

Appendix L- *In vitro* genotoxicity studies considered in the re-evaluation of E171 (EFSA ANS Panel, 2016)

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Tables summarising *in vitro* studies on TiO₂ considered in the re-evaluation of E171 (EFSA ANS Panel, 2016). The studies have been evaluated based on the criteria set in Appendix D.

*** indicates that more than one assay is investigated/indicates when papers belong to more than one table**

**** indicates that both *in vitro* and *in vivo* assays are investigated (Appendix M)**

Table 1: *in vitro* Gene mutation assays

Gene mutation (in mammalian cells)							
Test system/ Test object	Exposure conditions (concentration/ duration /metabolic activation)	Information on the characteristics of the test substance	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E	Result	Reliability/ Comments	Relevance of the result	Reference
Mammalian cell gene mutation assay mouse lymphoma L5178Y cells Ames test (Table 1), CA assay (Table 2), SCE assay (Table 4)*	Highest concentration: 1.6 µg/ml without S9	NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion.	Negative	Reliability: 4 Insufficient details reported	Low	Tennant et al. (1987) *
Mammalian cell gene mutation assay - thymidine kinase (Tk) locus mouse lymphoma L5178Y cells	1.56, 3.13, 6.25, 12.5, 25, 50, 100 µg/ml +/-S9 negative and positive controls included Exposure was not of only 4h, but	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion.	Inconclusive (negative with no evidence of TiO ₂ uptake) Authors report that TiO ₂ co-pelleted with the cells after the treatment period	Reliability: 3 No evidence of TiO ₂ uptake	Low	Myhr and Caspary (1991)

	continued up to 2 days expression period.			(4h), therefore the exposure continued up to 2 days expression period.			
Mammalian cell gene mutation assay - Tk locus mouse lymphoma L5178Y cells Ames test (Table 1), CA assay (Table 2), comet assay (Table 3)*	250-2000 µg/mL After 1 h incubation in the dark, the cells were exposed to UV/vis light for 50 min (5 J/cm ²) and then suspended in culture medium. Measurements were done after 11-13 days without S9 negative control included Positive control: methyl methanesulfonate (MMS)	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm [the Panel noted that the test material is described by the authors as anatase]	NSC: 3 No information on dispersion.	Inconclusive (negative with no evidence of TiO ₂ NPs uptake)	Reliability: 3 only high concentrations tested, lack of information on dispersion and cellular uptake	Low Study conducted only with UV/vis irradiation	Nakagawa et al. (1997)*
Mammalian cell gene mutation assay Hypoxanthine-guanine Phosphoribosyl transferase (HPRT) locus Human B-cell lymphoblastoid WIL2-NS cells	0, 26, 65 and 130 µg/mL Cells were treated for 6, 24 and 48 h. Then cells were grown in flasks for 7 days to allow mutations to be expressed Cells were plated and the plates were incubated at 37 °C for 14–27 days, and	TiO ₂ NPs (no further information)	NSC: 2 some but incomplete information provided, suggesting minor agglomeration	Positive: mutations (~ 2.5-fold increases at 130 µg/mL) data briefly described, but no table/figure reported	Reliability: 3 Data briefly described, but no table/figure reported	Low	Wang et al. (2007)*

Micronucleus assay (Table 2), Comet assay (Table 3)*	then clonal growth was scored without S9						
Gene mutation (<i>Spi</i>) assay gpt delta transgenic mouse primary embryo fibroblasts (MEF) Oxidative stress	0.1, 1, 10 and 30 µg/mL 3 days exposure without S9 No positive control	1) TiO ₂ NPs, anatase 5 nm 2) TiO ₂ NPs, anatase, 40 nm 3) TiO ₂ , anatase, 325 mesh	NSC: 2 sonication and indirect assessment of exposure to particles by flow cytometry	Positive: 5 and 40 nm significantly increased mutation yield at 0.1 µg/mL and above; the effect was concentration-related with TiO ₂ NPs (40nm), as reported by the authors, however, it is not supported by statistical analysis, nor by the visual inspection of the data. The effect was abrogated by the concurrent treatment with the endocytosis inhibitor Nystatin. Treatment of MEF cells with TiO ₂ NPs (40 nm), resulted in a concentration-dependent decrease in cell viability when analysed with MTT assay. Negative: TiO ₂ -325 mesh	Reliability: 2 No positive control was used. Results of statistical analysis not reported in detail, test system not validated for regulatory purpose.	Limited	Xu et al. (2009)

				<p>Oxidative stress: treatment of MEF cells with either TiO₂ NPs (5 nm) or TiO₂ NPs (40nm), (but not TiO₂ -325 mesh), resulted in a concentration-dependent induction of peroxy nitrite anions ONOO⁻. Particles endocytosis: after 24 h incubation, the cellular granularity of MEF cells exposed to TiO₂NPs and TiO₂ particles increased in a concentration-dependent effect.</p>			
<p>Mammalian cell gene mutation assay HPRT locus</p> <p>CHO-K1 cells</p> <p>Comet assay (Table 3)*</p>	<p>10, 20 or 40 µg/mL for 60 days</p> <p>without S9</p>	<p>TiO₂NPs, anatase, < 25 nm (XRD)</p>	<p>NSC: 1</p> <p>no information on dispersion but exposure confirmed by EM and Ti measurements</p>	<p>Negative: no significant increase in gene mutations. No effects on colony forming ability. No cytotoxicity (XTT assay). Cells exposure is demonstrated.</p>	<p>Reliability: 2</p> <p>No positive control; no cytotoxicity observed.</p>	<p>Limited</p>	<p>Wang et al. (2011)*</p>
<p>Mammalian cell gene mutation assay HPRT locus</p> <p>Chinese hamster lung fibroblasts (V79 cells)</p>	<p>0, 5, 20 and 100 µg/mL</p> <p>2h exposure</p> <p>Positive control: EMS</p> <p>without S9</p> <p>OECD TG 476 (1997)</p>	<p>TiO₂NPs, anatase, 75 nm</p>	<p>NSC: 2</p> <p>DLS measurement in cell media confirming agglomeration.</p>	<p>Positive: statistically significant and concentration-related increase in the mutation frequency of HPRT gene.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Chen et al. (2014)*, **</p>

Comet assay (Table 3)*							
Bacterial assays							
Rec-assay system M45 recombination-deficient strain of <i>Bacillus subtilis</i> Rapid Streak Method, -S9 (20h)	Not indicated	TiO ₂ (no further information)	NSC: 3 No information on dispersion	Negative	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials	Low	Kada et al. 1980
Bacterial reverse mutation assay (Ames test) <i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538 and <i>E. Coli</i> WP2 <i>uvrA</i> interlaboratory study	Up to 10 mg/plate With or without S9 preparations from uninduced and from Aroclor 1245-induced F344 rats, B6C3F1 mice, Syrian hamsters; Plate incorporation Positive and negative controls included	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion	Negative in all laboratories	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials	Low	Dunkel et al. 1985
Bacterial reverse mutation assay (Ames test) <i>S. typhimurium</i> /microsome	Highest concentration: 10000 µg/plate +/-S9	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion.	Negative	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials	Low	Tennant et al. (1987)*

<p>Bacterial reverse mutation assay (Ames test)</p> <p><i>S. typhimurium</i> TA100, TA98, and TA102 with and without UV</p>	<p>6750- 54000 µg/plate.</p> <p>The UV/vis light irradiation was performed for 10 min (1J/cm²) or 50 minutes (5 J/cm²). After irradiation, the suspension of bacteria and TiO₂ particles was mixed with top agar. Incubation for 48 hours without S9, plate incorporation method</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm [the Panel noted that the test material is described by the authors as anatase]</p>	<p>NSC: 3</p> <p>No information on dispersion.</p>	<p>Negative</p>	<p>Reliability 5</p> <p>Bacterial systems are not suitable for testing nanomaterials</p>	<p>Low</p>	<p>Nakagawa et al. (1997)*</p>
<p>Bacterial reverse mutation test (Ames test)</p> <p><i>S. Typhimurium</i> strains TA98, TA100, TA1535 and TA1537, and in <i>E. coli</i> strain WP2uvrA</p> <p>Chromosomal aberrations (CA) assay (Table 2)*</p>	<p>100, 333, 1000, 3333, and 5000 µg/plate</p> <p>+/-S9</p> <p>Duration of exposure not specified</p>	<p>TiO₂, rutile/anatase (79/21%), 90 wt% TiO₂, 7% alumina, and 1% amorphous silica (XRF), 140 nm (DLS)</p>	<p>NSC: 3</p> <p>No specific information on dispersion for the gentox studies.</p>	<p>Negative</p>	<p>Reliability 5</p> <p>Bacterial systems are not suitable for testing nanomaterials</p>	<p>Low</p>	<p>Warheit et al. (2007)*</p>

