Committee on Mutagenicity

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

Assessment of *in vitro* 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 vitro* papers?

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

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Committee on Mutagenicity

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

Assessment of *in vitro* 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 COM has been asked to provide an opinion on its genotoxicity.

Methodology

Screening and evaluation of papers

2. The *in vitro* 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 for a particular criterion,

a score of 1 was given. For example, a score of 1 was awarded if sufficient data were presented on crystalline form, agglomeration or dispersion method. More weight was placed on some criteria, such as inclusion of data on particle size, inclusion of positive controls and a valid number of replicates, and hence were given a score of 2 if the criterion was satisfied.

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 vitro* genotoxicity studies on TiO_2

NM characteristics

- Crystalline form (score=1)
- Particle size (primary and secondary size) (score = 2)
- Particle shape (score = 1)
- Agglomeration method (score = 1)
- Dispersion method and preparation samples (score = 1)

Study design characteristics

- Use of positive controls (score = 2)
- Number of replicates (score = 2)

Tier 2. Generic genotoxicity study design

When assessing papers based on generic genotoxicity study design, the papers were scored against the criteria outlined in

5. 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 replicates being >1, were given a higher weighting (see bold text in Table 2). Papers with a total score of 9 out of 13 and above proceeded 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 vitro* genotoxicity studies on TiO_2

Nanoparticle

- Source of nanoparticle (score = 1)
- Concentrations in exposure media (score = 1)
- Concentrations <500 µg/ml (score = 1)

Organism characteristics

- Cell model (score = 1)
- Duration of exposure (score = 1)
- Use of negative controls (score = 2)
- Use of positive controls (score = 2)
- Number of replicates >1 (score = 2)
- OECD-recommended cytotoxicity assay (score = 1)
- Statistical analysis (score = 1)

Tier 3. Detailed genotoxicity study design

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

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

Nanomaterial characteristics

- Primary and secondary size

Nanomaterial dispersion

- Method and surfactant

Test system

- Cell type
- Treatment
- S9
- CytoB regime
- No. of cells
- No. of replicates
- Total no. of cells
- Dose range
- 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

Nuclear/ cellular 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, as follows.

8. Only assays with OECD guidelines were included in the assessment, including assays for the formation of micronuclei (MN; OECD TG487), hypoxanthine phosphoribosyl transferase *(hprt)* gene mutations (OECD TG476), chromosomal aberrations (CA; OECD TG473), the mouse lymphoma

assay, and TK6 gene mutation assay (OECD 490). Other assays were excluded from further evaluation. Additional exclusion criteria included the lack of positive controls, no or incorrect cytotoxicity assays, use of inappropriate cell lines, inadequate duration of exposure, only high concentrations tested, insufficient number of cells assessed, and high spontaneous levels of genotoxicity.

9. 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 a good robust study with no 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 or irrelevant tests, meaning that they are not of sufficient quality for use in the assessment of the genotoxicity of TiO_2.

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

11. Many of the papers assessed and outlined below contained additional studies that were not evaluated as they do not have an OECD guideline, including the *in vitro* Comet assay for the detection of DNA strand breaks and the assessment of reactive oxygen species (ROS) using various methods such as the formation of 8-oxo-dG adducts. The results of these studies have, however, been used as potential indicators of mechanisms of action.

12. Overall, from a total of 191 papers that were initially assessed, 15 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).

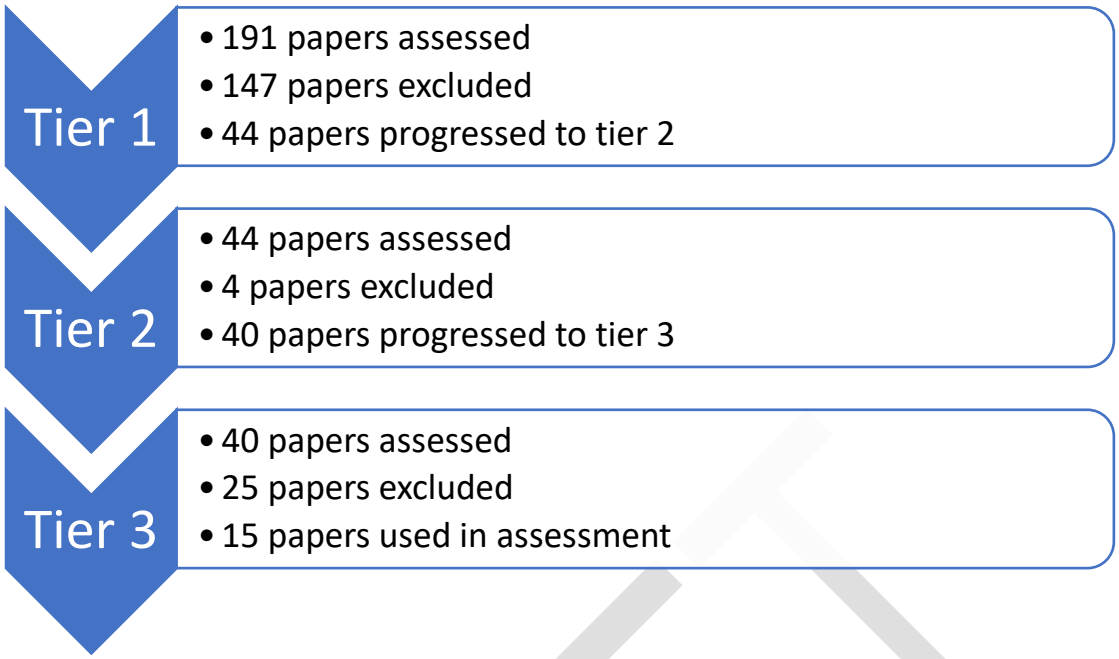

Figure 1 Summary of number of papers assessed in tier 1, 2 and 3

13. The 15 papers (reporting 16 assays) are summarised below together with a brief summary of the COM opinion for each paper. An overall summary at the end of the document draws a conclusion on the potential *in vitro* genotoxicity of TiO_2.

14. Seven of the 16 assays were considered as the most robust and were categorised as green, and 9 assays were categorised as amber. The number and type of assay in each RAG category is shown in Table 4.

