Committee on Mutagenicity

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MUT/2023/07

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

Assessment of in *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. The paper provided in Annex A gives an 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. 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 vitro* papers?

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

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

Committee on Mutagenicity

MUT/2023/07 – Annex A

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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.

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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 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); 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 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 (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 vitro* genotoxicity studies on TiO_2

NM characteristics

- Crystalline form
- **Particle size (primary and secondary size) and shape**
- Agglomeration method
- Dispersion method and preparation samples,

Study design characteristics

- **Use of positive controls**
- **Number of replicates**

Tier 2. Generic genotoxicity study design

5. When assessing papers based on the 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 replicates being >1 (see bold text in Table 2). Papers with a score of 9 out of 13 and above proceeded to tier 3 and were further evaluated by assessing the detailed genotoxicity study design.

Table 2: Assessment criteria for genotoxicity study design of *in vitro* genotoxicity studies on TiO2

Nanoparticle

- Source of nanoparticle
- Concentrations in exposure media (Doses <500 µg/ml)

Organism characteristics

- Cell model
- Duration of exposure
- **Use of negative/positive controls**
- **Numbers of replicates >1**
- OECD-recommended cytotoxicity assay
- 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. Such data were assessed using the exclusion criteria listed below, using expert judgement.

Table 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.

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) and chromosomal aberrations (CA; OECD TG473). Other assays

were excluded from further evaluation. Other exclusion criteria included the lack of positive controls, no or incorrect cytotoxicity assays, use of inappropriate cell lines, inadequate duration of exposure, high concentrations tested, insufficient number of cells assessed and high spontaneous levels of damage.

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 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 are not of sufficient quality for use in the assessment of genotoxicity of TiO_2.

10. A number of criteria such as the absence of appropriate controls, insufficient experimental details and irrelevant tests automatically led to these studies being graded as red (RAG rating) and not being 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, however, of these studies have been used as potential indicators of mechanisms of action.

12. Overall, from a total of 294 papers that were initially assessed, 20 papers were categorised as green or amber and were thereby considered to be relevant and of sufficient quality for use in the *in vitro* genotoxicity assessment of TiO_2 (Figure 1).

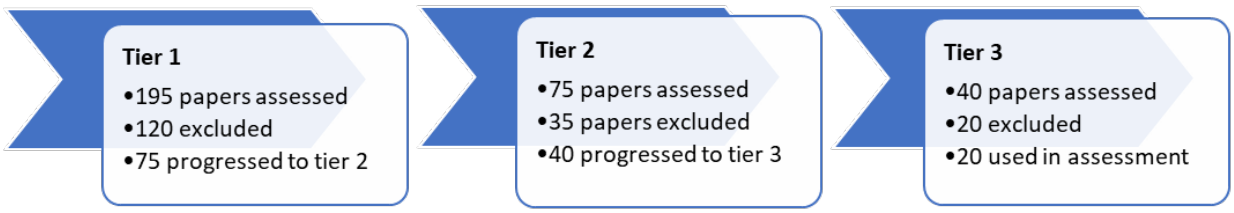

Figure 1 Summary of number of papers assessed in tier 1, 2 and 3

13. The 20 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 vitro* genotoxicity of TiO_2.

14. Nine of the 20 papers were considered as the most robust and were categorised as green and 11 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[Sb1]

Test	*Green category*	*Amber category*
MN	7	10
Hprt	2	1
CA	0	2

Note: some papers assessed several endpoints

‘Green’ papers – MN assay

Andreoli et al. (2018)

15. Andreoli et al. (2018) conducted a cytokinesis block micronuclei (CBMN) assay in peripheral blood mononuclear cells (PBMCs), using cytochalasin B (cytoB) and 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 hence more weight was put on such results.

16. The primary size of TiO_2 nanoparticles was 20-60 nm, 30x100 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 a 210 nm (50-1570 nm), 226 nm (50-3340 nm) or 328 (50-1770 nm), respectively when dispersed in Roswell Park Memorial Institute (RPMI) media without a surfactant. Two 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 and no toxicity was observed after any treatment with TiO_2.

17. The negative control (background) was 0.75-1% and the positive control was 5-8-fold higher than the background. No increase in MN formation was detected with either TiO_2 treatment protocol.

18. COM opinion – This study was considered robust and well-conducted and RAG assessed as green. A flow-based method was used which does not confirm cellular uptake. Hydrogen peroxide was used as a positive control, which is not a recommended positive reference control according to OECD TG487. However, it was positive in the formation of MN. The study detected induction of 8-oxo-2'-deoxyguanosine (8-oxodG) indicating oxidative damage for anatase and rutile and the mixture, suggesting a possible mechanism of action. Overall, no increase in MN formation was detected and the study was considered negative. [JK2][Sb3][Sb4]This study was cited in the EFSA review (EFSA, 2021).

Demir et al. (2015)

19. Demir et al. (2015) conducted a CBMN assay in human embryonic kidney (HEK293[JK5]) 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 nanoparticles for 48 hours. CytoB was added for the last 24 hours prior to harvesting.

20. Two sizes of anatase nanoparticles were tested. The primary sizes were 21 ± 2.8 nm or 50 ± 12 nm and the secondary sizes were 22.94 ± 0.3 nm and 50.72 ± 0.4 nm. For these measurements, TiO_2 NP were dispersed by ultrasonication in 0.05% bovine serum albumin (BSA). 1000 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.