<p>Bacterial reverse mutation assay (Ames test) S. typhimurium TA98, TA100, TA1535, TA1537 and TA102</p> <p>Cellular uptake of TiO₂ NPs</p> <p>Comet assay (Table 3)*</p>	<p>A pre-incubation assay using 0, 38.4, 76.8, 153.6, 307.2, 614.4, 1228.8, 2457.6 and 4915.2 µg/plate of TiO₂-NPs; without S9 activation; according to OECD TG 471; Positive controls included</p>	<p>TiO₂NPs, anatase, ellipsoidal shape (TEM), 10x 30 nm, minor axes 12.1 ± 3.2 nm</p>	<p>NSC: 3 Dispersion was considered but results are not reported. Internalisation was considered with TEM-EDS confirmation that NPs did not cross bacterial membrane</p>	<p>Negative Analysis of bacterial TiO₂ uptake showed that nanoparticles were not in bacterial cells, but only outside.</p> <p>Uptake in Salmonella: no</p>	<p>Reliability 5</p> <p>Bacterial systems are not suitable for testing nanomaterials</p>	<p>Low</p>	<p>Woodruff et al. (2012)*</p>
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BET: Brunauer–Emmett–Teller method; CA: chromosomal aberrations; DMEM: Dulbecco's Modified Eagle's medium; EM: electron microscopy; EMS: ethylmethanesulfonate; FBS: Fetal Bovine Serum HPRT: hypoxanthine-guanine phosphoribosyl transferase; IBO: interested business operator; ICP-AES: Inductively coupled plasma atomic emission spectroscopy MEF: mouse primary embryo fibroblasts; MMS: methyl methanesulfonate; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NPs: Nanoparticles; NSC: nanoscale considerations; ROS: reactive oxygen species; XTT: 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide; SCE: sister chromatid exchange; TEM: Transmission electron microscopy; Tk: thymidine kinase

Table 2: *In vitro* chromosomal aberrations/ mammalian cell micronucleus test

Test system/ Test object	Exposure conditions (concentration/ duration /metabolic activation)	Information on the characteristics of the test substance	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E	Result	Reliability/ Comments	Relevance of the result	Reference
Chromosomal aberration assay Chinese hamster ovary (CHO) cell line	Highest concentration: 25 µg/ml without S9	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion.	Negative	Reliability: 4 Insufficient details reported	Low	Tennant et al. (1987)*
Chromosomal aberration assay CHO cells SCE assay (Table 4)*	Concentrations tested: 15, 20, 25 µg/ml Exposure with metabolic activation (+S9): 2h Exposure without metabolic activation (-S9): 8h	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion. Highest concentration tested limited by solubility	Inconclusive negative with no evidence of TiO ₂ NPs uptake	Reliability: 3 No evidence of TiO ₂ NPs uptake, in addition some methodological limitations	Low	Ivett et al. (1989)*
Micronucleus (MN) assay CHO cells	Without S9: 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 10 µg/ml With S9: 0.25, 0.5, 1, 2.5, 5, 10 µg/ml	TiO ₂ (no further information)	NSC: 3 No information on dispersion. TiO ₂ was dissolved in DMSO (1% DMSO in medium).	Inconclusive negative with no evidence of TiO ₂ uptake	Reliability: 3 No evidence of TiO ₂ uptake	Low	Miller et al. (1995)

	48h sampling time. CytoB was not used						
Micronucleus assay (CBMN) Rat liver epithelial cell line	5, 10, 20 µg/cm ² with and without UV light After 1-hr of incubation, half of the slides were irradiated with an UV lamp (366 nm, 5 min). All the cultures were treated with CytoB (1 µg/ml) to prevent cytokinesis and further incubated for 20 hr (37°C). Without S9	1) TiO ₂ NPs (P25), anatase/rutile, 15-24 nm 2) TiO ₂ , 170 nm.	NSC: 3 No information on dispersion	Inconclusive negative with no evidence of TiO ₂ NPs uptake	Reliability: 3 no evidence of TiO ₂ NPs uptake High spontaneous frequencies of MN in untreated controls (53-71 MN/1000 binucleated cells, which is much higher than the limit, 2%, as indicated in Appendix D)	Low	Linnainmaa et al. (1997)
Chromosomal aberration assay Chinese hamster lung cell line (CHL/IU cells)	25- 800 µg/mL, w/o UV; 0.78- 28.5 µg/ml with UV. After 1 h incubation in the dark with TiO ₂ particles, the cells were exposed to UV/vis light for 50 min at 1.25 J/cm ² , 2.50 J/cm ² , and 5 J/cm ² After 20 h incubation and 2 h demecolcin (0.1 µg/ml),	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm [the Panel noted that the test material is described by the authors as anatase]	NSC: 3 No information on dispersion.	In the absence of UV/vis: Negative In the presence of UV/vis: Positive (minimum effective concentration 12.5 µg/mL) Statistically significant increase of structural aberrations (chromatid break and exchange).	Reliability: 2 Uptake was not directly demonstrated, however cytotoxicity is reported	Limited	Nakagawa et al. (1997)*

	<p>measurements were done. Negative and positive controls included.</p> <p>Without S9</p>			<p>Also, polyploidy is reported to increase at concentrations >6.25 µg/ml</p> <p>TiO₂ NPs (P25) were more than 50-times cytotoxic in the presence of UV/vis light than in the absence of it.</p>			
<p>Micronucleus assay (conventional and cytokinesis-block)</p> <p>CHO-K1 cells</p> <p>SCE assay (Table 4)*</p>	<p>5, 10, 15, 20 µM</p> <p>Cells treated for 18h without CytoB</p> <p>Cells treated with TiO₂ and CytoB for 24 h Triplicates.</p> <p>Giemsa staining.</p> <p>Without S9.</p> <p>No positive control. Cytotoxicity was determined by a colony forming assay.</p>	TiO ₂ (no further information)	<p>NSC: 1 no specific dispersion protocol, except confirming lack of microscopic precipitates, but dose related accumulation confirmed under the same exposure conditions.</p>	<p>Positive: statistically significant increases in MN observed up to 10 µM, without CytoB.</p> <p>Positive: concentration-related and statistically significant increase of MN in the presence of CytoB. Higher levels of MN (2.5- to 3-fold increases) observed compared to the treatment without CytoB. Cellular TiO₂ uptake was demonstrated.</p>	<p>Reliability: 2</p> <p>Lack of positive control CBPI not measured. Lack of information on dispersion is not considered as a major shortcoming for a positive result</p>	Limited	Lu et al. (1998)*

<p>Micronucleus assay</p> <p>Syrian hamster embryo fibroblasts (SHE)</p>	<p>0.5, 1, 5, 10 µg/cm² for 12, 24, 48, 66 and 72 h.</p> <p>Without CytoB</p> <p>Incubation of cells with CREST serum for 1h for kinetochores staining. Examined at least 100 MN for the presence of kinetochores.</p> <p>No positive control.</p> <p>Without S9.</p>	<p>1) TiO₂NPs, ≤ 20 nm (crystalline form and shape unknow)</p> <p>2) TiO₂, > 200 nm (crystalline form and shape unknow)</p>	<p>NSC: 3</p> <p>No information on dispersion</p>	<p>1) TiO₂NPs: Positive (1 and 5 µg/cm²). Induced micronuclei mainly from clastogenic and not aneugenic events. Cytotoxicity increased after exposure of cells to higher concentrations of TiO₂NPs (> 10.0 µg/cm²). (data not shown)</p> <p>2) TiO₂: Negative (data not shown)</p>	<p>Reliability: 2 for TiO₂NPs</p> <p>No positive control.</p> <p>Reliability: 2 for TiO₂</p> <p>Results are not reported in detail.</p> <p>No positive control</p>	<p>Limited (TiO₂NPs)</p> <p>Limited (TiO₂)</p>	<p>Rahman et al. (2002)</p>
<p>Micronucleus assay (CBMN)</p> <p>human B-cell lymphoblastoid WIL2-NS cells</p> <p>Cytotoxicity: MTT assay</p> <p>Population growth assay: trypan blue-dye exclusion</p>	<p>0, 26, 65 and 130 µg/mL</p> <p>Cells were treated for 6, 24 and 48 h.</p> <p>CytoB was added at 4.5 µg/ml and the cultures were incubated for another 26 h.</p> <p>Styrene oxide was used as positive control. Treatment with styrene oxide (0.2 mM) decreased</p>	<p>TiO₂NPs (no further information)</p>	<p>NSC: 2</p> <p>some but incomplete information provided, suggesting minor agglomeration</p>	<p>Positive statistically significant increase in MNBN cells after 6 h exposure. Increased incidence of MN~ 2.5-fold at 130 µg/mL. For exposure longer than 6h no data were collected at the highest concentration due to cytotoxicity. At the lower concentrations a statistically significant MN increase was</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Wang et al. (2007)*</p>