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

Test	*Green category*	*Amber category*	*Red category*
MN	5	8	22
Hprt	1	1	3
CA	1	0	2

Note: some papers assessed several endpoints

‘Green’ papers – Micronucleus (MN) assay

Andreoli et al. (2018)

15. Andreoli et al. (2018) conducted a cytokinesis block micronucleus (CBMN) assay in peripheral blood mononuclear cells (PBMCs), using cytochalasin B (cytoB) and performed according to OECD TG487 with minor modifications. $1x10^6$ PBMC were suspended in medium with phytohaemaglutinin (PHA) to stimulate lymphocyte proliferation. Cells were treated using two different protocols. In protocol 1, cells were treated with PHA for 24 hours, then with TiO_2 (anatase, rutile or a mixture of both, at concentrations of 0, 10, 50, 100 or 200 µg/mL) for 20 hours, after which cytoB was added for 28 hours (48-hour total treatment time). In protocol 2, cells were treated PHA for 43.5 hours and TiO_2 for 30 minutes, after which cytoB was added for 28 hours (28.5-hour total treatment time). Protocol 1 was considered the more robust methodology and hence more weight was put on these results.

16. The primary size of the commercial TiO_2 nanoparticles was 20-60 nm, 30 x-100 nm and 45-252 nm for the anatase, rutile and mix, respectively, with a secondary size of 328 nm (70-2130 nm), 283 nm (50-2730 nm) and 303 nm (40-2450 nm), respectively, when dispersed by ultrasonication in MilliQ H_2O, and 210 nm (50-1570 nm), 226 nm (50-3340 nm) and 328 (50-1770 nm), respectively, when dispersed in RPMI media without a surfactant. One thousand binucleated cells/concentration were analysed for MN formation per experiment and two independent experiments were conducted (total of 2000 binucleated cells/concentration). Hydrogen peroxide (500 µM) was used as a positive control. For cytotoxicity assessment, the cytokinesis block proliferation index (CBPI) was calculated, but no toxicity was observed after any treatment with TiO_2.

17. The MN frequency in the negative control (background) was 0.75-1% and the positive control was 5- to 8-fold higher than the background.

18. Following treatment with TiO_2, no increase in MN formation was detected with either treatment protocol.

19. COM opinion – This study was considered robust and well conducted with minor deficiencies. A flow-based method was used which does not confirm cellular uptake. The highest dose (200 µg/mL) exceeded the concentration recommended in the OECD guidelines for testing nanomaterials but despite this, was still negative. Hydrogen peroxide was used as a positive control, which is not a recommended positive control according to OECD TG487; however, it induced an unequivocal positive response (5- to 8-fold increase over background). Despite these reservations, the study was given a RAG rating of green.

20. The study detected induction of 8-oxo-2'-deoxyguanosine (8-oxodG) by anatase and rutile TiO_2 and the mixture, indicating oxidative damage which did not translate into a genotoxic signal.

21. Overall, no increase in MN formation was detected and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Demir et al. (2015)

22. Demir et al. (2015) conducted a CBMN assay in human embryonic kidney (HEK293) cells and mouse embryonic fibroblast (NIH/3T3) cells using cytoB. $5x10^5$ HEK293 or NIH/3T3 cells were treated with 10, 100 or 1000 µg/ml anatase TiO_2 nanoparticles for 48 hours. CytoB was added for the last 24 hours prior to harvesting.

23. Two sizes of anatase nanoparticles were tested. The primary sizes of these were 21 ± 2.8 nm and 50 ± 12 nm and the secondary sizes were 22.94 ± 0.3 nm and 50.72 ± 0.4 nm. For these measurements, TiO_2 nanoparticles were dispersed by ultrasonication in 0.05% bovine serum albumin (BSA). One thousand binucleated cells/concentration were scored for MN in duplicate (total of 2000 binucleated cells/concentration). Mitomycin C (MMC; 0.3µM) was used as the positive control. For cytotoxicity assessment, CBPI was calculated.

24. The MN frequency in the negative control (background) was 0.55% in HEK293 cells and 0.6% in NIH/3T3 cells; the positive controls induced approximately 8- and 6-fold higher Mn levels than background in HEK293 cells and NIH/3T3 cells, respectively.

25. Following treatment with TiO_2, no genotoxicity or cytotoxicity was detected between 10-100 µg/mL in either cell line for both sizes of nanoparticles. MN formation was only increased at the highest TiO_2 concentration (1000 µg/ml) in both cell lines.

26. COM opinion – This study showed robust methodology. HEK293 is a non-standard cell line and is not recommended by OECD, but background MN frequency was in line with that seen in OECD-recommended cells. The doubling time of the cells is 24-48/36 hours. No evidence of nuclear uptake was provided. The increased MN formation observed at the highest concentration (1000 µg/mL) in both cell lines was not considered to be relevant for genotoxicity assessment, as this is an extremely high concentration that would not be considered appropriate for testing nanomaterials (OECD, 2022). This concentration was therefore discounted from any assessment of effect. Despite these reservations, the study was given a RAG rating of green.

27. A Comet assay ± formamidopyrimidine DNA glycosylase (Fpg) in the study gave no evidence of oxidative DNA damage or cell transformation between 10-100 µg/ml.

28. Overall, no increase in MN formation was detected at the relevant concentration range and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Di Bucchianico et al. (2017)

29. Di Bucchianico et al. (2017) carried out a CBMN assay in a human bronchial epithelial cell line (BEAS-2B) using cytoB according to OECD TG487. $6x10^4$ cells were treated with three different TiO_2 nanoparticles: uncoated anatase (NM-100; 50-150 nm), coated (no further information given)

anatase (NM-101; 5-8 nm) and coated rutile (NM-103; 20-28 nm) at doses of 1, 5 or 15 µg/ml for 20 hours, after which cytoB was added for 28 hours.

30. MN were also evaluated using flow cytometry without cytoB, in which cells were treated with1, 5, 15 or 30 µg/mL for 48 hours.

31. Nanoparticles were dispersed using ultrasonication in 0.05 % BSA. 2000 binucleated cells were analysed for MN formation. Mitomycin C (0.05 µg/mL) was used as a positive control. Cytotoxicity was assessed by calculating the reduction in replication index (RI) of the treated cells compared to the negative control. The number of apoptotic, necrotic and mitotic cells per 1000 cells was also evaluated as a measure of cytotoxicity and cell proliferation.