21. The negative control (background) was 0.55% in HEK293 cells and 0.6% in NIH/3T3 cells, and the positive controls were approximately 8-or 6-fold higher than the background, respectively. No genotoxicity or cytotoxicity was detected between 10-100 µg/ml in either cell lines for both sizes of nanoparticles. MN formation was only increased at the highest TiO_2 concentration (1000 µg/ml) in both cell lines.

22. COM opinion – This study showed robust methodology and was RAG assessed as green. Non-standard cell lines were used but background MN frequency was in line with that seen in OECD-recommended cells. 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. A Comet assay ± formamidopyrimidine DNA glycosylase (Fpg) in the study gave no evidence of oxidative DNA damage [AP6][Sb7] or cell transformation between 10-100 µg/ml. Overall, no increase in MN formation was detected and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Di Bucchianico et al. (2017)

23. 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: 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.

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

25. Nanoparticles were dispersed using ultrasonication in 0.05 % BSA. 2000 binucleated cells were analysed for MN formation. MMC (0.05 µg/ml) was used as a positive control. Cytotoxicity was assessed by calculating the reduction of 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.

26. Cytotoxicity was minimal with a background of approximately 1% (for both methods employed) and the positive control was 8-9-fold higher than the background (for the manual vs flow method, respectively). 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. A weak positive formation of MN was observed in the low dose region only, i.e., at doses of 1 and 5 µg/ml in the rutile (NM-103)-treated cells using both methods.

27. COM opinion – This study was well conducted and showed robust methodology and was RAG assessed as green. The cell line used in the study was not, however, listed as recommended by OECD but the background frequency of MN was in line with OECD-recommended cell lines. The Litron kit method normally requires 10000 cells to be scored, whereas in the study, 2000 nuclei were scored. The methodology used to show cellular uptake is not reliable. The number of replicates was unclear, although figures contained error bars.

28. A Comet assay was conducted ±Fpg in the study. This was positive after 3 hours with both NM-100 and NM-103 while after 24 hours only NM-101 was positive. This was indicative of oxidative damage. Overall, the study was

weakly positive for the formation of MNat lower doses but only in rutile-treated cells. This study was cited in the EFSA review (EFSA, 2021).

Prasad et al. (2013) change to amber?

29. 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.

30. 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 which have been previously used in TiO_2 genotoxicity studies: keratinocyte growth media (KGM) supplemented with 0.1% BSA (KB); 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). Methyl methanesulphonate (MMS; 100µM) was used as a positive control. Cytotoxicity was measured by CBPI.

31. The negative control measured manually was 2-2.2% and the positive control showed a 3.5-fold increase over the background. There did not appear to be cytotoxicity at any tested concentration. There was no firm evidence of cellular uptake although the method used was not appropriate for uptake analysis. Only TiO_2 prepared in KF media gave a dose-related significant increase in the formation of MN. This media was considered by the authors to be that which facilitated the lowest amount of agglomeration, the greatest amount of nanoparticle cellular interaction, and the highest population of cells accumulating in S phase.

32. COM opinion – The study design and conduct were considered reasonable and the study was RAG assessed as green[Sb8]. The cell line used in the study was not, however, recommended by the OECD but the background frequency of MN was in line with OECD-recommended cell lines.

33. Only TiO_2 prepared in KF media gave a dose-related increase in the formation of MN. While this was statistically significant, the level of induction was very low from approximately 2% (background) to 2.8% (20 µg/ml) and 3% at 50 µg/ml and 3.8% at 100 µg/ml. 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 the OECD.

34. A [Sb9]Comet assay was 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). Overall, despite the level of MN induction being low, the study was considered to be negative. This study was cited in the EFSA review (EFSA, 2021).

Srivastava et al. (2013) change to amber?

35. Srivastava et al. (2013) conducted a CBMN study with cytoB, in a human lung alveolar cancer cell line, A549. Cells (number not given) were exposed to the TiO_2 nanoparticles at concentrations of 1, 5, 10 or 50 µg/ml for 24 hours after which cytoB was added for 22 hours.

36. TiO_2 nanoparticles, with the primary size < 25 nm and secondary size 434 nm in complete media were sonicated in Dulbecco's Modified Eagle Medium (DMEM) with 10% serum with no surfactant. No further details about the nanoparticles were given. One thousand binucleated cells were analysed for MN formation. Ethyl methanesulphonate (EMS; 6 mM) was used as a positive control. Cell cytotoxicity was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assays. Transmission electron microscopy (TEM) confirmed uptake of nanoparticles into the cytoplasm.

37. Cytotoxicity was less than 20% and the negative control was 0.5% and positive control was 6-fold higher than the background. A dose-related 2-3-fold increase in MN formation was observed at 10 and 50 µg/ml, respectively.

38. COM opinion – This was considered to be a good [JK10][Sb11]quality study and was RAG assessed as green[Sb12], although there was limited information on the actual nanoparticle studied. Moreover, a non-standard cell line was used as well as non-standard cytotoxicity tests (MTT and LDH). [MOU13][AP14][JK15][Sb16]In further studies also described in this paper, ROS induction was detected at 6, 12 and 24 hours at doses of 10 and 50 µg/ml while apoptosis was also noted at 48 hours in a dose-dependent manner. p53 activation and p21 expression were also observed. With the caveat of the study limitations, there was evidence of a dose-related increase in MN formation and this was considered a positive result. This study was cited in the EFSA review (EFSA, 2021).

Stoccoro et al., (2016 and 2017) change to amber?