<p>Comet assay (Table 3)*</p>	<p>viability and population growth and induced MNBN cells in WIL2-NS cells.</p> <p>Without S9</p>			<p>observed up to 48h. Also a statistically significant increase in the frequency of nucleoplasmic bridge was observed. CBPI: statistically significant decrease at 48h.</p> <p>Statistically significant increase of apoptotic cells for 6h exposure at all concentrations tested and for 24 h at 65 µg/ml. No increase in apoptotic cells was observed with FACS analysis.</p> <p>MTT assay: Statistically significant decrease in cell viability at 130 µg/mL (6, 24 and 48h, time-dependent).</p> <p>Inhibition of population growth (trypan blue-dye exclusion): concentration-dependent and time-dependent.</p>			
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Micronucleus assay (CBMN) human peripheral blood lymphocytes SCE assay (Table 4)*	Exposure for 72 h (CytoB was added after 44 h). 1000 binucleated lymphocytes were examined per concentration. Without S9 No positive control.	TiO ₂ (no further information)	NSC: 3 No information on dispersion	Positive: concentration-related and statistically significant increases in micronuclei	Reliability: 2 Cytotoxicity was not measured. No positive control was used.	Limited	Turkez and Geyikoglu (2007)*
Chromosomal aberrations assay CHO cells	750, 1250, and 2500 µg/mL for 4 h (without S9,4h), 25, 50, and 100 µg/ml (without S9, 20h), 62.5, 125, and 250 µg/ml (+S9, 4h) 200 cells analysed for each concentration.	TiO ₂ , rutile/anatase (79/21%), 90 wt% TiO ₂ , 7% alumina, and 1% amorphous silica (XRF), 140 nm (DLS)	NSC: 3 No specific information on dispersion for the genotoxicity studies.	Negative TiO ₂ particles did not induce structural or numerical chromosome aberrations in this study. Concentrations were selected based on inhibition of the mitotic activity.	Reliability: 3 Data briefly described, but no table/figure reported. Positive controls used are not listed. The experiment was not repeated, and only single cultures were used for each treatment.	Low	Warheit et al. (2007)
Micronucleus assay (CBMN) human bronchial epithelial BEAS-2B cells Comet assay (Table 3)*	1, 5, 10, 20, 40, 60, 80, and 100 µg/cm ² for 24, 48, or 72 h exposure. CytoB was added to the cell cultures simultaneously with the particles	1)TiO ₂ , rutile, < 5000 nm 2) TiO ₂ NPs, anatase, < 25 nm	NSC: 2 high level of agglomeration observed, presence of nanoparticles in dispersion measured by TEM but data not reported	TiO ₂ NPs anatase: Equivocal Positive at 10 and 60 µg/cm ² induced a statistically significant increase of micronucleated cells (no concentration dependency) after 72h exposure	Reliability: 1 CytoB was added to cell cultures at the same time of particles.	Limited Not all criteria for a clearly positive result are met.	Falck et al. (2009)*

	<p>Positive control: Mitomycin C</p> <p>Without S9.</p>			<p>without effect on CBPI.</p> <p>TiO₂: Negative. A decrease of MN was observed. No effects on CBPI.</p>			
<p>Micronucleus assay (CBMN)</p> <p>CHO-K1 cells</p> <p>SCE assay (Table 4)*</p>	<p>0.5, 1, 5, 10 µg/mL For 24h exposure CytoB and TiO₂ were added at the same time</p> <p>1000 binucleated cells, per slide, were scored. Test repeated 3 times. without S9</p>	<p>TiO₂NPs, "complex" shape (TEM), 20±7 nm (TEM) (crystalline form unknow)</p>	<p>NSC: 1 limited information but exposure confirmed by EM</p>	<p>Positive: statistically significant increase of MN frequencies at 0.5 and 1 µg/mL, but not at 5 µg/mL. At 10 µg/mL TiO₂, nuclei (and eventually MN) were covered by NPs, thus preventing MN scoring.</p>	<p>Reliability: 2</p> <p>CBPI was not measured; no positive control. Possible interference of particles in the scoring of MN.</p>	<p>Limited</p>	<p>Di Virgilio et al. (2010)*</p>
<p>Micronucleus assay (CBMN)</p> <p>Human negroid cervix carcinoma HEp-2 cells (HeLa derivative).</p> <p>Cytotoxicity: Neutral Red and MTT assays (2h, 4h, 24h)</p> <p>Comet assay (Table 3)*</p>	<p>10, 20 and 50 µg/ml for 2 h exposure. CytoB was added after the treatment and incubated overnight.</p> <p>Positive control: Mitomycin C without S9</p>	<p>TiO₂NPs, anatase (no further information)</p>	<p>NSC: 2 partial information on dispersion and stability provided</p>	<p>Positive: statistically significant increase in MN only at 50 µg/ml. Precipitation observed at 100 µg/ml.</p> <p>Cytotoxicity: statistically significant decrease in cell viability at 2h and 4h at 100 µg/ml and at 24h at concentrations up to 20 µg/ml</p>	<p>Reliability: 2</p> <p>CBPI was not measured; Limited number of cells scored</p>	<p>Limited</p>	<p>Osman et al. (2010)*</p>

<p>Micronucleus assay (CBMN)</p> <p>human epidermal cell line (A431).</p> <p>MTT and Neutral Red uptake assays (cytotoxicity)</p> <p>Flow cytometry and TEM (Cellular uptake)</p> <p>Comet assay (Table 3)*</p>	<p>0.008, 0.08, 0.8, 8 and 80 µg/ml for 6 h exposure</p> <p>CytoB was added after the treatment and cells were incubated for additional 18h. Ethyl methanesulfonate (6 mM) was used as positive control</p> <p>without S9</p>	<p>TiO₂NPs, anatase, 50 nm (TEM)</p>	<p>NSC: 1 size distribution measured in the media confirming a level of agglomeration and exposure confirmed by EM and flow cytometry</p>	<p>Positive: statistically significant induction in MN formation was observed after 6 h exposure at 0.8 µg/ml and higher concentrations; no effect on CBPI.</p> <p>Cytotoxicity was tested by MTT and Neutral Red uptake assays: mild significant cytotoxic response (after 48h exposure)</p> <p>Cellular uptake was measured by Flow cytometry and TEM: significant increase in cellular uptake was observed</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Shukla et al. (2011)*</p>
<p>Micronucleus assay (CBMN)</p> <p>A549 human lung carcinoma cells</p> <p>Cytotoxicity: MTT assay</p>	<p>50, 100, 200 µg/ml 24 h exposure, then cytoB was added and cells cultured for additional 24h; Positive control: etoposide</p> <p>MTT assay: 25, 50, 75, 100 µg/ml</p>	<p>1)TiO₂NPs,anatase, 12 nm (TEM) 2) TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1 limited information on dispersion but cell internalisation of NPs confirmed by EM</p>	<p>Negative</p> <p>Cytotoxicity (MTT) measured only up to 100 µg/ml after 48h of exposure, but not at 200 µg/ml</p> <p>Cellular uptake was demonstrated</p>	<p>Reliability 2</p> <p>Shortcomings in the reporting of results (MN/slide instead of %MNBN cells). CBPI was not measured.</p>	<p>Limited</p>	<p>Jugan et al. (2012)*</p>

Comet assay (Table 3), γ H2AX foci and The base excision repair (BER) (Table 4)*	Colony formation: 1, 10, 100 μ g/ml Intracellular accumulation: 50 μ g/ml						
Micronucleus assay Syrian hamster embryo (SHE) cells Comet assay (Table 3)*	5, 10, and 50 μ g/cm ² for 24 h. Positive control: Methyl methanesulfonate (MMS). Without S9 1000 cells/slide were analysed CytoB was not used.	1) TiO ₂ NPs, anatase, 14 \pm 4 nm (TEM) 2) TiO ₂ , anatase, 160 \pm 48 nm (TEM), 3) TiO ₂ , rutile, 530 \pm 216 nm (TEM) 4) TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 level of dispersion measured in the media (suggesting high level of agglomeration) and confirmation of cellular uptake by EM but only at 1 μ g/cm ²	Negative. All particles induced moderate cytotoxicity (a decrease in relative increase in cell count (RICC) of <50%). Cellular uptake was demonstrated	Reliability: 2 decrease of MN frequency at the high concentration may be due to the presence of particles on the slide which, according to the authors, disturbed MN scoring.	Limited	Guichard et al. (2012)*
Micronucleus assay (CBMN) human lung epithelial cells (BEAS-2B) Comet assay (Table 3)*	10 -100 μ g/ml 24 h then CytoB for 18 h Positive control: methyl methanesulfonate Without S9 3 different types of treatment media: (a) KB, which had a low concentration of protein (0.1% BSA); (b) DM, which had a lower level of	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Three dispersion protocols and level of dispersion reported for each concentration, confirming high agglomeration and cell internalisation assessed by flow cytometry and dark-field microscopy	Cells treated with TiO ₂ NPs in KF medium (lower level of agglomeration compared to KB and DM media): Positive , statistically significant induction of MN (concentration-related). TiO ₂ NPs in KB or DM: Negative , no increases in MN	Reliability: 1	High	Prasad et al. (2013)*

Cellular uptake	protein (0.6% BSA) plus 0.001% surfactant; (c) KF, which contained 10% FBS			<p>formation could be shown for BEAS-2B cells treated with any concentration. CBPI showed no difference in treatment medium on cytostasis; however, there was an effect of concentration.</p> <p>Direct relationship between concentration of TiO₂NPs delivered to the cells and side scatter detected by the flow cytometer. Cells treated with KB and DM showed lower side-scatter values at all concentrations compared to the KF medium</p> <p>The rank order of agglomeration size in treatment media based on the DLS (dynamic light scattering) data at 0 and 24 h was KB > DM > KF. Results confirmed also by SEM (scanning</p>			
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				electron microscopy)			
Micronucleus assay (CBMN) HepG2 cells Comet assay (Table 3)*	1, 10, 20, 40 and 80 mg/ml for 6h, then addition of CytoB and incubation for 20h positive control: EMS (ethyl methanesulfonate-) 2000 binucleate cells from each concentration. Cytotoxicity (MTT, NRU) Cellular uptake demonstrated Without S9	TiO ₂ NPs, anatase, 30-70 nm (TEM)	NSC:1 DLS confirmation of agglomeration in the media and cell internalisation confirmed by flow cytometry and EM	Positive Statistically significant increase of MN cells at all concentrations compared to the negative control. However, without a concentration effect relationship. A large number of particles were also seen at these concentrations on the slides, which might have interfered with scoring of MN.	Reliability: 3 CBPI was not measured. Possible interference of particles in the scoring of MN.	Low	Shukla et al. (2013)*
Micronucleus assay (CBMN) Human lung cancer cell line A549 Internalization of TiO ₂ NPs Cytotoxicity (MTT and LDH)	1–50 µg/ml for 24h, then addition of CytoB and incubation for 20h Positive control: EMS Without S9 1–100 mg/ml for 6h, 24h, 48h	TiO ₂ NPs, anatase, 25 nm	NSC: 1 DLS size confirming high agglomeration and cell internalisation confirmed by EM	Positive Statistically significant and concentration related increase at 10–50 µg/ml for 24 h Minimum of 1000 bi-nucleated cells were scored. internalization of TiO ₂ NPs concentration-related. The particles adhered on	Reliability: 2 Cytotoxicity (CBPI) was not measured.	Limited	Srivastava et al. (2013)