32. Cytotoxicity was minimal. The MN frequency in the negative control (background) was approximately 1% (for both methods employed) and the positive control was 8- to 9-fold higher than background (for manual vs flow method, respectively).

33. Following treatment with TiO_2, uncoated and coated anatase (NM-100 and NM-101, respectively) were both negative for MN formation in both methods, apart from 1 µg/mL NM-101 in the flow cytometry method which showed a small increase in MN formation. Low doses of NM-103 also showed a small increase in MN formation in both methods (2-fold increase at 1 µg/mL (manual) and 1.5-fold increase (flow method).

34. COM opinion – This study was well conducted and showed robust methodology. BEAS-2B is a non-standard cell line and is not recommended by OECD, but background MN frequency was in line with that seen in OECD-recommended cells. The doubling time of the cells is 26 hours. The Litron kit method normally requires 10,000 cells to be scored, whereas in this study only 2000 nuclei were scored. Flow-based measures are not reliable to show cellular uptake. The number of replicates was unclear, although figures did contain error bars. Despite these reservations, the study was given a RAG rating of green.

35. There were indications of oxidative damage as the Comet assay, conducted ±Fpg, was positive after 3 hours with both NM-100 and NM-103, while after 24 hours only NM-101 was positive.

36. Overall, the study was weakly positive (1.5-2-fold increase) for the formation of MN at lower doses, but only in rutile-treated cells. This study was cited in the EFSA review (EFSA, 2021).

Li et al. (2017)

37. Li et al. (2017) conducted an MN assay without cytoB in human lymphoblastoid TK6 cells using two methods. Test 1 used flow cytometry and test 2 used manual microscopy. As TiO_2 emits fluorescence which could interfere with flow cytometry, results from test 1 were not considered further. In test 2, $3x10^5$ cells were exposed to synthesised anatase TiO_2 nanoparticles at concentrations of 100 and 200 µg/mL.

38. The primary size of TiO_2 nanoparticles was 8.9-15.3 nm and the secondary size was 860-892 nm. The samples were prepared by probe sonication in sterilised water. Two thousand binucleated cells were analysed for MN formation. MMC was used as the positive control (0.01 µg/mL) . Cytotoxicity was assessed by relative increase in cell counts (RICC) and relative population doubling (RPD).

39. In the manual assay (test 2), RICC ranged from 58-73% and RPD from 54-77% at 200 and 100 µg/mL, respectively. The MN frequency in the negative control was 1.85% and the positive control was 2.5-fold higher than background.

40. Following treatment with TiO_2, a significant increase in frequency of MN was detected (1.5- and 2-fold increase at 100 and 200 µg/ml, respectively).

41. COM opinion – The manual analysis method in test 2 was well conducted. However, only data for the lowest dose (100 µg/mL) was considered, as the highest dose (200 µg/mL) exceeded the concentration recommended in the OECD guidelines for testing nanomaterials and was

therefore discounted. Despite these reservations, the study was given a RAG rating of green. No information was available from this study to aid in the understanding of any mechanism of action. No cellular uptake was reported.

42. Overall, test 2 indicated a 1.5-fold increase in MN formation which is not considered to be biologically relevant, hence the study was considered negative. A 2-fold increase was observed at the highest dose, which was discounted due to it being higher than OECD guidelines. This study was cited in the EFSA review (EFSA, 2021).

Unal et al. (2021)

43. Unal et al. (2021) conducted a CBMN assay with cytoB and a CA assay (see section 56) in human peripheral blood lymphocytes (HPBLs) isolated from whole blood from three non-smoking females. The cells (number not given) were exposed to TiO_2 nanopowder ((CAS 13463-67-7; rutile/anatase) at concentrations of 20, 40, 60, 80 or 100 µg/mL for 48 hours and cytoB was added after 44 hours.

44. The primary size of TiO_2 nanopowder was 10-360 nm but secondary size was not stated. Stock nanoparticle suspensions were ultrasonicated in distilled water, diluted to final concentrations and then sonicated again. Three thousand binucleated cells were analysed for MN. Mitomycin C (0.2 µg/ml) was used as a positive control. Cytotoxicity was measured by CBPI (called Nuclear Division Index (NDI)).

45. There was no change in the NDI. The MN frequency in the negative control was 0.13% and the positive control was 20-fold higher than the background.

46. Following treatment with TiO_2, no significant increases in MN formation were observed at any dose. There was no evidence of uptake of nanoparticles.

47. <u>COM opinion</u> – This study was regarded to be good quality, although some of the study details are unclear; i.e. whether PHA was used to induce cell division (but as the positive control shows a 20-fold increase over

background, it is assumed that it was). There is no evidence of uptake. Despite these reservations, the study was given a RAG rating of green.

48. Further experiments in this study included a Comet assay that showed increased DNA damage only at the highest concentration, which did not result result in fixed genetic damage (MN).

49. Overall, no increase in MN formation was detected and the study was considered negative. This study was identified during the recent literature search.

‘Green’ papers – *hprt* assay

Kazimirova et al. (2020)

50. Kazimirova et al. (2020) conducted a *hprt* forward mutation test in V79 cells according to OECD TG476. $1x10^6$ cells/dish were exposed to an anatase/rutile TiO_2 nanoparticles mix at concentrations of 3, 5 or 75 µg/cm^2 for 24 hours. After 24 hours, cells were washed and reseeded for 8 days, after which they were replated with 6-TG to detect mutation frequency.

51. The primary size of TiO_2 nanoparticles was 21 nm (15-60nm) and secondary size was 228 ± 3.2 nm as measured using dispersion procedure (DP) 1, and 184 ± 3.5 nm using DP2. For DP1, the samples were prepared by ultrasonication in PBS and 10% FBS, and in DP2 samples were suspended in culture medium with HEPES buffer without FBS, and sonicated. Methyl methanesulphonate (MMS; 0.1 mM) was used as a positive control. Cytotoxicity was measured by determining plating efficiency (PE).