39. 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 was used as a benchmark chemical.

40. 7.5×10^5 cells (BALB/3T3) 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 the cells were harvested after 72 hours.

41. The primary sizes of TiO_2 nanoparticles were 83.5, 57.5 or 155.6 nm (uncoated, citrated or silicate, respectively) and secondary sizes were 1608, 68.3 or 563.2 nm (uncoated, citrated or silicate coated, respectively).

42. 7.5×10^4 cells (A549) 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.

43. The primary sizes of TiO_2 nanoparticles were 1608, 91.3 or 563.2 nm (uncoated, citrated or silicate coated, respectively).

44. 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 [JK17][Sb18]were analysed for MN formation in each assay. MMC (0.1 µg/ml) was used as a positive control. Cell cytotoxicity was assessed by CBPI. TEM was used to evaluate cytoplasmic uptake.

45. Cytotoxicity was < 20% in NIH-3T3 cells whereas in A549 cells cytotoxicity was <20% apart from with citrate-coated nanoparticles, which reduced cytotoxicity to approximately 50%). The negative control was 1% in both cell lines and the positive control was 60-fold higher than background in A549 cells and 5-folder higher in NIH-3T3 cells.

46. In BALB/3T3 cells, an increase in 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 treatments with 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 of 2%, 3% and 4% was observed for 32, 64 and128 µg/ml, respectively, compared with 1% for the negative control.

47. COM opinion – These combined studies were considered to be robust and were both RAG assessed as green[Sb19]. Non-standard cell lines were used but the background frequency of MN was in line with OECD-recommended cell lines. In BALB/3T3 cells, positive results for MN formation were only detected in citrate-coated nanoparticles. Further studies with a Comet assay with Fpg suggested ROS involvement. In A549 cells, increased formation of MN was seen with all treated nanoparticles and was considered positive. The Comet with Endo III and Fpg included as additional studies in these papers also showed oxidative DNA damage[AP20][Sb21]. Fluorescence in situ hybridization (FISH) analysis suggested possible aneuploidy in MN with TiO_2 (but very weak) and all nanoparticles increased DNA methylation. Overall, these studies showed increased formation of MN and were considered positive. These studies were cited in the EFSA review (EFSA, 2021).

‘Green’ papers – *hprt* assay

Jain et al. (2017) change to amber?

48. Jain et al. (2017) carried out a *hprt* forward mutation test in Chinese Hamster lung fibroblasts (V79) according to OECD TG476. $1x10^5$ cells/well were exposed to anatase nanoparticles at concentrations of 1, 10, 25, 50 or 100 µg/ml for 6 hours using either a culture plate method or a soft agar method. After 6 hours, cells were washed and reseeded for 7 days after which time cells were replated with 6-thioguanine (6-TG) to determine cloning efficiency.

49. The primary size of TiO_2 nanoparticles was < 25 nm and secondary size was 176.2 nm in DMEM. The nanoparticle samples were probe sonicated in DMEM containing 10% foetal bovine serum (FBS). EMS (50 µg.ml) was used as a positive control. Cell cytotoxicity was assessed by cloning efficiency (although no data were given), MTT assay, propidium iodide (PI) uptake.

50. Approximately 65% MTT reduction[JK22][Sb23][Sb24] and approximately 20% dead cells (PI) were observed at the highest genotoxic dose (100 µg/ml) at 6 hours. The negative control was approximately 7.7% in[JK25] the culture plate method and 2.7% (small colonies) and 0.7% (large colonies) in the agar method. Positive controls were 6-fold (plate method) and approximately 8-fold (small colonies) and 10-fold (large colonies) (agar method) over background. A dose-related increase in *hprt* mutations was observed (although this was only statistically significant at 50 and 100 µg/ml) using both methods.

51. COM opinion – This was considered a well-conducted study with consistent mechanism of action data and was RAG assessed as green. In further studies, significant dose-related increases were seen in intracellular ROS formation and an increase in DNA damage in a Comet assay and hence a consistent association was considered with the *hprt* results. Overall, a dose-related increase in *hprt* mutations was seen in this study and it was considered a positive study. This study was cited in the EFSA review (EFSA, 2021).

Kazimirova et al. (2020)

52. Kazimirova et al. (2020) conducted a *hprt* forward mutation test in V79 cells according to OECD TG476. $1x10^6$ cells/dish were exposed to anatase/rutile 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.

53. The primary size of TiO_2 nanoparticles was 21 nm (15-60nm) and secondary size 228 ± 3.2 nm using dispersion procedure (DP) 1 and 184 ± 3.5 nm using DP2. In 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. MMS (0.1 mM) was used as a positive control. The cytotoxicity was measured by determining both the relative growth activity (RGA) and the plating efficiency (PE). Cytotoxicity was assessed by cloning efficiency.

54. There were no substantial effects of exposure time (24, 48 and 72 h), on cytotoxicity although a tendency for a lower RGA at longer exposure was observed. No significant difference in PE values were seen. The negative control was approximately $2x10^5$ [JK26]and the positive control was approximately 6-fold higher than the background.

55. There was no evidence was found of increased *hprt* mutation frequency in cells treated with TiO_2 in spite of evidence of uptake of NPs by cells.

56. COM opinion – This was considered to be a well-conducted assay using robust methodology and was RAG assessed as green. No further studies from the paper were available to aid consideration of a mechanism of action. Overall, no evidence of increased mutation was seen in this study and it was considered negative[AP27][Sb28]. This study was cited in the EFSA review (EFSA, 2021).