<p>Oxidative markers: ROS, GSH, LPO, catalase</p> <p>Apoptosis markers (RT-PCR and western immunoblotting)</p>	<p>1–50 µg/ml for 6h, 12h for 24h</p> <p>1–50 µg/ml for 24h and 48h</p>			<p>the cell surface when incubated for 30 min and subsequently internalized in small vacuoles at cortical cytoplasm in extending incubations and reached to deep cell center near the mitochondria and Golgi apparatus in larger vacuoles over 48 h of exposure</p> <p>Cytotoxicity: statistically significant and concentration-related.</p> <p>Oxidative markers: statistically significant and concentration related:</p> <ul style="list-style-type: none"> -ROS and LPO increase -GSH and catalase decrease <p>Apoptosis: statistically significant increase of apoptotic cells especially at 48h; Increased expression (mRNA</p>	<p>Oxidative markers: The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism</p>		
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				and protein) of: P53, p21, caspase-3; decrease of bcl2; no changes of bax			
Micronucleus assay (CBMN) human peripheral lymphocytes	20 – 300 mg/ml for 6h, then addition of CytoB and incubation for 24h Positive control: MMC Without S9 2000 binucleate cells and 1000 mononucleate cells from 2 separate cultures.	1)TiO ₂ NPs (NM-102), anatase, 21-22 nm 2) TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 Dispersion protocol considering also stability confirming agglomerates in the nano range	Equivocal NM-102: small (1.6% MNBN cells vs 1.05% in negative control), but statistically significant increase at 125 mg/ml. However, not concentration-related NM-105: Inconclusive (negative with no evidence of TiO ₂ NPs uptake) No effects on cell viability and cell cycle progression	For NM-102 Reliability: 2 no analysis of particle uptake For NM-105 Reliability: 3	NM-102: Limited NM-105: Low relevance	Tavares et al. (2014)

BET: Brunauer–Emmett–Telle method; CA: chromosomal aberrations; CBMN: Cytokinesis block micronucleus; CBPI: cytokinesis-block proliferation index; CREST: Immunofluorescent antikinetochore staining; CytoB: Cytochalasin B; DMSO: Dimethyl sulfoxide; EM: electron microscopy; EMS: ethylmethanesulfonate; FACS: Fluorescence-activated cell sorting; GSH: reduced glutathione; IBO: interested business operator; ICP: Inductively coupled plasma; LDH: Lactate dehydrogenase; LPO: Lipid peroxidase; MMC: Mitomycin C; MN: Micronucleus; MNBN: Micronucleated binucleated; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide ;NPs: Nanoparticles; NSC: nanoscale considerations; ROS: reactive oxygen species; RICC: Relative increase in cell counts; ROS: reactive oxygen species; SCE: sister chromatid exchange; TEM: Transmission electron microscopy

Table 3: In vitro DNA damage (Comet assay)

Test system/ Test object	Exposure conditions (concentration/ duration /metabolic activation	Information on the characteristics of the test substance	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E	Result	Reliability/ Comments	Relevance of the result	Reference
<p>Comet assay</p> <p>mouse lymphoma L5178Y cells</p> <p>Photogenotoxicity of TiO₂ was investigated.</p>	<p>WA, WR and TP-3: 50-3200 µg/mL</p> <p>p-25: 12.5 - 800µg/mL</p> <p>with and without UV/vis light (the spectra of generated UV/vis light was almost identical to natural sunlight)</p> <p>After 1 h incubation in the dark with TiO₂ particles, the cells were exposed to UV/vis light for 50 min at 0.61</p>	<p>1) TiO₂NPs (P25), anatase/rutile, 15-24 nm [the Panel noted that the test material is described by the authors as anatase]</p> <p>2) TiO₂, anatase, 255 nm [WA]</p> <p>3) TiO₂, rutile, 255 nm [WR]</p> <p>4) TiO₂, rutile, 420 nm [TP-3]</p>	<p>NSC: 3</p> <p>No information on dispersion.</p>	<p>Positive: WA induced DNA damage at 3200 µg/mL. In presence of UV/vis the effect was observed also at lower concentrations.</p> <p>P25 and TP-3 induced increase of mean tail length only when cells were UV/vis irradiated and</p>	<p>Reliability: 2</p> <p>No positive control was used.</p> <p>No statistical analysis of results (it seems that the experiment was not repeated, and only single cultures were used for each treatment).</p>	<p>Limited</p>	<p>Nakagawa et al. (1997)*</p>

	<p>J/cm², 1.25 J/cm², 2.50 J/cm², and 5 J/cm². Then incubated for 20 hours.</p> <p>Part of the cell cultures were not exposed to UV/vis light irradiation, as control.</p> <p>No positive control</p> <p>without S9</p>			<p>exposed to TiO₂ at concentrations of 12.5 and 200 µg/mL, respectively</p> <p>Negative: WR (50–3200 µg/mL) with or without UV/vis irradiation</p> <p>Cell survival was reduced at concentrations that elicited photogenotoxicity</p>			
<p>Comet assay</p> <p>Human B-cell lymphoblastoid WIL2-NS cells</p> <p>Apoptosis assay (flow cytometry)</p> <p>Cytotoxicity: MTT assay</p>	<p>0, 26, 65 and 130 µg/mL</p> <p>Cells were treated for 6, 24 and 48 h.</p> <p>Positive control: Styrene oxide</p> <p>Without S9</p>	TiO ₂ NPs (no further information)	NSC: 2 some but incomplete information provided, suggesting minor agglomeration	<p>Positive: 3-fold significant (P < 0.05) increases in %tail DNA at 65 µg/mL, 24 h exposure, and 5-fold increases of Olive tail moment at same concentration.</p> <p>Apoptosis assay (Flow cytometry): no statistically significant increase in apoptosis observed.</p> <p>Cell viability: statistically</p>	Reliability: 3	Low	Wang et al. (2007)*

				significant decrease at 130 µg/mL (mainly after 24 and 48h exposure)			
Comet assay +/- Fpg (DNA damage and oxidative lesions) human alveolar type II-like cell line A549	40 and 20 µg/cm ² (40 and 80 µg/mL) for 4 h. without S9 For analyses of oxidative DNA lesions, the results in comet assay with and without the enzyme formamidopyrimidine DNA glycosylase were compared No positive control	1)TiO ₂ NPs, rutile/anatase, 20–100 nm (TEM) 2) TiO ₂ , rutile, 300–1000 nm (TEM)	NSC: 2 limited information provided confirming a level of agglomeration	1) TiO ₂ NPs Positive: statistically significant increases in DNA damage (at 40 µg/cm ²); 2) TiO ₂ Positive: statistically significant increases in DNA damage (treated at 20 and 40 µg/cm ²) For oxidative DNA damage, no significant increases in oxidised purines were observed for both TiO ₂ NPs and TiO ₂ .	Reliability: 2 Single exposure time. No positive control. For each sample 35 cells were examined in duplicates	Limited	Karlsson et al. (2009)
Comet assay Oxidative stress (8-oxo-dGuo and ROS) human lung fibroblasts (IMR-90)	2, 5, 10 and 50 µg/cm ² 24 h exposure Without S9;	TiO ₂ NPs, anatase, < 100 nm), 91 nm (DLS)	NSC: 2 partial information and indication that exposure was confirmed by EM	Comet assay: Negative. Results reported as Olive tail moment only. Cellular uptake was demonstrated.	Reliability: 3 Single exposure time	Low	Bhattacharya et al. (2009)

and human bronchial epithelial cells (BEAS-2B)	No positive control Results reported as Olive tail moment only		but data not provided	Oxidative stress: Positive, increased oxidative damage (8-hydroxy 2'-deoxyguanosine (8-OH-dG)) and intracellular generation of ROS Decrease cell viability at 50 µg/cm ² in IMR 90 cells.	No positive control was used. No information on number of cells scored. Results reported as Olive tail moment only.		
Comet assay human bronchial epithelial BEAS-2B cells Viability: Trypan blue exclusion assay after a 48-h recovery	1, 5, 10, 20, 40, 60, 80, and 100 µg/cm ² 24, 48, or 72 h exposure. Concentrations selected based on viability assay Positive control: hydrogen peroxide 100 cells per sample (two replicates, each with 50 cells/slide) were analysed. Measure of % tail DNA without S9	1)TiO ₂ , rutile, < 5000 nm 2) TiO ₂ NPs, anatase, < 25 nm	NSC: 2 high level of agglomeration observed, presence of nanoparticles in dispersion measured by TEM but data not reported	TiO ₂ NPs: Positive with limited evidence TiO ₂ : Positive Viability: Treatment with TiO ₂ NPs anatase decreased cell viability starting at 80 µg/cm ² with all treatment times. A 50% reduction in viable cell count was obtained after 72-h treatment at around 120 µg/cm ²	Reliability: 2	Limited	Falck et al. (2009)*
Comet assay	10, 20, 50	TiO ₂ NPs, anatase (no	NSC: 2	Positive: concentration-	Reliability: 2	Limited	Osman et al. (2010)*