52. There were no substantial effects of exposure time (24, 48 and 72 h), on cytotoxicity, although a tendency for a lower Relative Growth Activity at longer exposure was observed. No significant difference in PE values were seen. The *hprt* mutation frequency in the negative control was approximately $2x10^5$ and the positive control was approximately 6-fold higher than background.

53. Following treatment with TiO_2, there was no evidence of increased *hprt* mutation frequency in cells treated with TiO_2 in spite of evidence of uptake of nanoparticles by cells.

54. COM opinion – This was considered to be a well-conducted assay using robust methodology and the study was given a RAG rating of green.

55. Overall, no evidence of increased mutation was seen and it was considered negative. This study was cited in the EFSA review (EFSA, 2021).

'Green' papers – CA assay

Unal et al. (2021)

56. Unal et al. (2021) conducted a CBMN assay with cytoB (see section 43) and a CA assay in HPBLs isolated from whole blood from three non-smoking females. The cells (number not given) were exposed to TiO_2 nanopowder (no further information given) at concentrations of 20, 40, 60, 80 or 100 µg/mL for 24 and 48 hours, with colchicine added 2 hours before harvest.

57. Three hundred metaphases per treatment were analysed. Mitomycin C (0.2 µg/mL) was used as a positive control. Cytotoxicity was measured by Mitotic Index (MI) in the CA assay.

58. At 24 hours the MI was significantly lower at 60 and 80 µg/ml (reduced by 20% at both concentrations compared to a negative control) but was still within the range of control values. There was no evidence of cytotoxicity at 48 hours. The CA in the negative control at both 24 and 48 hours was 1.3%, excluding gaps, and the positive control was 17- and 13-fold higher than the background, respectively.

59. Following treatment with TiO_2, a significant increase in CA was observed at all doses at 24 hours (3.6- to 5.6-fold increase over background), At 48 hours, similar results were obtained, but were significant only at 20, 80 and 100 µg/mL (3.6-, 2.9- and 2.9-fold increase). There was no evidence of uptake of nanoparticles.

60. COM opinion – This study was regarded to be good quality, although some of the study details are unclear; i.e. whether PHA was used to induce cell division (but as the positive control shows a 20-fold increase over background, it is assumed that it was). No dose response was observed as CA frequency decreased with increasing TiO_2 concentration. There was no evidence of uptake. Despite these reservations, the study was given a RAG rating of green.

61. Further experiments in this study included a Comet assay, which showed increased DNA damage only at the highest concentration.

62. Overall, the study was considered positive as there was a significant increase in CA at all doses at 24 hours, although there was no positive dose response as CA frequency decreased with increasing TiO_2 concentration. At 48 hours similar results were obtained but were significant only at 20, 80 and 100 µg/mL. This study was identified during the recent literature search.

'Amber' papers – MN assay

Kazimirova et al. (2019)

63. Kazimirova et al. (2019) conducted a CBMN assay in TK6 lympoblastoid cells and lymphocytes from human volunteers.

64. $2x10^5$ TK6 cells were treated with 3, 15 or 75 µg/cm^2 anatase/rutile TiO_2 nanoparticle mix for 4 or 24 hours. CytoB was added for the last 24 hours prior to harvesting, resulting in total treatment times of 28 or 48 hours.

65. Fourteen volunteers also participated in the study (ages 40-50 years old, eight women and six men) but were not considered further.

66. The primary size of the anatase/rutile nanoparticle mix was 21 nm (15-60 nm) with a bimodal distribution of 102 ± 15 nm and 285 ± 67 nm when dispersed by ultrasonication in PBS and FBS.

67. 2000 binucleated cells per concentration were analysed for MN formation in two independent experiments. Micronuclei in 1000 mononucleated cells per concentration were also analysed. Mitomycin C (0.3

µM) was used as the positive control. For cytotoxicity assessment, CBPI was calculated.

68. The number of MN in the negative control (background) was 1.5 after 28 hours (4 hour treatment) and 4 after 48 hours (24 hour treatment), corresponding to frequencies of 0.075% and 0.2 %, respectively, and the positive control induced a level approximately 24-fold higher than the background.

69. Following treatment with TiO_2, no cytotoxicity and no increases in MN frequency were detected.

70. COM opinion – This study showed acceptable methodology. However, only the 24 hour treatment time was considered appropriate as the 4 hour treatment time followed by the addition of cytoB for 24 hours was considered a co-treatment. Data showed large variability (even when expressed as SEM of duplicates) which suggests a lack of reproducibility, and TK6 cells had high background MN levels at 48 hours. Due to these observations, the study was given a RAG rating of amber.

71. Overall, no increase in MN formation was detected and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Kurzawa-Zegota et al. (2017)

72. Kurzawa-Zegota et al. (2017) conducted a CBMN assay with cytoB in HPBLs. The blood lymphocytes were collected from 3 different cohorts of patients: group 1, healthy patients (n=20); group 2, polyposis coli patients (n=19); group 3, colon cancer patients (n=20). For the purpose of this review, only data from healthy volunteers is considered. 400 µL whole blood was incubated with PHA for 24 hours, then exposed to anatase titanium nanoparticles at concentrations of 10, 40 or 80 µg/mL for 20 hours, after which time cytoB was added (at 44 hours). Cells were harvested at 72 hours.

73. The primary size of TiO_2 nanoparticles was <25 nm and the secondary size increased with time and dose and varied with the media used, ranging from 104.2 nm in water to 1303 nm in RPMI. The samples were prepared by

ultrasonication in water, PBS or RPMI 1640 (although it is unclear which preparation was used for cell treatment). Two thousand binucleated cells were analysed for MN formation. Cytotoxicity was determined by CBPI. Mitomycin C (0.4 µM) was used as a positive control.

74. No cytotoxicity was observed in any cohort. The frequency of MN in the negative control for group 1 was 0.36. The positive control was 11-fold higher than the negative control. No uptake was reported.

75. Following treatment with TiO_2, group 1 had significantly increased MN formation at the highest dose (6-fold increase over background).

76. COM opinion – The approach used in the study appears to be relevant. However, there was insufficient information on the population used, it was unclear how the nanoparticles were suspended for the MN assay, and there was no direct evidence of uptake. Due to these observations, the study was given a RAG rating of amber.