Summary - wait for final list of studies

57. xxxxxxxxxxxxxxx

‘Amber’ papers – MN assay

Bioreliance (2021)

58. The Titanium Dioxide Manufacturers’ Association Bioreliance (2021) carried out a CBMN assay with cytoB in human peripheral blood lymphocytes (HPBLs) according to OECD TG 487. The cells (number not given; 0.5 ml) were exposed to TiO_2 E171-E anatase at concentrations of 0.3, 1, 2, 3, 10 or 30 µg/ml for 4 hours, with and without S9 mix, after which time cytoB was added for 20 hours. Cells were also continuously treated with TiO_2 and co-administered cytoB, but such results were disregarded.

59. The primary size of TiO_2 nanoparticles was 70 nm at x10, 110 nm at x50 and 180 nm at x90. The secondary size was not reported. The samples were prepared by vortexing in sterile water and water filtration media (PFL) with 15% fluidized bed crystallization (FBC) in culture media as a surfactant. 2000 binucleated cells/concentration were analysed for MN formation. MMC (0.4 µg/ml) was used as the positive control for assays with S9, while cyclophosphamide (CP; 7.5 µg/ml) and vinblastine (VIN; 5 ng/ml) were used without S9. Cytotoxicity was assessed by CBPI.

60. The negative control was 0.15% at 4 hours, with and without metabolic activation and 0.2% after 24 hours without metabolic activation. The positive controls were 16-, 8- and 5.5-fold higher than controls for MMC, CP and VIN, respectively. Cellular uptake was conducted [JK29]by TEM, but no results were reported. No increased MN formation was detected in any of the experiments.

61. COM opinion – The methodology is considered sub-optimal due to the addition of cytoB after 4 hours and subsequently being left for 20 hours. Therefore, the study was RAG rated amber. No further information was available from this study to aid consideration of any mechanism of action. Overall, no evidence of genotoxicity was seen in this study and the study was considered negative. This study was cited in the EFSA review (EFSA, 2021).

Kurzawa-Zegota et al. (2017)

62. Kurzawa-Zegota et al. (2017) conducted a CBMN assay with cytoB in HPBLs. The blood lymphocytes were collected from 3 different groups of patients: group 1. Healthy patients (n=20); group 2. Polyposis coli patients (n=19); group 3. Colon cancer patients (n=20). 400 µl whole blood was incubated with PHA for 24 hours, then exposed to anatase 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.

63. The primary size of TiO_2 nanoparticles was <25 nm [Sb30]and the secondary size increased with time, dose and varied with the media used, ranging from 104.2 nm in water to 1303 nm in RPMI[Sb31][Sb32]. The samples were prepared by ultrasonication in water, PBS or RPMI1640 (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. MMC (0.4 µM) was used as a positive control.

64. No cytotoxicity was observed in any cohort. The negative control for group 1, 2 and 3 was 0.36%, 1.03%, and 1.02% respectively. The positive control was 11-, 2.5- and 4-fold higher than the negative control for group 1, 2 and 3, respectively. No uptake was reported.

65. The results of the CBMN assay showed group 1 had significantly increased MN formation at the highest dose (6-fold increase over background), while dose-related increases were seen in groups 2 (1-, 1.3- and 2.13-fold increase) and 3 (1.25-, 1.68- and 2.3- fold increase).

66. <u>COM opinion</u> – The approach appears to be relevant while sensitivity may be a problem as is the low number of cells counted[JK33][Sb34]. This study was considered RAG status amber as there was insufficient information on the population used, it was unclear how the nanoparticles were suspended in the MN assay and there was no direct evidence of uptake. There was a significant increase in DNA strand breaks detected by a Comet assay in group 1 and dose-related increases in group 2 and especially in group 3. In a MN-FISH assay[JK35][Sb36], there was a dose-related increase particularly for MN

without centromeres in all groups. Overall, this study indicated 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).

Li et al. (2017) change to green?

67. 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 microscopy. $3x10^5$ cells were exposed to synthesised anatase at concentrations of 10, 50, 100, 200, 400 or 800 µg/ml in test 1, and 100 and 200 µg/ml in test 2.

68. 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. Ten thousand binucleated cells were analysed for MN formation in test 1 and 2000 in test 2. X-rays were used as the positive control in test 1 and MMC (0.01 µg/ml) in test 2. Cytotoxicity was assessed by several methods: For test 1; relative increase in cell counts (RICC), relative population doubling (RPD) and relative nuclei to bead ratios (RNBR) were used and for test 2 only RICC and RPD were used.

69. No cytotoxicity was detected. The negative control was 0.24% for test 1 and 1.85% for test 2. The positive control was 10-15-fold the background in test 1 and 2.5fold the background in test 2. No cellular uptake was reported. The results of the study from test 1; were not possible to interpret due to TiO_2 interference with the flow cytometry analysis and for test 2, a significant increase in frequency of MN was detected (1.5- and 2-fold increase at 100 and 200 µg/ml, respectively).

70. COM opinion –In study 1, as TiO_2 emits fluorescence, this could interfere with flow cytometry and therefore the results are difficult to interpret. However, study 2 was well conducted. The study was considered as RAG status amber [Sb37]overall since the manual analysis method in study 2 was interpretable. No further information was available from this study to aid consideration of any mechanism of action. Test 1 was insufficient to draw an

interpretation, however, test 2 indicated a significant increase in MN formation and was considered positive. This study was cited in the EFSA review (EFSA, 2021).