<p>Human negroid cervix carcinoma HEp-2 cells (HeLa derivative)</p> <p>Cytotoxicity: Neutral Red and MTT assays (2h, 4h, 24h)</p>	<p>and 100 µg/ml for 4 h exposure (test repeated 3 times)</p> <p>Negative control: untreated Positive control: Hydrogen peroxide</p> <p>50 cells from each concentration were analysed; Three independent experiments.</p> <p>without S9</p>	<p>further information)</p>	<p>partial information on dispersion and stability provided</p>	<p>dependent statistically significant increase in DNA damage (based on OTM and % tail DNA). Concentrations were based on viability test: 70 and 85% viability at all concentrations at 100 µg/ml, 65% viability.</p>	<p>only 50 cells analysed for each tested concentration</p>		
<p>Comet assay +/- Fpg (formamidopyrimidine DNA glycosylase-modified Comet; detection of oxidative DNA base damage, 8-oxodG)</p> <p>human epidermal cell line (A431).</p> <p>Cytotoxicity tested by MTT and Neutral Red uptake assays.</p> <p>Cellular uptake</p>	<p>0.008, 0.08, 0.8, 8 and 80 µg/ml for 6h exposure</p> <p>Positive control: Hydrogen peroxide (25 µM)</p> <p>Images from 50 random cells (25 from each replicate slide) were analysed for each experiment. The experiment (and not the cell) was used as the experimental unit for data analysis</p> <p>without S9.</p>	<p>TiO₂NPs, anatase, 50 nm (TEM)</p>	<p>NSC: 1 size distribution measured in the media confirming a level of agglomeration and exposure confirmed by EM and flow cytometry</p>	<p>Positive: statistically significant induction in DNA damage based on OTM and % tail DNA from 0.8 µg/mL TiO₂NPs, + Fpg and from 8 µg/mL TiO₂ -Fpg.</p> <p>Cytotoxicity: Mild significant cytotoxic response after 48h exposure. No significant cytotoxicity after 6 and 24 h exposure</p>	<p>Reliability: 2</p> <p>Limited number of cells analysed</p>	<p>Limited</p>	<p>Shukla et al. (2011)*</p>

<p>measured by flow cytometry and transmission electron microscopy</p> <p>Measurement of intracellular ROS</p>				<p>Cellular uptake: Significant increase</p> <p>Reduced glutathione level with increase in lipid hydroperoxides and ROS.</p>			
<p>Comet assay</p> <p>CHO-K1 cells</p> <p>Cell viability: XTT assay (24h and 48h)</p> <p>Cell cycle analysis: PI (flow cytometry) at 2 or 60 days</p> <p>Internalisation of Nano-TiO₂ and titanium content analysis</p>	<p>0, 10, 20 or 40 µg/mL for 60 days Duplicate cultures</p> <p>Solvent: DMSO No positive control</p> <p>75 nuclei per sample were scored (tail length)</p>	<p>TiO₂NPs, anatase, < 25 nm (XRD)</p>	<p>NSC: 1 no information on dispersion but exposure confirmed by EM and Ti measurements</p>	<p>Comet: Negative, no significant increase in tail length (results reported as log transformed)</p> <p>XTT, Trypan Blue exclusion and colony-forming assays: no effect on cell viability.</p> <p>Cell cycle: at 2-day exposure no effect. The percentage of cells in the G2/M phase was significantly increased at all concentrations examined at 60 days.</p> <p>TEM analysis demonstrated that</p>	<p>Reliability: 3</p> <p>No positive control; No cytotoxicity observed; Only chronic exposure; Results reported as log transformed; The protocol was not well described</p>	<p>Low</p>	<p>Wang et al. (2011)*</p>

<p>Cellular ROS (flow cytometry analysis)</p> <p>Cellular free ROS</p>				<p>nano- TiO₂NPs aggregates were internalized by CHO cells that were chronically exposed. Titanium levels were significantly higher in the two-day treatments at the two highest concentrations in comparison to the 60-day treatments at those same concentrations.</p> <p>ROS increased in a concentration - dependent manner. Average differences in ROS were significantly greater in the two-day treatments when compared to the 60-day treatments, which is consistent with Titanium content analysis.</p> <p>TiO₂NPs also significantly increased the production of ROS in the absence of cells.</p>	<p>ROS: The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism.</p>		
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				Superoxide dismutase quenched the ROS signal produced by TiO ₂ NPs alone, suggesting one of the principal ROS produced is superoxide			
Comet assay Human peripheral blood lymphocytes Cytotoxicity: Trypan blue	20, 50,100 and 200 µg/mL for 24 h Negative control: PBS Positive control: MMS For each sample, two slides with 50 randomly selected cells each were counted (total of 100 cells) Measure of OTM and tail length	TiO ₂ NPs, anatase, 15-30 nm (TEM)	NSC: 2 specific dispersion protocol mentioned, and EM used for confirming internalisation of NPs, but the reporting is insufficient and suggest large agglomeration and limited cellular uptake	Inconclusive (negative with no evidence of TiO ₂ NPs uptake). As reported by the Authors: "The rate of cells with NPs transferred to the cytoplasm was low. In 100 counted lymphocytes, intracytoplasmatic TiO ₂ -NPs could be demonstrated in five cells. Mainly large-sized particle aggregates up to 500 nm in diameter were seen, and NP invasion into the nucleus was observed in one cell". No significant cytotoxicity	Reliability: 3	Low	Hackenberg et al. (2011)

<p>Comet assay</p> <p>A549 human lung carcinoma cells</p> <p>Quantification of 8-oxodG and other oxidized bases by HPLC-tandem mass spectrometry (HPLC-MS/MS)</p> <p>Cytotoxicity: MTT assay</p> <p>Colony formation assay</p> <p>Intracellular accumulation: TEM</p> <p>ROS production (H₂DCFDA assay)</p>	<p>0, 100 µg/ml 4hr, 24hr and 48hr exposure</p> <p>No positive control At least 50 comets per slide were analysed; TriPLICATE</p> <p>8-oxodG: 100 µ/ml 4hr, 24hr and 48hr exposure</p> <p>MTT assay: 25, 50, 75, 100 µg/ml</p> <p>Colony formation: 1, 10, 100 µg/ml</p> <p>Intracellular accumulation: 50 µg/ml 4h exposure</p> <p>ROS (H₂DCFDA assay): 100 µg/ml TiO₂-A12, -A140, -R20 and P25 (TiO₂-A25) GSH content: 100 µg/ml 4, 8, 16 or 24 h exposure</p>	<p>1)TiO₂NPs, anatase, 12 nm (TEM) [A12] 2) TiO₂NPs, rutile, 21 nm (TEM) [R20] 3) TiO₂NPs (P25), anatase/rutile, 15-24 nm 4) TiO₂, anatase, 142 nm (TEM) [A140]</p>	<p>NSC: 1 limited information on dispersion but cell internalisation of NPs confirmed by EM</p>	<p>Positive at: 4h with all TiO₂ NPs 24h with TiO₂ NPs [A12], TiO₂ NPs (P25) and TiO₂ NPs [R20] 48h with TiO₂ NPs [A12]</p> <p>8-oxodG: TiO₂ NPs [A12], TiO₂ NPs (P25): Positive, oxidised bases after 4h, 24h and 48h TiO₂ NPs [R20]: Positive, oxidised bases after 24h and 48h TiO₂-A140: Negative</p> <p>TiO₂-NPs, both rutile and anatase, with diameters < 100 nm exerted more pronounced toxic effects than TiO₂-NPs with diameters >100 nm. Diameter rather than crystalline phase was found to be the major parameter influencing TiO₂-</p>	<p>Reliability: 2</p> <p>One concentration tested; No positive control.</p>	<p>Limited</p>	<p>Jugan et al. (2012)*</p>
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				<p>NPs cytotoxic potential. No significant changes in colony formation. Accumulation of the smallest NPs (TiO₂ NPs [A12], P25 and [R20]) observed in the cytoplasm, and TiO₂ NPs [A12] also observed in the nucleus of cells. For larger NPs, cytoplasmic accumulation was also observed, but NPs were never observed in cell nuclei.</p> <p>ROS increase with all TiO₂ NPs Decrease of reduced GSH: TiO₂ NPs [A12] and [R20] for 24 h</p>			
Comet assay human amnion epithelial (WISH) cells	0.625 to 20 µg/ml for 6h Positive control: EMS Measurement of OTM, tail length and tail intensity	TiO ₂ NPs, rutile, 30 nm (TEM)	NSC: 1 particle size distribution in the media reported for the second highest concentration and exposure confirmed by EM.	Equivocal Statistically significant increase of tail intensity at 10 and 20 µg/ml (up to 5-fold at the highest concentration, however borderline	Reliability: 3 Cytotoxicity measured only up to 10 µg/ml, but not at 20 µg/ml	Low	Saquib et al. (2012)