77. There was, additionally, a significant increase in DNA strand breaks detected by Comet assay in group 1 and dose-related increases in group 2 and especially in group 3. In a MN-FISH assay, there was a dose-related increase particularly for MN without centromeres in all groups.

78. Overall, this study indicated that TiO_2 significantly increased MN formation in a range of human lymphocytes and was considered positive. This study was cited in the EFSA review (EFSA, 2021).

Osman et al. (2018)

79. Osman et al. (2018) conducted a CBMN assay with cytoB in HPBLs from healthy volunteers and from a group of volunteers with respiratory disease. For the purpose of this review, only data from healthy volunteers is considered. The cells (number not given) were exposed to PHA for 24 hours and then treated with anatase nanoparticles at a concentration of 5 and 10 µg/mL for 20 hours. CytoB was added at 44 hours and cells were harvested at 72 hours.

80. The primary size of TiO_2 nanoparticles was 40-70 nm but the secondary size was not determined. The samples were prepared by suspension in DMSO, filtration through a 0.2 µm syringe filter and then sonicated. One thousand binucleated cells were analysed for MN formation. Mitomycin C (0.4 µM) was used as a positive control. Cytotoxicity was determined by NDI.

81. No cytotoxicity was observed. The MN frequency in the negative control was 0.2% and the positive control showed a 15-fold increase over background in the healthy group.

82. No significant increase in MN formation was seen in the healthy controls when exposed to either of the TiO_2 doses. No cellular uptake data was stated.

83. COM opinion – This study focussed on the comparison of DNA damage seen in HPBLs from healthy controls and in those from patients with lung disease following exposure of the cells to TiO_2. Only data from healthy individuals were considered in this report. The experimental data and details of the TiO_2 nanoparticles used were limited, only 1000 binucleated cells were counted, only two concentrations of TiO_2 were examined, and there was no analysis of uptake. Due to these observations, the study was given a RAG rating of amber.

84. Overall, no evidence of genotoxicity was seen in healthy volunteers and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Prasad et al. (2013)

85. Prasad et al. (2013) conducted a CBMN assay with cytoB in BEAS-2B cells. $5x10^4$ cells/cm^2 were treated with TiO_2 in the different media at doses of 10, 20, 50 or 100 µg/ml for 24 hours, after which cytoB was added for 18 hours.

86. TiO_2 nanoparticles (86% anatase and 14% rutile; primary particle size 27.5 nm (range 14.2-64.6 nm) were prepared in three different media that

have been previously used in TiO_2 genotoxicity studies: keratinocyte growth media (KGM) supplemented with 0.1% BSA (KB); a medium that mimics BAL by containing phosphate buffered saline (PBS) supplemented with 0.6% BSA and 0.001% surfactant (referred to as dispersion medium (DM)); or KGM media supplemented with 10% foetal bovine media (KF). The nanoparticles were probe sonicated for dispersal. One thousand binucleated cells were analysed for MN formation in two independent experiments (total of 2000 binucleated cells/concentration). MMS (100µM) was used as a positive control. Cytotoxicity was measured by CBPI.

87. The MN frequency in the negative control was 2-2.2% and the positive control showed a 3.5-fold increase over background. There did not appear to be cytotoxicity at any tested concentration.

88. Following treatment with TiO_2, only TiO_2 prepared in KF medium gave a dose-related significant increase in the formation of MN. This medium was considered by the authors to be that which facilitated the lowest amount of particle agglomeration, the greatest amount of nanoparticle cellular interaction, and the highest population of cells accumulating in S phase.

89. COM opinion – The study design and conduct were considered reasonable. However, BEAS-2B is a non-standard cell line and is not recommended by OECD. The doubling time of the cells is 26 hours. There was a high background frequency of MN (>2 %), which was considered to be at the upper limit of acceptability. Only TiO_2 prepared in KF media gave a dose-related increase in MN formation. While this was statistically significant, the level of induction was very low, increasing from approximately 2% (background) to 2.8% at 20 µg/mL, 3% at 50 µg/ml and 3.8% at 100 µg/mL (maximum 1.7-fold increase). The low incidence of MN may be due to the exposure duration being less than one cell cycle, rather than 1.5-2 cycles as recommended by OECD. There was no firm evidence of cellular uptake, although the method used was not appropriate for uptake analysis. Due to these observations, the study was given a RAG rating of amber.

90. A Comet assay was also conducted and this was only significantly positive for DNA damage in KB media at the highest dose, although the increase over background was also very low despite being statistically significant (2% (background) increasing to approximately 3.5% at 100 µg/ml).

91. Overall, due to the level of MN induction being low (<2-fold), the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

Stoccoro et al., (2016 and 2017)

92. CBMN formation, with cytoB, was studied in mouse BALB/3T3 cells (Stoccoro et al., 2016) and human A549 cells (Stoccoro et al., 2017). TiO_2 nanoparticles (84% anatase and 16% brookite crystal phase) were treated in different ways: pristine (uncoated), silicate coated, or sodium citrate coated. Aeroxide P25 (a fine-particulate pure TiO_2) was used as a benchmark material.

93. 7.5×10^5 BALB/3T3 cells were treated with anatase at concentrations of 10, 20 or 40 µg/cm^2 (32, 64, 128 µg/mL) for 48 hours. CytoB was added after 44 hours and the cells were harvested after 72 hours.

94. The primary sizes of the TiO_2 nanoparticles were 83.5, 57.5 or 155.6 nm (uncoated, citrate coated or silicate coated, respectively) and secondary sizes were 1608, 68.3 or 563.2 nm (uncoated, citrated or silicate coated, respectively) (Stoccoro et al., 2016).

95. 7.5×10^4 A549 cells were treated with anatase at concentrations of 10, 20, 40 µg/cm^2 (32, 64, 128 µg/mL) for 48 hours. CytoB was added after 44 hours and cells were harvested after 72 hours.

96. The primary sizes of the TiO_2 nanoparticles were 1608, 91.3 and 563.2 nm (uncoated, citrated and silicate coated, respectively) (Stoccoro et al., 2017).

