bw	**Species/strain/sex**	**Route and duration of administration**	**Endpoint**	**Result**	**RAG**	**Reference**
Anatase	12.1 ± 3.2 nm	0.5-50	5 male B6C3F1 mice	i.v. 3 days	Pig A	Neg	A	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	Neg	A	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 γ-H2AX	Neg	A	Relier et al. (2017)

142. 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.

143. In three studies exposure to the test material was via the oral route, in a further three studies the test animals were dosed i.v., in two studies exposure was by i.p. injection, and in one study exposure was by intratracheal instillation.

144. A positive result for *in vivo* genotoxicity was obtained in the intratracheal instillation study, with the effect being associated with oxidative stress. One of the i.p. studies gave positive results in both the MN assay and the Comet assay, with the effects also being attributed to oxidative stress; the other i.p. study similarly yielded positive results in the MN assay (associated Comet assay deemed invalid) but only at the highest dose. Positive results were obtained in two of the three oral studies; in one case this only occurred in the highest dose group. All three i.v. studies were negative.

145. The only study deemed to have used robust methodology (Donner et al, 2016) used the MN assay to detect MN-RET in peripheral blood cells. No biologically or toxicologically relevant increases in MN-RET frequency was 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.

146. Results from the oral studies are considered most relevant. These studies variously utilised the MN, Comet and CA assays. Positive results were obtained for the CA assay in two studies (only at the highest dose in one of these), in the MN assay in one study at the highest dose, and for the Comet assay in the one valid study, only at the highest dose. As noted above, the only robust oral study (which utilised the MN assay) yielded negative results.

Abbreviations

A/G	Albumin/globulin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
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
EM	Electron Microscopy
ENU	N-ethyl-N-nitrosourea
GPx	Glutathione Peroxidase
GSH	Glutathione
i.p.	Intraperitoneal
i.v.	Intravenous
ICR	Institute for Cancer Research
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 oxidative stress
SD	Sprague Dawley (rats)
SEM	Scanning electron microscope
SOD	Superoxide dismutase
TEM	Transmission Electron Microscopy
TiO_2	Titanium dioxide (E171)
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.

Committee on Mutagenicity

MUT/2023/08 – Annex B

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

Assessment of in *in vivo* studies of TiO_2 genotoxicity

Screening spreadsheet IN VIVO TiO2 screening - not available publicly.

IEH Consulting under contract supporting the UKHSA COC and COM Secretariat
October 2023