<p>Cytotoxicity: - MTT -neutral red uptake (NRU)</p>	<p>0.625 to 10 µg/ml for 24h</p>		<p>Both constituent particles and agglomerates observed</p>	<p>effects up to 10 µg/ml)</p>			
<p>Catalase activity</p>	<p>0.625 to 10 µg/ml for 24h</p>			<p>Cytotoxicity: 42.5% and 24.5% decline in cell viability at 10 µg/mL in NRU and MTT assays, respectively More than 85% of the analysed cell sections exhibited internalized TiO₂NPs aggregates.</p>			
<p>GSH level</p>	<p>0.625 to 10 µg/ml for 24h</p>			<p>Catalase activity: Statistically significant decrease at 5 and 10 µg/ml</p>			
<p>ROS production</p>	<p>0.625 to 10 µg/ml for 24h</p>			<p>GSH level: Statistically significant decrease at 5 and 10 µg/ml</p>			
<p>Cell cycle progression (propidium iodide (PI) staining, FACS analysis)</p>	<p>0.625 to 10 µg/ml for 24h</p>			<p>ROS production: Statistically significant increase at 5 and 10 µg/ml</p>			
				<p>Cell cycle progression:</p>			

				Statistically significant induction of G2/M cell cycle arrest			
<p>Comet assay - standard assay and -modified Comet assay with endonuclease III (EndoIII) - modified Comet assay with human 8-hydroxyguanine DNA-glycosylase (hOGG1)</p> <p>TK6 cells (human lymphoblastoid cells)</p> <p>Cell viability in TK6 cells treated with TiO₂-NPs evaluated using the Trypan blue dye exclusion assay.</p> <p>Cellular uptake of TiO₂ NPs.</p>	<p>0, 50, 100, 150 and 200 µg/mL TiO₂-NPs in 24h</p> <p>Positive control: MMS</p> <p>Triplicate cultures</p> <p>100 cells scored for each slide</p> <p>Measure of Comet tail lengths, width and intensity, (%) DNA in the tail</p>	<p>TiO₂NPs, anatase, ellipsoidal shape (TEM), 10x 30 nm, minor axes 12.1 ± 3.2 nm</p>	<p>NSC: 1 some information on dispersion and confirmation of NPs internalisation by TEM with EDS identification of Ti</p>	<p>Comet assay: Negative</p> <p>Oxidative DNA damage: negative</p> <p>TiO₂NPs concentration-dependent cytotoxicity observed in TK6 cells. Uptake of TiO₂NPs by TK6 cells was demonstrated. Cell viability around 55% at 200 µg/mL</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Woodruff et al. (2012)*</p>
<p>Comet assay</p> <p>Syrian hamster embryo (SHE) cells</p>	<p>0, 10, 25, and 50 µg/cm² for 24 h. Positive control: MMS without S9 100 cells per slide were analysed</p> <p>Determination of % DNA in tail</p>	<p>1)TiO₂NPs, anatase, 14 ±4 nm (TEM) 2) TiO₂, anatase, 160 ± 48 nm (TEM), 3) TiO₂, rutile, 530 ± 216 nm (TEM)</p>	<p>NSC: 2 level of dispersion measured in the media (suggesting high level of agglomeration) and confirmation of cellular uptake</p>	<p>Positive with only moderate cytotoxicity. At the highest concentration all TiO₂ particles caused increased DNA damage.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Guichard et al. (2012)*</p>

<p>Cytotoxicity based on the relative cell count (24 or 72h exposure)</p> <p>Cellular uptake</p>	<p>1 $\mu\text{g}/\text{cm}^2$ for 24 h.</p>	<p>4) TiO_2NPs (P25), anatase/rutile, 15-24 nm</p>	<p>by EM but only at $1\mu\text{g}/\text{cm}^2$</p>	<p>TiO_2 NPs (P25) was the only test material that induced a significant effect at all concentrations. The highest levels of DNA damage were obtained with anatase TiO_2, with no significant difference between TiO_2NPs and TiO_2. Rutile TiO_2 induced significant DNA damage at the highest concentration. However, all relevant materials increased DNA damage. Concentration-dependent cytotoxicity. TiO_2NPs were significantly more cytotoxic than TiO_2. Cellular uptake was demonstrated: TiO_2NPs or TiO_2 are able to penetrate cells in the form of individual particles and agglomerates</p>			
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<p>Acellular ROS assay</p> <p>Intracellular ROS assay</p>	<p>Dilutions of particle suspensions (250 µg/ml)</p> <p>1, 5, 10 µg/cm² for 72 h.</p>			<p>Acellular ROS assay: Anatase TiO₂ NPs and TiO₂ NPs (P25) produced the highest level of ROS. ROS activity was found to be significantly stronger for the anatase and rutile nanoparticles than for their micrometer counterparts. Intracellular ROS assay: Statistically significant increase. Anatase TiO₂ induced the highest ROS increase compared to the other particles.</p>	<p>Acellular ROS assay: The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism</p> <p>Intracellular ROS assay: The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism</p>		
<p>Comet assay +/- Fpg (DNA oxidation lesions: 8-oxodG)</p> <p>TK6 cells</p> <p>Cytotoxicity: trypan blue exclusion</p>	<p>0.12, 0.6, 3, 15, and 75 µg/cm² which correspond to 0.57, 2.9, 14.4, 72.0, and 360.2 µg/ml NPs</p> <p>2 h and 24 h treatments</p> <p>positive controls:</p> <p>- H₂O₂</p>	<p>TiO₂NPs (NM-105), anatase/rutile, 15-24 nm</p>	<p>NSC: 1 for DP1 NanoTest protocol a level of agglomeration observed</p> <p>NSC: 2 for DP2 Large agglomeration confirmed</p>	<p>DP1 (TiO₂NPs , NanoTest project): negative (% tail intensity) both alkaline and Fpg modified (2h and 24h)</p> <p>DP2 (TiO₂NPs): Comet: Negative</p>	<p>Reliability 1</p>	<p>High</p>	<p>Magdol enova et al. (2012)</p>

<p>Proliferation activity (cells counted at 24h intervals)</p>	<ul style="list-style-type: none"> - photosensitizer Ro 19-8022 plus visible light - without S9 <p>Determination of % tail intensity</p>		<p>Zeta potential - 30.2 mV</p> <p>2 dispersion protocols used:</p> <p>1) DP1: Dispersion of TiO₂NPs in foetal bovine serum (FBS) in PBS was sonicated for 15 min at 100 Watt (cycle: 100%). The dispersion was cooled during sonication with an ice/water bath in order to prevent heating of the dispersion. NP containing media were immediately added to the cells.</p> <p>2) DP2: TiO₂ NPs suspended in culture medium containing HEPES buffer without FBS were sonicated using an ultrasonic probe sonicator for 3 min at 60 W (on ice and water mixture to allow the cooling down of the solution). Within 2 min after sonication and</p>	<p>Oxidative stress: positive, statistically significant increase in the level of Fpg sites after 2 h exposure to the highest concentration</p> <p>DP1: "more stable (up to 2 days) bimodal dispersion with two peaks more or less in the nanosized range".</p> <p>DP2: "large agglomerates and less stable dispersion"</p> <p>No effect of the different dispersion protocols on proliferation activity.</p>			
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			<p>directly after 10 s of vortexing, the solution was divided into 10 microcentrifuge tubes and stored at 20°C for further use. Immediately before use TiO₂NPs were thawed, vortexed for 10 s before being immediately sonicated for 1 min (on ice and water mixture) at 60 W, and added to the cell culture medium NP-containing media were immediately added to the cells.</p> <p>Hydrodynamic diameter measured after DP1 and DP2: more efficient DP1 (stability 2 days while agglomerated for DP2)</p>				
Comet assay +/- Fpg	0.12, 0.6, 3, 15, and 75 µg/ cm ²	TiO ₂ NPs (NM-105),	NSC: 2	Comet: Equivocal 2h exposure: statistically	Reliability 2	Limited	

<p>Cos-1 monkey kidney fibroblasts</p> <p>-Proliferation activity</p> <p>- Plating efficiency assay</p>	<p>2h and 24 h NPs exposure</p> <p>Positive controls:</p> <ul style="list-style-type: none"> - H₂O₂ - photosensitizer Ro 19-8022 plus visible light <p>without S9</p> <p>Proliferation activity: cells exposed for 24h and counted at 24 h intervals for 2–3 days</p> <p>plating efficiency: cells exposed to TiO₂ for 24h and cultivated for approximately 10 days allowing cells to form colonies.</p>	<p>anatase/rutile, 15-24 nm</p>	<p>Large agglomeration confirmed</p> <p>Only TiO₂NPs dispersed with DP2 (“large agglomerates and less stable dispersion”) was tested</p>	<p>significant increase only at 75 µg/ cm² without Fpg.</p> <p>24h exposure:</p> <ul style="list-style-type: none"> - statistically significant increase only at 75 µg/ cm² (in the presence of cytotoxicity) without Fpg <p>Oxidative stress:</p> <ul style="list-style-type: none"> - statistically significant increase only at 3 µg/ cm² for 24h with Fpg <p>-After 24h exposure: concurrent decrease of proliferation activity and plating efficiency at 75 µg/ cm²</p> <p>Authors: “However, induction of DNA damage at this dose could have been due to the cytotoxicity observed with the plating efficiency and proliferation”</p>	<p>Equivocal results</p>		
<p>Comet assay</p>	<p>0, 0.12, 0.6, 3, 15, and 75 µg/ cm²</p>	<p>TiO₂NPs (NM-105),</p>	<p>NSC: 1 for DP1</p>	<p>Equivocal DP1: negative</p>	<p>Reliability 2</p>	<p>Limited</p>	