97. A549 cells were dispersed in complete cell culture medium while citrate and 0.05% BSA were used for dispersion of BALB/3T3 cells. 1000 binucleated cells were analysed for MN formation in each assay. Mitomycin C

(0.1 µg/ml) was used as a positive control. Cell cytotoxicity was assessed by CBPI. TEM was used to evaluate cytoplasmic uptake.

98. Cytotoxicity was < 20% in BALB/3T3 cells and A549 cells, apart from citrate-coated nanoparticles which reduced cytotoxicity to approximately 50%. The MN frequency in the negative control was 1% in both cell lines; the positive control was 60-fold higher than background in A549 cells and 5-fold higher in BALB/3T3 cells.

99. Following treatment with TiO_2, in BALB/3T3 cells an increase in the formation of MN was only observed in citrate-coated TiO_2 nanoparticles at the highest dose (3% compared to 1% negative control), while the assay was negative for uncoated and silica-coated nanoparticles. In A549 cells, however, formation of MN was increased with all treated nanoparticles (except for the lowest dose of silicate-coated TiO_2). A dose-dependent increase in MN (2%, 3% and 4%) was observed with 32, 64 and128 µg/mL, respectively, compared with 1% for the negative control.

100. COM opinion – These combined studies were considered to be robust. However, BALB/3T3 cells and A549 cells are non-standard cell lines and are not recommended by OECD, but background MN frequencies were in line with that seen in OECD-recommended cells. The doubling time of BALB/3T3 cells is 18 hours and A549 cells is >22 hours. Only 1000 binucleated cells were counted and the fold-increase with the positive control in A549 cells was high. Due to these observations, the study was given a RAG rating of amber.

101. A Comet assay with Fpg in BALB/3T3 cells suggested ROS involvement. The Comet assay with Endo III and Fpg in A549 cells also showed oxidative DNA damage. Fluorescence in situ hybridization (FISH) analysis suggested possible aneuploidy in MN with TiO_2 (but very weak) and all nanoparticles increased DNA methylation.

102. In BALB/3T3 cells, positive results for MN formation were only detected in citrate-coated nanoparticles. In A549 cells, increased formation of MN was seen with all treated nanoparticles and was considered positive. Overall, these studies showed increased formation of MN and were

considered positive. These studies were cited in the EFSA review (EFSA, 2021).

Vales et al. (2015)

103. Vales et al. (2015) conducted a 4-week chronic CBMN assay with cytoB in BEAS-2B cells. 5.5 x 10^5 cells were exposed to NM-102 (anatase) at concentrations of 1, 10 or 20 µg/mL for up to 3 weeks. TiO_2 was added every 4 days and cells were subcultured weekly. For MN analysis, cells were harvested after 48 hours, 1 or 3 weeks following cytoB co-treatment (no further details were available).

104. The primary size of TiO_2 nanoparticles was 21.7 nm and secondary size was 575.9 nm. The samples were prepared by pre-wetting in ethanol, dispersed in 0.05% BSA and sonicated. Two thousand binucleated cells were analysed for MN. Mitomycin C (150 ng/ml) was used as a positive control. Transmission electron microscopy (TEM) was used to detect cellular uptake into the vacuoles and the nuclear surface. Cytotoxicity was detected by CBPI.

105. The MN frequency in the negative control was 1.7% at 48 hours and 1 week, and 1.2% at 3 weeks; the positive control was 5-fold higher than background at 48 hours and 3 weeks, and 3-fold higher after 1 week.

106. Following treatment with TiO_2, there was no increase in MN formation at 48 hours, 1 week or 3 weeks.

107. COM opinion – This is a good quality study which was reasonably well-conducted. BEAS-2B is a non-standard cell line and is not recommended by OECD, but background MN frequency was in line with that seen in OECD-recommended cells. The doubling time of these cells is 26 hours. As well as the standard short-term exposure, non-standard long-term exposures (1 and 3 weeks) were used. Due to these observations, the study was given a RAG rating of amber.

108. Further studies reported in the paper (intracellular ROS measurement) showed no induction of ROS.

109. Overall, no increase in MN formation was detected and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Vieira et al. (2022)

110. Vieira et al. (2022) conducted a CBMN assay with cytoB in the human intestinal cell lines Caco-2 and H29-MTX-E12, according to OECD 487. $1.5x10^5$ (Caco-2) or $0.5x10^5$ cells (H29-MTX-E12) were exposed to NM-102 (anatase), NM-103 (rutile, coated with hydrophobic Al) and NM-105 (81.5% anatase and 18.5% rutile mix) at concentrations of 0.14, 1.4, and 14 µg/mL for 52 hours (Caco-2) or 72 hours (H29-MTX-E12). CytoB was added after 24 hours.

111. The primary size of TiO_2 nanoparticles was between 22 and 30 nm and the secondary size was 20.4-25.7 nm. The samples were prepared by pre-wetting in ethanol, adding BSA/water and probe sonicating. The stock dispersions were used either immediately, for the static digestion process using a standardised static INFOGEST 2.0 in vitro digestion protocol (resulting in the digested samples to mimic human digestion) or directly (corresponding to the undigested samples) after dilution in cell culture medium. Two thousand binucleated cells were analysed for MN formation. Mitomycin C (0.3 µg/mL) was used as a positive control. Cytotoxicity was measured by CBPI/RI (data not shown).

112. No decreases in CBPI or RI were reported in either cell line after TiO_2 exposure. In Caco-2 cells, the MN frequency in the negative undigested control was 1% and in the digested control was 1.5-2%. In H29-MTX-E12 cells, the MN frequency in the negative undigested control was 0.75-1.5% and in the digested control was 0.8-2.5%. The positive control in Caco-2 cells induced a 3.2-fold higher level of MN compared to the undigested control, and in H29-MTX-E12 cells was 6-8-fold higher than the undigested control.

113. Following treatment with TiO_2, a significant increase in MN formation was only observed with 14 µg/mL undigested NM-105 (1.6-fold compared with controls), but this was not considered biologically relevant. There was no evidence of cellular uptake.

114. COM opinion – The study methodology used a number of non-standard procedures. Caco-2 and H29-MTX-E12 are non-standard cell lines and are not recommended by OECD, but background MN frequencies were in line with those seen in OECD-recommended cells. The doubling time of Caco-2 cells is 23 hours and H29-MTX-E12 cells is approximately 24 hours. No cellular uptake was confirmed. It was unclear how the *in vitro* digestion protocol (INFOGEST) is validated for use in these assays since increases were observed in the 'digested' controls. It is also not clear what the historical ranges would be under these conditions. Due to these observations, the study was given a RAG rating of amber.