Osman et al. (2018)

71. Osman et al. (2018) conducted a CBMN assay with cytoB in HPBLs from healthy volunteers as well as from a group of volunteers with respiratory disease[JK38]. For the purpose of this review, only data from healthy volunteers will be considered. The cells (number not given) were exposed to PHA for 24 hours then treated with anatase 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.

72. 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 sonicated. One thousand binucleated cells were analysed for MN formation. MMC (0.4 µM) was used as a positive control. Cytotoxicity was determined by Nuclear Division Index (NDI).

73. No cytotoxicity was observed. The negative control was 0.2% and the positive control showed a 15-fold increase over background in the healthy group and a 3.5-fold increase in the patient group. No cellular uptake data was stated. The only significant increase in MN formation was observed at 10 µg/m in the respiratory disease patient group (an approximate 1.4fold increase). No significant increase in MN formation was seen in the healthy controls when exposed to either of the TiO_2 doses.

74. <u>COM opinion</u> – This study was considered as being of little relevance to the assessment of genotoxicity. The experimental data and details of the TiO_2 nanoparticle used were limited, [JK39][Sb40][JW41]and there was no analysis of uptake hence the study was RAG rated amber. Moreover, only two concentrations of TiO_2 were examined, the highest concentration being quite low, and the study focussed on the comparison between the response to TiO_2 exposure between healthy controls and patients with lung disease. 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).

Shukla et al. (2011)

75. Shukla et al. (2011) conducted a CBMN assay with cytoB in a human epidermal cell line, A431, according to OECD TG 487. $7x10^4$ cells were exposed to anatase at concentrations of 0.008, 0.08, 0.8, 8 or 80 µg/ml (0.0025 to 25 µg/cm^2) for 6 hours and co-treated with cytoB for a further 18 hours.

76. The primary size of TiO_2 nanoparticles was 50 nm and the secondary size in culture media was 171.4 nm and 124.9 nm in water. The nanoparticles were freshly prepared using a 160 µg/ml TiO_2 stock suspension in DMEM with 10% FBS and probe sonicated. 2000 binucleated cells were analysed for MN formation. EMS (6mM) was used as a positive control. TEM was used to determine uptake. Cytotoxicity was determined by CBPI.

77. There was no cytotoxicity observed. The negative control was 0.93% and the positive control was 2.9-fold higher than the background. A small increase in MN formation was observed at 0.8, 8 and 80 µg/ml (1.6-, 1.7- and 1.71-fold above control respectively).

78. COM opinion – Reservations[Sb42] have been expressed about the robustness of this study. Uptake appeared unlikely given the short exposure time in the absence of cytoB, as <1.5 cell cycles were achieved as recommended by OECD. The levels of MN formation by TiO_2 were low albeit significant (max 1.7-fold increase). The selected cell line was unusual although there were appropriate positive/negative control levels for MN. The small MN increases and Comet responses observed in further studies (see below), could be due to limited exposure time without cytoB. Further studies conducted by the authors included a Comet assay, Fpg assay and ROS generation using several methods. At 8 and 80 µg/ml statistically significant, concentration-related increases were seen in a number of markers of oxidative stress and DNA damage. The results indicated a small increase in MN formation; therefore, it was considered, subject to the limitation of the

study, a positive result. This study was cited in the EFSA review (EFSA, 2021).

Tavares et al. (2014) change to red?

79. Tavares et al. (2014) conducted a CBMN assay in HPBLs according to OECD TG487. The cells (number not given) were exposed to four types of TiO_2 particles (NM-102 (anatase), NM-103 (rutile (hydrophobic)), NM-104 (rutile (hydrophilic)) and NM-105 (rutile-anatase (15-85%)) at concentrations 5, 15, 45, 125 or 250 µg/ml for 6 hours and then cytoB was added for a further 24 hours.

80. The primary sizes of TiO_2 nanoparticles were 20.8, 21.39, 19.0 and 20.0 nm, respectively and secondary sizes were following agglomeration was approximately 100 nm. The samples were prepared by pre-wetting with ethanol and BSA and sonicating. Two thousand binucleated cells were analysed. MMC (0.075 and 0.167 µg/ml) was used as a positive control. Cytotoxicity was detected by CBPI.

81. No cytotoxicity was observed[Sb43].. The negative control was 0.7% (range 0.5-1% due to individual done variability) and the positive control was 3.8-fold higher than the background. An increase in MN formation was seen with NM-102 (1.4-fold higher than background at 125 µg/ml), NM-103 (1.7- and 1.5-fold higher at 5 and 45 µg/ml, respectively), NM-104 (2- and 1.9-fold higher at 15 and 45 µg/ml, respectively) (but not NM-105) although no dose response was observed.

82. COM opinion – There were a number of variations in this study. There was a difference in the MN formation response for the different nanoparticles and for the positive and negative controls as well as there being no dose response. There also appeared to be variation between the different donors. Therefore, this study was RAG assessed as amber[JK44][Sb45]. The increase in MN formation in three out of four nanoparticles indicated a positive result for genotoxicity noting the caveats of the study limitations. This study was cited in the EFSA review (EFSA, 2021).

Unal et al. (2021)

83. Unal et al. (2021) conducted a CA assay (see below) and a CBMN assay with cytoB in HPBLs. The cells (number not given) were exposed to nanopowder[AP46][Sb47] (no further information given) at concentrations of 20, 40, 60, 80 or 100 µg/ml for 48 hours and cytoB was added after 44 hours.