EUE human embryonic epithelial cells	For 24 h NPs exposure Positive controls: - H ₂ O ₂ - photosensitizer Ro 19-8022 plus visible light without S9	anatase/rutile, 15-24 nm	NanoTest protocol a level of agglomeration observed NSC: 2 for DP2 Large agglomeration confirmed	DP2: statistically significant only at 75 µg/cm ²	Unclear reporting of positive control results; No information on cytotoxicity at the highest tested concentration		
Comet assay Human embryonic kidney cells (HEK293) Human peripheral blood lymphocytes (HPBL) Cell viability assay: Fluorescein diacetate (FDA)/ ethidium bromide (EB)	0, 1, 10, 100 µg/ml 3h Positive control: EMS Without S9	1) TiO ₂ NPs, 2.3 nm (crystalline form and shape unknow) 2) "ionic" form of Ti from Sigma Aldrich, relevance of test material not clear for the evaluation of E171	NSC: 3 No information on dispersion in the cell media or PBS	Comet assay: - TiO ₂ NPs: positive Statistically significant increase of DNA damage (tail intensity and tail moment) only at 100 µg/ml in both cell lines, in absence of cytotoxicity -"ionic" forms of Ti: negative in both cell lines No cytotoxicity observed.	Reliability: 1	High	Demir et al., 2013
Comet assay Endo III or Fpg included Human embryonic kidney cells (HEK293)	0, 100 µg/ml 3h Positive control: EMS Without S9	TiO ₂ NPs, 2.3 nm (crystalline form and shape unknow)		Positive TiO ₂ NPs: statistically significant increase of DNA damage (tail intensity and tail moment) in	Reliability: 1	High	

Human peripheral blood lymphocytes (HPBL)				both cell lines at 100 µg/ml Damage detected with Endo III treatment was higher than with Fpg treatment, indicating greater induction of damage to pyrimidines than purines. No cytotoxicity observed.			
Comet assay human lung epithelial cells (BEAS-2B) Cytotoxicity: trypan blue Cellular uptake Cell cycle (PI staining)	0, 10 -100 µg/ml 24h Positive control: MMS Without S9 3 different types of treatment media: (a) KB, which had a low concentration of protein (0.1% BSA); (b) DM, which had a lower level of protein (0.6% BSA) plus 0.001% surfactant; (c) KF, which contained 10% FBS	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Three dispersion protocols and level of dispersion reported for each concentration, confirming high agglomeration and cell internalisation assessed by flow cytometry and dark-field microscopy	Positive Weak, but statistically significant and concentration-dependent increase in DNA damage (% tail DNA) in all three treatment media, regression analysis statistically significant (p = 0.0006, R ² = 0.38). -Direct relationship between concentration of nanoparticles delivered to the cells and side scatter detected by the flow cytometer. Cells treated	Reliability: 1	High	Prasad et al. (2013)*

				<p>with KB and DM showed lower side-scatter values at all concentrations compared to the KF medium. The rank order of agglomeration size in treatment media based on the DLS (dynamic light scattering) data at 0 and 24 h was KB > DM > KF. Results confirmed also by SEM (scanning electron microscopy)</p> <p>-No cytotoxicity observed Results from positive control are not shown -Cells treated with TiO₂ NPs in KF medium elicited a significant concentration-dependent increase of cells in S-phase</p> <p><u>Authors:</u> "the medium</p>			
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				composition influences both agglomeration size and, consequently, nanoparticle uptake”.			
Comet assay +/- Fpg HepG2 cells Cellular uptake (FACS analysis) Cytotoxicity (MTT and NRU)	0, 1, 10, 20, 40 and 80 µg/ml for 6 h Positive control: H ₂ O ₂ Analysis of 50 comets (25 from replicate slides) was carried out for each experiment. without S9 OTM and %DNA tail are reported.	TiO ₂ NPs, anatase, 30-70 nm (TEM)	NSC: 1 DLS confirmation of agglomeration in the media and cell internalisation confirmed by flow cytometry and EM	Positive Statistically significant increase in DNA damage observed from 20 µg/ml in the absence of Fpg and from 10 µg/ml in the presence of Fpg. Statistically significant, increase in oxidative DNA damage (tail DNA values statistically significantly higher with Fpg treatment than without); Cellular uptake: significant and concentration-related after 6h. TiO ₂ NPs in cytoplasm and nucleus	Reliability: 2 Limited number of comets analysed.	Limited	Shukla et al. (2013)*

<p>Oxidative stress markers: GSH, LPO (lipid peroxidation), ROS</p> <p>Apoptosis markers: -mitochondrial membrane potential; -Annexin V binding assay -immunoblotting of heat shock proteins, p53, cytoC, Bax, caspase-3, caspase-9, Apaf-1, Bcl-2</p>	<p>0, 1, 10, 20, 40, 80 µg/mL for 6h, 24h, 48h</p> <p>0, 20, 40, 80 µg/mL for 24h</p> <p>0, 20, 40, 80 µg/mL for 48h</p>			<p>Cytotoxicity: statistically significant increase > 20µg/ mL at 24h and 48h</p> <p>Oxidative stress, statistically significant and concentration related: -ROS and LPO increase -GSH decrease</p> <p>Apoptosis: -alteration in the mitochondrial membrane integrity -increase of apoptotic and necrotic cells -increased expression of pro-apoptotic proteins</p>	<p>Oxidative stress markers: The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism</p>		
<p>Comet assay</p> <p>Chinese hamster lung fibroblasts (V79 cells)</p>	<p>0, 5, 10, 20 and 100 µg/mL 6h, 24h</p> <p>Positive control: MMS 100 cells analysed/ slide/treatment Triplicate experiments</p>	<p>TiO₂NPs, anatase, 75 nm</p>	<p>NSC: 2 DLS measurement in cell media confirming agglomeration</p>	<p>Equivocal Only increase in the percentage of DNA in the tail at 100 µg/mL after 24 h exposure.</p> <p>Cell viability: significantly decreased in all TiO₂NPs -exposed</p>	<p>Reliability: 2</p> <p>Statistical unit is not clearly reported. No analysis of particles uptake.</p>	<p>Low</p> <p>Shortcomings and equivocal results</p>	<p>Chen et al. (2014)*, **</p>

	Cytotoxicity assay: 0, 5, 10, 20, 50 and 100 µg/mL 6h, 24h, 48h Measured by Cell Counting Kit-8 assay			groups at 24 and 48 h, but no obvious change was found at 6 h; time-dependent, but not concentration-dependent cytotoxicity.			
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BET: Brunauer–Emmett–Teller method; DLS: Dynamic light scattering; DMEM: Dulbecco's Modified Eagle's medium; DMSO: Dimethyl sulfoxide EM: Electron Microscopy; EMS: ethylmethanesulfonate; FACS: Fluorescence-activated cell sorting; Fpg: enzyme formamidopyrimidine glycosylase; FBS: Fetal Bovine Serum; GSH: reduced glutathione; H₂DCFDA: 2',7'-dichlorodihydro-fluorescein diacetate acetyl ester; HPLC-MS/MS: High performance Liquid Chromatography with tandem mass spectrometry; MMS: methylmethanesulfonate ; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NPs: Nanoparticles; NSC: nanoscale considerations; OTM: Olive tail moment; PBS: Phosphate-buffered saline PI: propidium iodide; ROS: reactive oxygen species; TEM: Transmission electron microscopy; UV/Vis: Ultra violet/Visible; ; XTT: 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide;

Table 4: Other *in vitro* assays

Sister Chromatid Exchange (SCE)							
Test system/ Test object	Exposure conditions (concentration/ duration /metabolic activation	Information on the characteristics of the test substance	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure),	Result	Reliability/ Comments	Relevance of the result	Reference

			assigned according to Appendix E				
Sister chromatid exchange (SCEs) assay Chinese hamster ovary (CHO) cell line	Highest concentration: 25 µg/ml without S9	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion.	Negative	Reliability: 5 Concentrations tested are not reported. OECD test guideline 479 was deleted in 2014 because the mechanism of formation of SCE is not elucidated	Low SCE not validated for hazard assessment	Tennant et al. (1987)*
Sister chromatid exchange assay CHO cells	TiO ₂ NPs tested with and without metabolic activation, which was a mixture of S9 (from liver of Aroclor 1254-treated rats), NADP, isocitric acid. Negative and positive controls included. Highest concentration: 25 µg/ml (313µM) Concentrations tested: 2.5, 8.3, 25.0 µg/ml	TiO ₂ NPs, anatase (Unitane® 0-220), particle size > 100 nm	NSC: 3 No information on dispersion. Highest concentration tested limited by solubility	Inconclusive (negative with no evidence of TiO ₂ uptake)	Reliability: 5 OECD test guideline 479 was deleted in 2014 because the mechanism of formation of SCE is not elucidated	Low SCE not validated for hazard assessment	Ivett et al. (1989)*