115. A Comet assay and oxidative stress studies were also described in this paper. These similarly gave mixed results, but generally there was no induction of ROS.

116. Overall, the results showed no evidence of a dose response, and only small, inconsistent increases, that were not biologically relevant, were observed; hence the study was considered negative. This study was identified during the recent literature search.

'Amber' papers – *hprt* assay

Vital et al. (2022)

117. Vital et al. (2022) conducted a *hprt* assay with cytoB in V79 cells according to OECD TG476. $3x10^5$ cells were exposed to NM-100 (anatase) at concentrations of 1, 3, 10, 30, and 75 µg/cm^3 for 24 hours.

118. The primary size was 110 nm and the secondary size was 256.7-341.3 nm in culture media at 0 hours and 218.0-260.6 nm at 24 hours. The samples were prepared using the Nanogenotox dispersion protocol (no further details provided) in which a stock suspension of 2.56 mg/ml NM-100 was sonicated. MMS (0.1 mM) was used as a positive control. Cytotoxicity was assessed using plating efficiency.

119. In the *hprt* assay, cytotoxicity was 30% at the highest concentration. The *hprt* mutation frequency in the negative control was 9.59 x 10^{-6} and the positive control was 4-fold higher than the background.

120. Following treatment with TiO_2, no evidence of uptake was reported. Significantly increased numbers of mutations were detected at low doses (1, 3, 10 µg/cm^3), but increases were not significant at higher doses.

121. COM opinion – The study was of good design. However, the plating efficiency was 40% at the lowest concentration and 30% at the highest concentration. No uptake data were reported. Due to these observations, the study was given a RAG rating of amber.

122. Overall, there were some significant increases in *hprt* mutations at the lower doses but not the higher doses (lack of dose-response relationship) but overall, the study was considered positive. This study was identified during the recent literature search.

Summary

123. Following the screening of papers cited in the EFSA opinion (EFSA, 2021) as described in the methodology section, and a further assessment of newer literature (2021–2023; Annex 1), 15 papers, reporting 16 assays, were identified to be of sufficient quality to warrant further assessment.

124. Regarding the *in vitro* genotoxicity of TiO_2, the studies considered to be of sufficient quality included three genotoxicity assays, namely CBMN (green=5; amber=8), *hprt* (green=1; amber=1) and CA (green=1; amber=0), all of which are recognised by the OECD and other international regulatory bodies. 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.

125. An overall summary of the data is presented in **Error! Reference source not found.** and results of each study are summarised in Table 5 to **Error! Reference source not found.**.

126. Of the six green studies deemed to have used robust methodology only one reported an increase in MN frequency, in BEAS-2B cells following treatment with coated rutile TiO_2 (Di Bucchianico et al., 2017). The same study also reported negative results with coated and uncoated anatase. All other 'green' studies showed no increase in MN frequency following TiO_2 treatment in various cell lines or HPBLs. Kazimirova et al. (2020) also reported no increase in hprt mutations in V79 cells following treatment with an anatase/rutile mixture, although Unal et al. (2021) reported an increase in CA in HPBLs following treatment with TiO_2 nanopowder.

127. From the 'amber' papers, an increase in MN frequency was reported in HPBLs following treatment with anatase TiO_2 (Kurzawa-Zegota et al., 2017), and in BALB/3T3 cells following treatment with a citrate coated anatase/brookite mix (Stoccoro et al., 2016) and in A549 cells with uncoated, citrate coated and silica coated anatase/brookite mix (Stoccoro et al., 2017). All other 'amber' studies showed no increase in MN frequency following TiO_2 treatment in various cell lines or HPBLs. Vital et al. (2022) showed an increase in hprt mutations in V79 cells following anatase treatment.

Table 5 Summary of the 'Green' MN results

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Anatase Rutile Mixture	20-60 nm 30x100 nm 45-252 nm	10-200 µg/mL (200 µg/mL data excluded from interpretation*)	PBMCs	Yes	Neg	Andreoli et al. (2018)
Anatase	21 or 50 nm	10-100 µg/mL (1000 µg/mL data excluded from interpretation*)	HEK293 NIH/3T3	No	Neg	Demir et al. (2015)
Uncoated anatase (NM-100)	50-150 nm 5-8 nm	1-15 µg/mL	BEAS-2B	No	Neg (uncoated anatase) Neg (coated anatase) Pos (coated rutile) (parallel Comet assay assessment +/-	Di Bucchianico et al. (2017)

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Coated anatase (NM-101) Coated rutile (NM-103)	20-28 nm				fpg was suggestive of oxidative damage)	
Anatase	8.9-15.3 nm	100 µg/mL (200 µg/mL data excluded from interpretation*)	TK6	Yes	Neg	Li et al. (2017)
Rutile/anatase nanopowder	10-360 nm	20-100 µg/mL	HPBLs	Yes	Neg	Unal et al. (2021)

*higher doses excluded from interpretation as they exceeded those recommended in OECD test guidelines

Table 6 Summary of the 'Green' *hprt* results

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Anatase/rutile mix	21 nm	3-75 µg/cm^2	V79	Yes	Neg	Kazimirova et al. (2020)

Table 7 Summary of the 'Green' CA results

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Rutile/anatase nanopowder	10-360 nm	20-100 µg/mL	HPBLs	Yes	Pos	Unal et al. (2021)

Table 8 Summary of the 'Amber' MN results

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Anatase/rutile mix	21 nm (15-60 nm)	3, 15 or 75 µg/cm^2	TK6	No	Neg	Kazimirova et al. (2019)
Anatase	<25 nm	10-80 µg/mL	HPBLs	Yes	Pos	Kurzawa-Zegota et al. (2017)
Anatase	40-70 nm	10-100 µg/mL	HPBLs	Yes	Neg	Osman et al. (2018)
86% anatase, 14 % rutile	27.5 nm	10-100 µg/mL	BEAS-2B	No	Neg	Prasad et al. (2013)
84% anatase, 16% brookite Pristine (uncoated) Silicate coated	83.5 nm 57.5 nm 155.6 nm	32-128 µg/mL	BALB/3T3	No	Neg (uncoated) Pos (citrate coated) (Comet assay with Fpg suggested ROS involvement) Neg (silicate coated)	Stoccoro et al. (2016)