84. The primary size of TiO_2 nanopowder was 10-360 nm but secondary size was not stated. Stocks were ultrasonicated in distilled water diluted to final concentrations and then sonicated again. Three thousandbinucleated cells were analysed for MN. MMC (0.2 µg/ml) was used as a positive control. Cytotoxicity was measured by CBPI (called NDI).

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

86. There was no evidence of uptake of nanoparticles. There were no significant increases in MN formation observed at any dose.

87. COM opinion – This study is regarded as of medium quality, the study details are unclear and it is unknown whether PHA was used to induce cell division. [Sb48]There is no evidence of uptake. Therefore, this study was RAG assessed as amber. Further experiments in this study included a Comet assay, CA and sister chromatid exchange (SCE). Within the limitation of the study, a negative result for MN was observed. This study was identified during the recent literature search.

Vales et al. (2015)

88. 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 24 hours[Sb49][JW50], 1 or 3 weeks following cytoB co-treatment (no further details were available).

89. 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. MMC (150 ng/ml) was used as a positive control. TEM was used to detect cellular uptake into the vacuoles and the nuclear surface. Cytotoxicity was detected by CBPI.

90. The negative control was 1.7% at 24 hours and 1 week, and 1.2% at 3 weeks and the positive control was 5-fold higher than background at 24 hours[Sb51], and 3 weeks, and 3-fold higher after 1 week. The results showed no increase in MN formation at 24 [JK52][Sb53]hours, 1 week or 3 weeks.

91. COM opinion – This is a good quality study which was reasonably well-conducted, but, as well as the standard short-term exposure, also used a non-standard long-term exposure (1 and 3 weeks) and despite using a non-standard cell line (BEAS-2B[AP54][Sb55]). Therefore, it has been RAG assessed as amber. Further studies reported in the paper also showed no induction of ROS. Within these limitations, the results indicated no increase in MN formation and was considered a negative result. This study was cited in the EFSA review (EFSA, 2021).

Vieira et al. (2022)

92. Vieira et al. (2022) conducted a CBMN assay with cytoB in human intestinal cell lines, Caco-2 and H29-MTX-E12 cells 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.

93. 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. Two thousand binucleated cells were analysed for MN formation. MMC (0.3 µg/ml) was used as a positive control. Cytotoxicity was measured by CBPI/RI but the data were not shown.

94. No decreases in CBPI or RI were reported in either cell line. In Caco-2 cells, the negative undigested control was 1% and the digested control was 1.5-2%. In H29-MTX-E12 cells, the negative undigested control was 0.75-1.5% and the digested control was 0.8-2.5%. The positive control in Caco-2 cells was 3.2-fold higher than the undigested control and in H29-MTX-E12 cells, was 6-8-fold higher than the undigested control.

95. There was no evidence of cellular uptake. 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.

96. COM opinion – This study methodology used a number of non-standard procedures. Non-standard cell lines were used and 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. Therefore, this study was RAG assessed as amber. Comet assay and oxidative stress studies were also conducted in this paper and similarly gave mixed results, however, generally there was no induction of ROS. The results showed no evidence of a dose response and only small, inconsistent increases, that were not biologically relevant,were observed and hence considered a negative result. This study was identified during the recent literature search.

Vital et al. (2022)

97. Vital et al. (2022) conducted a *hprt* assay (see below) and CBMN assay with cytoB in V79 cells according to OECD TG487. $3x10^5$ cells were exposed to NM-100 (anatase) at concentrations of 1, 3, 10, 30, and 75 µg/cm^3 for 24 hours after which time cytoB was added for 24 hours.

98. The primary size of TiO_2 nanoparticles 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 standardized Nanogenotox dispersion protocol to disperse NM-100 and

99. NM-212 by sonication, at a concentration of a 2.56 mg/mL stock dispersion. MMS (0.1 mM) was used as a positive control. Two thousand binucleated cells were analysed for MN formation. Cytotoxicity was assessed using CBPI and RI.

100. In the MN assay, no cytotoxicity was measured. There was a high background level of MN as the negative control was 3%, and the positive control was 9-fold higher than background. No increased frequency of MN formation was detected.

101. COM opinion – In general it was considered that this study was of good design. It was considered, however, that V79 cells were not an ideal choice, [Sb56]there were no uptake data reported and the negative and positive controls showed high levels of MN as the negative control was 3%[MOU57] and the positive control was 9-fold higher than background. [MOU58][Sb59]Overall, the RAG was assessed as amber. No increases, in MN formation were detected and hence this study is considered negative. This study was identified during the recent literature search.

Amber' papers – CA assay

Patel et. (2017)

102. Patel et al. (2017) conducted a CA assay in HPBLs. The cells (number not given) were exposed to a mix of anatase and rutile at concentrations of 25, 75, 125 µM (the highest concentration was equivalent to approximately 10 µg/ml) for 22 hours.

103. The primary size of TiO_2 nanoparticles was 20-25 nm and secondary size was 255-650 nm). The nanoparticles were prepared by suspending in deionized water with no surfactant. Two hundred metaphases were analysed. MMC (concentration not given) was used as a positive control.

104. There was no cytotoxicity assessment. The negative control was 0.1% and the positive control was 5-fold higher than the background. No cellular uptake data was stated. The top two doses of TiO_2 gave significant positive increases in CA, of 2-fold and 3-fold, respectively.