	<p>Exposure without metabolic activation(-S9): 25h</p> <p>Exposure with metabolic activation (+S9): 2h</p>						
<p>Sister chromatid exchange assay</p> <p>CHO-K1 cells</p>	<p>1, 2 and 5 µM for 24 h. Colcemid 0.2 µg/ml was added during the last 2-h incubation.</p> <p>Experiment repeated 3 times. Without S9. No positive control.</p>	<p>TiO₂ (no further information)</p>	<p>NSC: 1 no specific dispersion protocol, except confirming lack of microscopic precipitates, but concentration related accumulation confirmed under the same exposure conditions.</p>	<p>Positive: concentration-related and statistically significant increases in SCE.</p> <p>At the highest concentration 5 mM, the SCE frequency increase was 1.59 fold compared to the negative control.</p> <p>Cytotoxicity (colony forming assay) was measured: no cytotoxicity observed up to 20 µM.</p> <p>Intracellular TiO₂ accumulation was measured (0-10 µM for 24h): TiO₂ accumulated in</p>	<p>Reliability: 5</p> <p>OECD test guideline 479 was deleted in 2014 because the mechanism of formation of SCE is not elucidated</p>	<p>Low</p> <p>SCE not validated for hazard assessment</p>	<p>Lu et al. (1998)*</p>

				CHO-K1 cells in a concentration-dependent manner			
Sister chromatid exchange assay human peripheral blood lymphocytes	1, 2, 3, 5, 7.5 and 10 μ M Cells were exposed for 72 h. 5-bromo-2'-deoxyuridine at 20 μ M was added at the beginning of the cultures Colcemid (0.5 μ g/ml) was added 1.5h before harvest Without S9 No positive control.	TiO ₂ (no further information)	NSC: 3 No information on dispersion	Positive: concentration-related and statistically significant increases in SCE. Oxidative stress markers were measured in erythrocytes: TiO ₂ induced a concentration dependent significant decrease of the levels of antioxidant enzymes in erythrocytes, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase	Reliability: 5 OECD test guideline 479 was deleted in 2014 because the mechanism of formation of SCE is not elucidated	Low SCE not validated for hazard assessment	Turkez and Geyikoglu (2007)*
Sister chromatid exchange assay Cytotoxicity: Neutral Red and MTT assays	1, 5, 10, 25 μ g /ml For 24 h exposure without S9	TiO ₂ NPs, "complex" shape (TEM), 20 \pm 7 nm (TEM) (crystalline form unknow)	NSC: 1 limited information but exposure confirmed by EM	Positive: SCE significantly increased at 1 and 5 μ g/mL TiO ₂ NPs. Highest concentration (10	Reliability: 5 OECD test guideline 479 was deleted in 2014 because	Low SCE not validated for hazard assessment	Di Virgilio et al. (2010)*

CHO-K1 cells	100 metaphases were scored per treatment			and 25 µg/mL) could not be measured due to cytotoxic effects. Cytotoxicity: statistically significant decrease in cell viability. At 25 µg/mL decrease in cell viability seems about 20% compared to control. The authors indicate absence of metaphases at higher concentrations. Uptake of TiO ₂ NPs by CHO-K1 cells was measured by TEM: TiO ₂ NPs (50µg/ml) agglomerates on both the surface and inside of CHO-K1 cells, without entering the nucleus	the mechanism of formation of SCE is not elucidated.		
DNA damage response (including γH2AX, BER)							
γH2AX foci A549 human lung carcinoma cells	50, 100, 200 µg/ml 24-hr exposure	1)TiO ₂ NPs, anatase, 12 nm (TEM) 2) TiO ₂ NPs (P25),	NSC: 1 limited information on dispersion but cell internalisation of	Negative	Reliability: 2 Study may be indicative of DNA damage although γH2AX	Limited	Jugan et al. (2012)*

		anatase/rutile, 15-24 nm	NPs confirmed by EM		assay is not a standardised test. The method is not validated for regulatory purposes.		
The base excision repair (BER) and nucleotide excision repair abilities (NER) of A549 cells exposed to TiO ₂ -NPs were assessed using the newly developed multiplexed excision/synthesis assay	100 µg/ml 24-hr and 48-hr exposure	1)TiO ₂ NPs, anatase, 12 nm (TEM) [A12] 2) TiO ₂ NPs, rutile, 21 nm (TEM) [R20] 3) TiO ₂ NPs (P25), anatase/rutile, 15-24 nm 4) TiO ₂ , anatase, 142 nm (TEM) [A140]	NSC:1 limited information on dispersion but cell internalisation of NPs confirmed by EM	after 24 h exposure TiO ₂ NPs [A12] and - TiO ₂ [A140] drastically decreased cellular excision/ repair ability of all the tested lesions. BER and NER pathways were inhibited to the same extent. In cells exposed to TiO ₂ NPs [R20], the inhibition was also significant, but less marked. Conversely, TiO ₂ NPs (P25) slightly increased the repair ability of all the tested lesions. This difference might be due to NPs diameter, however, other parameters are also determinant since TiO ₂ [A140] is as efficient as TiO ₂ NPs [A12] in	Reliability: 5 The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism.	Low	Jugan et al. (2012)*

				inhibiting DNA repair ability. After 48 h of exposure the inhibition was generalized, and even TiO ₂ NPs (P25) caused the inhibition of repair ability of all the tested lesions. The more active NPs were TiO ₂ NPs [A12] and - TiO ₂ NPs [R20] at this late time point.			
<p>γH2AX (western blot and immunofluorescence)</p> <p>A549 human lung carcinoma cells</p> <p>Cellular uptake of TiO₂ NPs (FACS analysis)</p>	<p>100, 250, 500, 750, 1000 µg/ml for 1h</p> <p>250, 500, 750, 1000 µg/ml TiO₂ NPs or TiO₂ MPs for 1h</p>	<p>1) TiO₂NPs, anatase, 5 nm</p> <p>2) TiO₂, anatase, 5,000 nm</p>	<p>NSC: 2 two protocols, one including coating with BSA and internalisation measured by flow cytometry</p>	<p>concentration-dependent increase of γH2AX foci especially with TiO₂ NPs</p> <p>TiO₂ NPs uptake in a concentration-dependent manner. Uptake higher with TiO₂ NPs than with TiO₂.</p> <p>TiO₂ NPs (also TiO₂) were taken-up equally in all cell phases. The generation of γH2AX was proportional to the incorporation</p>	<p>Reliability: 2</p> <p>Study may be indicative of DNA damage although γH2AX assay is not a standardised test. The method is not validated for regulatory purposes.</p>	<p>Limited</p>	<p>Toyooka et al. (2012)</p>
<p>γH2AX and cell cycle (FACS analysis)</p>	<p>TiO₂ NPs 500 µg/ml for 1h</p> <p>Positive control: H₂O₂</p>						

				of TiO ₂ particles. TiO ₂ NPs more readily induce DSBs than TiO ₂ . The generation of γH2AX by TiO ₂ NPs was independent of the formation of ROS.			
DSBs were investigated with a biased sinusoidal field gel electrophoresis (BSFGE) system	50, 100, 250 µg/ml TiO ₂ NPs or TiO ₂ MPs for 1h			According to the authors γH2AX generation by treatment with TiO ₂ was due to the formation of DSBs (inhibitors used to confirm).	Reliability: 2 Test not validated for regulatory purposes.	Limited	
ROS production was investigated using the 6-carboxy- 2,7' - diclorodihydrofluorescein diacetate, di(acetoxy ester) (DCFH-DA)	50, 100, 250, 500, 750, 1000 µg/ml nano-TiO ₂ for 1h Positive control: H ₂ O ₂			Slight increases of ROS were observed at 750 and 1000 µg/ml which were, however, not statistically significant.	Reliability: 5 The study does not address a genotoxicity endpoint, but it may be considered regarding the mechanism.	Low	
Effects of surface coating (BSA) on generation of γH2AX was also studied				incorporation of TiO ₂ NPs by A549 cells after incubation for 1h was attenuated. BSA-coated TiO ₂ NPs caused no γH2AX generation, but after 8h incubation, γH2AX was generated	Reliability: 2 Study may be indicative of DNA damage although γH2AX assay is not a standardised test. The method is not validated for	Limited	

				with both uncoated and BSA-coated TiO ₂ ; however, the generation after treatment with uncoated TiO ₂ was remarkable	regulatory purposes		
Human neonatal foreskin fibroblast cells (BJ) γH2AX immunofluorescence Cytotoxicity (PicoGreen assay)	10, 25, 50, 100, 250, 500, 1000 µg/mL 24h 10 and 500 µg/mL	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 Limited information (DLS size 255.8 ± 2.65 nm in complete medium with no indication of conditions) confirming large agglomeration	γH2AX: statistically significant increase and concentration related. cytotoxicity observed > 100 µg/mL and concentration-related	Reliability: 2 Study may be indicative of DNA damage although γH2AX assay is not a standardised test. The method is not validated for regulatory purposes	Limited	Setyawati et al. (2013)

BET: Brunauer–Emmett–Teller method; DLS: Dynamic light scattering; DMEM: Dulbecco's Modified Eagle's medium; FACS: Fluorescence-activated cell sorting; NADP: Nicotinamide adenine dinucleotide phosphate; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NPs: Nanoparticles; NSC: nanoscale considerations; ROS: reactive oxygen species; SCE: sister chromatid exchange; TEM: Transmission electron microscopy;