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
Sodium citrate coated						
84% anatase, 16% brookite Pristine (uncoated) Silicate coated Sodium citrate coated	83.5 nm 57.5 nm 155.6 nm	32-128 µg/mL	A549	Yes, but not extensively validated	Pos (uncoated) Pos (citrate coated) (Comet with Endo III and Fpg in showed oxidative DNA damage. (FISH analysis suggested possible aneuploidy in MN with TiO_2 (but very weak) and all nanoparticles increased DNA methylation) Pos (silicate coated)	Stoccoro et al., (2017)
NM-102 (anatase)	21.7 nm	1-20 µg/mL	BEAS-2B	No	Neg	Vales et al. (2015)

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
NM-102 (anatase) NM-103 (rutile, coated with hydrophobic Al) NM-105 (81.5% anatase and 18.5% rutile mix)	22-30 nm	0.14-14 µg/mL	Caco-2 H29-MTX-E12	Yes, but not extensively validated	Neg	Vieira et al. (2022)

Table 9 Summary of the 'Amber' *hprt* results

Test material	Primary size	Conc.	Cell type	OECD recommended cells	Result	Reference
NM-100 (anatase)	110 nm	1-75 µg/cm^3	V79	Yes	Pos	Vital et al. (2022)

COM opinion

128. After reviewing the in vitro genotoxicity studies performed to date on TiO2, we note the following points:

i. There were five in vitro studies of the highest quality (labelled “green” here) that used TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. All four “green” studies that used anatase TiO_2 nanoparticles reported negative results for the MN endpoint. Of the two green studies that used rutile TiO_2 nanoparticles, one was negative and the other was weakly positive for MN induction in a non-standard cell line but only at the two lowest doses used (1 and 5 mg/ml) (Di Bucchianico et al 2017). Two green studies used TiO_2 nanoparticles of mixed anatase/rutile form and both were negative for MN induction.

ii. There were two green studies that both used anatase/rutile TiO_2 nanoparticles in either the hprt gene mutation assay or CA assay. The TiO_2 nanoparticles were negative in the hprt assay. In the CA assay, the TiO_2 nanoparticles were positive, but the CA frequency decreased with increasing TiO_2 concentration, and despite the significant induction of CA, this study was negative with the micronucleus assay.

iii. There were eight amber studies (i.e., ones that contained some suboptimal aspects) that used TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. Four studies used anatase TiO_2 nanoparticles and three of these were negative for micronuclei induction. The one positive study reported a dose-dependent increase in micronuclei induction in lymphocytes from healthy individuals. All three studies that used nanoparticles of mixed anatase/rutile TiO_2 were negative for micronuclei induction. Two studies that used anatase/brookite TiO_2 nanoparticles reported positive results for micronuclei induction.

iv. The one amber study on hprt mutations was positive at low anatase TiO_2 nanoparticle doses but not at higher doses (Vital et al. 2022).

v. Some “green” studies included other assays (e.g. Comet assay) to provide mechanistic information but results were inconsistent, showing

either no increase (Demir et al., 2015), or an increase in oxidative DNA damage (Di Bucchianico et al., 2017) but only at the highest dose (Unal et al., 2021). Andreoli et al., 2018 and Stoccoro et al., 2017 showed ROS involvement.

129. Overall, the COM opinion is that there is little evidence that TiO_2 nanoparticles are genotoxic in vitro, with the limited number of positive studies all reporting no dose-response effects with significant effects being observed at the lowest doses used. There is also a lack of replication of study outcomes using the same nanoparticle in different labs.

130. 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. The studies identified in this report are not representative of E171, where the fraction of nanoparticulate is <50% and according to the recent "Guidance on the implementation of the Commission Recommendation 2022/C 229/01 on the definition of nanomaterial" (https://data.europa.eu/doi/10.2760/143118), E171 would fall under the definition of a NM, hence we need GLP studies with E171 to definitively assess the hazard.

131. We also note that there is a dearth of high-quality datasets available with well documented nanomaterial characteristics where the relevant OECD test guidelines have been followed.

Abbreviations

ANS Panel	EFSA Panel on Food Additives and Nutrient Sources added to Food
BEAS-2B	Bronchial epithelial cell line
BSA	Bovine serum albumin
CBMN	Cytokinesis block micronuclei
CBPI	Cytokinesis block proliferation index
CP	Cyclophosphamide
DMEM	Dulbecco's Modified Eagle Medium
EFSA	European Food Safety Authority
EMS	Ethyl methanesulphonate
FBC	Fluidized Bed Crystallization
FISH	Fluorescence in situ hybridization
Fpg	Formamidopyrimidine DNA glycosylase
HEK	Human embryonic kidney
HPBL	Human peripheral blood lymphocytes
Hprt	Hypoxanthine phosphoribosyl transferase
LDH	Lactate Dehydrogenase
MI	Mitotic Index
MMC	Mitomycin C
MMS	Methyl methanesulphonate
MN	Micronuclei
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NDI	Nuclear Division Index
OECD	Organisation for Economic Co-operation and Development
8-oxodG	8-oxo-2’-deoxyguanosine

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PFL	Water Filtration Media
PHA	Phytohaemoglutinin A
RI	Replication index
RICC	Relative increase in cell counts
RNBR	Relative nuclei to bead ratio
ROS	Reactive oxygen species
RPD	Relative population doubling
RPMI / RPMI 1640	Roswell Park Memorial Institute 1640 Medium
SCE	Sister chromatid exchange
SEM	Standard error of the mean
TEM	Transmission electron microscopy
6-TG	6-thioguanine
TiO_2	Titanium dioxide (E171)
VIN	Vinblastine

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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 vitro") AND PUBYEAR > 2020 AND PUBYEAR > 2020: 39

PubMed:

"titanium dioxide"[Title/Abstract] AND nanoparticle[Title/Abstract] AND genotox*[Title/Abstract] AND "in vitro"[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.