105. COM opinion – This study methodology was appropriate and reasonable. However, evidence of uptake would have made the paper more convincing and so the study was RAG assessed as amber. The concentrations used in the study were low compared with other studies, but gave a relatively large genotoxic response despite only analysing 200 metaphases per concentration (and not 300 as recommended in OECD TG473). A Comet assay conducted in this study also detected significant DNA damage at 75 and 125 µg/ml. Overall, this study showed evidence of significant CA at doses of 75 and 125 µg/ml associated with DNA damage detected by the Comet assay and it was considered positive. This study was cited in the EFSA review (EFSA, 2021).

Unal et al. (2021)

106. Unal et al. (2021) conducted a CA assay and CBMN study with CytoB (see above) in HPBLs. The cells (number not given) were exposed to nanopowder (no further information given) at concentrations of 20, 40, 60, 80 or 100 µg/ml for 24 and 48 hours.

107. The primary size was 10-360 nm but secondary size was not stated. Stocks were ultrasonicated in distilled water diluted to final concentrations and then sonicated again. Three hundred were analysed. MMC (0.2 µg/ml) was used as a positive control. Cytotoxicity was measured by Mitotic Index (MI) in the CA assay.

108. At 24 hours the MI was significantly lower [JK60][Sb61]only at 60 and 80 µg/ml (reduced by 20% at both concentrations compared to a negative control) but was still within the acceptable limit of controls. There was no evidence of cytotoxicity at 48 hours. The negative control at both 24 and 48 hours was 1.3% and the positive control was 17- and 13-fold higher than the background, respectively.

109. There was no evidence of uptake of nanoparticles. A significant increase in CA was observed at all doses at 24 hours (3.6-5.6-fold increase over background), but no dose response was observed as CA frequency decreased with increasing TiO_2 concentration. At 48 hours similar results were

obtained, but were significant at 20, 80 and 100 µg/ml only (3.6-, 2.9- and 2.9-fold increase).

110. COM opinion – This study is regarded as of medium quality. The study details are unclear such as whether PHA was used to induce cell division and [Sb62]there is no evidence of uptake. Therefore, this study was RAG assessed as amber. Further experiments in this study included a Comet assay, MN and SCE. Within the limitation of the study, a positive result for CA was observed. This study was identified during the recent literature search.

'Amber ' papers – *hprt* assay

Vital et al. (2022)

111. Vital et al. (2022) conducted a *hprt* assay and CBMN assay (see above) 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.

112. 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 in which a stock of 2.56 mg/ml was sonicated. MMS (0.1 mM) was used as a positive control. Cytotoxicity was assessed using plating efficiency.

113. In the *hprt* assay, cytotoxicity was 30% at the highest concentration. The negative control was 9.59×10^{-6} and the positive control was 4-fold higher than the background. There was no evidence of uptake reported. Significantly increased mutations were detected at low doses (1, 3, 10 µg/cm^3) but increases were not significant at higher doses.

114. COM opinion – In general it was considered that this study was of good design. It was considered, however, that V79 cells [JW63]were not an ideal choice and there were no uptake data reported. Overall, the RAG was assessed as amber. There were some increases in *hprt* mutants significant at lower doses and therefore the study was considered positive. This study was identified during the recent literature search.

Summary

115. Twenty papers were identified following screening of papers cited in the EFSA opinion (EFSA, 2021) as described in the methodology section and further assessment of newer literature (2021 – 2023; Annex 1) to be of sufficient quality to warrant further assessment. Regarding the *in vitro* genotoxicity of TiO_2. The studies included three genotoxicity assays, namely MN, *hprt* and CA assays, all of which are [JK64]recognised by the OECD and other international regulatory bodies. Studies were assessed as red, amber or green by assessing the genotoxicity study design, using the criteria outlined in Table 3.

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

Table 5 Simple summary of the *in vitro* genotoxicity test results

Test	*Positive*	*Some positive results*	*Positive results with limitations* *[MOU65][AP66] [AP67][JK68]* [Sb69]	*Negative*
CBMN	7	3	3	5
Hprt	1	-	-	2
CA	2	-	-	-
Further evidence from other studies e.g. ROS, Comet, SCE compared with the results of MN, *hprt*, CA				
Positive for genotoxicity test	10	3	3	-
Negative for genotoxicity test	-	-	-	7

Table 6 Summary of the 'Green' MN results[Sb70]

Test material	Size	Conc.	Cell type	OECD recommended[Sb71] cells	Endpoint	Result	RAG	Reference
Anatase Rutile Mixture	20-60 nm 30x100 nm 45-252 nm	10-200 µg/ml	PBMCs	Yes	MN	Neg	G	Andreoli et al. (2018)
Anatase	21 or 50 nm	10-1000 µg/ml	HEK293 NIH/3T3	No	MN	Neg	G	Demir et al. (2015)
Uncoated anatase (NM-100)	50-150 nm 5-8 nm	1-15 µg/ml	BEAS-2B	No	MN	Pos (rutile)	G	Di Bucchianico et al. (2017)

Test material	Size	Conc.	Cell type	OECD recommended[Sb71] cells	Endpoint	Result	RAG	Reference
Coated anatase (NM-101) Coated rutile (NM-103)	20-28 nm							
86% anatase and 14 % rutile	27.5 nm	10-100 µg/ml	BEAS-2B	No	MN	Pos	G	Prasad et al. (2013)
Unknown	<25 nm	1-50 µg/ml	A549	Yes, but not extensively validated	MN	Pos	G	Srivastava et al. (2013)
84% anatase and 16% brookite Pristine (uncoated)	83.5 nm 57.5 nm 155.6 nm	32-128 µg/ml	A549	Yes, but not extensively validated	MN	Pos	G	Stoccoro et al., (2017)

Test material	Size	Conc.	Cell type	OECD recommended[Sb71] cells	Endpoint	Result	RAG	Reference
Silicate coated Sodium citrate coated								
84% anatase and 16% brookite Pristine (uncoated) Silicate coated Sodium citrate coated	83.5 nm 57.5 nm 155.6 nm	32-128 µg/ml	BALB/3T3	No	MN	Pos (citrate coated)	G	Stoccoro et al., (2016)

Table 7 Summary of the 'Amber' MN results

Test material	**Size**	**Conc.**	**Cell type**	**OECD recommended cells**	**Endpoint**	**Result**	**RAG**	**Reference**
E171-E anatase	70 nm at x10 110 nm at x50 180 nm at x90	0.3-30 µg/ml	HPBLs	Yes	MN	Neg	A	Bioreliance (2021)
Anatase	<25 nm	10-80 µg/ml	HPBLs	Yes	MN	Pos	A	Kurzawa-Zegota et al. (2017)
Anatase	8.9-15.3 nm	10-800 µg/ml 100-200 µg/ml	TK6	Yes	MN	Pos	A	Li et al. (2017)

Test material	Size	Conc.	Cell type	OECD recommended cells	Endpoint	Result	RAG	Reference
Anatase	40-70 nm	10-100 µg/ml	HPBLs	Yes	MN	Neg	A	Osman et al. (2018)
Anatase	171.4 nm	0.008-80 µg/ml	A431	No	MN	Pos	A	Shukla et al. (2011)
(NM-102 (anatase) NM-103 (rutile (hydrophobic)) NM-104 (rutile (hydrophilic)) NM-105 (rutile-anatase (15-85%))	20.8 nm 21.39 nm 19.0 nm 20.0 nm	5-250 µg/ml	HPBLs	Yes	MN	Pos	A	Tavares et al. (2014)

Test material	**Size**	**Conc.**	**Cell type**	**OECD recommended cells**	**Endpoint**	**Result**	**RAG**	**Reference**
Nanopowder	10-360 nm	20-100 µg/ml	HPBLs	Yes	MN	Neg	A	Unal et al. (2021)
NM-102 (anatase)	21.7 nm	1-20 µg/ml	BEAS-2B	No	MN	Neg	A	Vales et al. (2015)
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 / No	MN	Neg	A	Vieira et al. (2022)
NM-100 (anatase)	110 nm	1-75 $\mu g/cm^3$	V79	Yes	MN	Neg	A	Vital et al. (2022)

Table 8 Summary of the 'Amber' CA results

Test material	**Size**	**Conc.**	**Cell type**	**OECD recommended[Sb72] cells**	**Endpoint**	**Result**	**RAG**	**Reference**
Anatase and rutile mix	20-25 nm	25-125 µg/ml	HPBLs	Yes	CA	Pos	A	Patel et. (2017)
Nanopowder	µg/ml	20-100 µg/ml	HPBLs.	Yes	CA	Pos	A	Unal et al. (2021)

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

Test material	**Size**	**Conc.**	**Cell type**	**OECD recommended[Sb73] cells**	**Endpoint**	**Result**	**RAG**	**Reference**
Anatase	< 25 nm	1-100 µg/ml	V79	Yes	*hprt*	Pos	G	Jain et al. (2017)
Anatase/rutile mix	21 nm	3-75 µg/cm^2	V79	Yes	*hprt*	Neg	G	Kazimirova et al. (2020)

Table 10 Summary of the 'Amber' *hprt* results

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

117. The[JK74] results of[JK75] the *in vitro* genotoxicity studies selected for their robust methodology were inconclusive. In the majority of the studies, however, some positive results were observed[MOU76]. Any limitations or caveats to these results were noted in the narrative describing the papers.

118. These papers also contained additional but non-regulatory experiments on the role of oxidative stress and DNA interactions which may offer insight into mechanisms of action. Table 2 lists these further studies such as Comet and SCE assays and ROS studies which also suggested mechanisms of action adding to the results of the genotoxicity tests. Note that further studies were not conducted in all papers.

119. In a number of *in vitro* studies only weakly positive MN formation was observed at concentrations too high to be considered realistically relevant, while in other tests, positivity was dependent on the preparation of nanoparticles (such as chemical coatings) or the media in which they were prepared.

120. In other studies, weakly increased MN formation was seen in one cell line, Balb/3T3, with one of three coated nanoparticles while positive MN formation was observed in all three coated particles in a different cell line (A549) (Stoccoro et al. (2016, 2017).

121. In conclusion the results of the *in vitro* genotoxicity studies are inconclusive and it is suggested that some of this variability may be due to the non-biological conditions in studies; such as differences in the samples used and their preparation, and experimental procedures; such as the media selected and the presence/ absence of surfactants[MOU77]. It may be concluded, from the assessment of these relevant studies that are weighed as sufficient quality, that *in vitro* genotoxicity of TiO_2 cannot be excluded.

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
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.

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MUT/2023/07 – Annex B

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

Assessment of in *in vitro* studies of TiO_2 genotoxicity

Screening spreadsheet IN VITRO TiO2 screening not available publicly.

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