Annex X - SCCS and EFSA analysis of studies on TiO2 genotoxicity - Taken into consideration

In vitro/In vivo	Type of assay	Reference authors_year	Evaluation by EFSA or SCCS	Test system/Test object	Exposure conditions (concentration/duration/metabolic activation)	TiO2 material tested	Test substance	Information on the characteristics of the test substance	Presence of nanofraction in fine TiO2 proved/possible	Cellular uptake	Results	Route of exposure	Genotoxicity result	Reliability/comments	Relevance [for EFSA - only data of high or limited relevance taken]	Crystalline Phase	Shape of Particle	Particle size, nominal particle size given by manufacturer (size range/ distribution) [nm]	Particle size measured by the authors of the publication (size range/ distribution) [nm]	Aspect ratio value (SD)	Crystal structure, ratio of anatase/rutile	Specific Surface area [m2/g] by BET method	Hydrodynamic diameter [nm] by DLS in distilled water	Hydrodynamic diameter [nm] by DLS, in saline, culture media, etc.	Zeta-potential [mV] at pH 7 in distilled water	Zeta-potential [mV] in saline, culture media	Polydispersity Index (PdI) in distilled water	Polydispersity Index (PdI) in saline, culture medium	Chemical composition of particles	Purity of particles [%]	Surface chemistry Coating	Impurities of concern	Other
in vitro	Comet in vitro	Vignard et al., 2023	SCCS	TR146 squamous cell carcinoma from human buccal cells	**nanoparticles**: E171, anatase **type of assay:** alkaline comet assay with a Comet SCGE assay kit **guideline:** **treatment time:** 2 h **concentrations:** 100 µg/ml **FBS serum:** 0 **S9 mix:** not used **biological medium for NPs dispersion:** **cytotoxicity assessment:** Cells exposed to 25 uM of etoposide as a positive control, or 5, 50 or 100 ug/ml of food-grade TiO2 (E171) and TiO2 test materials for 2 h. Cells were then washed with PBS and incubated in fresh culture medium for 72 h. Alamar blue assay. TEER measured immediately or within 48 h. **positive control:** etoposide 50 uM **methodology:** at least 60 cells were analyzed per sample using OpenComet software; at least three independent experiments. **statistics**: one-way or two-way analysis of variance (ANOVA) followed by the appropriate post hoc test **Additional measurements:** E171 particle translocation in vivo through the pig buccal mucosa and in vitro on human buccal TR146 cells;antibodies directed against cH2AX and 53BP1 (DNA damage biomarkers); oxidative stress	E171 equivalent	E171 equivalent, anatase, from French commercial supplier	**Suspension stability**: in ultrapure water (pH 7.75) and in TR146 cell culture medium (Ham's F12, pH 7.54) **Cell internalisation** : TEM; npSCOPE analyses combining SE, STIM and SIMS imaging; confocal microscopy **Sonication**: sonicated in ultrapure water (1 mg/ml) placed in an ice bath for 1 min at 40% amplitude (VCX 750-230 V, Sonics Materials) to obtain a stable dispersion of TiO2 particles for 15 days at 4oC.	possible (TEM image)	Confirmed by TEM; npSCOPE analyses combining SE, STIM and SIMS imaging; confocal microscopy	**any statistical increase at any concentration** : Yes **result**: positive **result for positive control**: 2.5 fold increase **cytotoxicity**: In proliferating cells, the tested TiO2 samples induced a significant cytotoxic activity at all tested concentrations compared to nontreated cells, with a dose-response tendency. In contrast, after cell differentiation, no viability drop was observed in any tested condition. **TEER**: Regardless of the time point tested during the 48 h after treatment, no alterations were detected at any dose, suggesting that epithelial barrier permeability and monolayer integrity were not affected. **Oxidative stress**: no increase at 5, 50 or 100 ug/mL **Internalisation**: Electron dense (TiO2) particles were isolated or recovered as small aggregates and then larger agglomerates of submicron-sized particles mixed with NPs into the cytoplasm. Conformed by npSCOPE analyses.		Positive	2	Limited	anatase		55% of the NPs by number were 20 to 440 nm for a mean size of 105 ± 45 nm					297.9 ± 5.1	318.7 ± 14.9	- 32.1 ± 0.79	- 8.97 ± 0.54	0,21	0,4					
in vitro	Other genotoxicity endpoints - H2AX	Vignard et al., 2023	SCCS	TR146 squamous cell carcinoma from human buccal cells	**nanoparticles**: E171, anatase **type of assay:** staining for gamma-H2AX **guideline:** **treatment time:** 2 h with direct measurement after the exposure OR 22 h thereafter **concentrations:** 5, 50, 100 µg/mL **FBS serum:** **S9 mix:** not used **biological medium for NPs dispersion:** **cytotoxicity assessment:** Cells exposed to 25 uM of etoposide as a positive control, or 5, 50 or 100 ug/ml of food-grade TiO2 (E171) and TiO2 test materials for 2 h. Cells were then washed with PBS and incubated in fresh culture medium for 72 h. Alamar blue assay. TEER measured immediately or within 48 h. **positive control:** etoposide 50 uM **methodology:** oroliferating cells were treated with etoposide or different concentrations of TiO2, and the gH2AX signal was quantified immediately or after 22 h of recovery in fresh culture medium **statistics:** **Additional measurements:** E171 particle translocation in vivo	E171 equivalent	E171 equivalent, anatase, from French commercial supplier	**Suspension stability**: in ultrapure water (pH 7.75) and in TR146 cell culture medium (Ham's F12, pH 7.54) **Cell internalisation** : TEM; npSCOPE analyses combining SE, STIM and SIMS imaging; confocal microscopy **Sonication**: sonicated in ultrapure water (1 mg/ml) placed in an ice bath for 1 min at 40% amplitude (VCX 750-230 V, Sonics Materials) to obtain a stable dispersion of TiO2 particles for 15 days at 4oC.	possible (TEM image)	Confirmed by TEM; npSCOPE analyses combining SE, STIM and SIMS imaging; confocal microscopy	**any statistical increase at any concentration** : Yes concentration-dependent increase, significant at 50 and 100 ug/mL immediately (2x increase), and 50 after 22 h (1.6x) **result**: positive **result for positive control**: 9x (immediately) and 3.7x (after 22h) increase **cytotoxicity**: In proliferating cells, the tested TiO2 samples induced a significant cytotoxic activity at all tested concentrations compared to nontreated cells, with a dose-response tendency. In contrast, after cell differentiation, no viability drop was observed in any tested condition. **TEER**: Regardless of the time point tested during the 48 h after treatment, no alterations were detected at any dose, suggesting that epithelial barrier permeability and monolayer integrity were not affected.		Positive	1	Limited the test treated as complementary in WoE	anatase		55% of the NPs by number were 20 to 440 nm for a mean size of 105 ± 45 nm					297.9 ± 5.1	318.7 ± 14.9	- 32.1 ± 0.79	- 8.97 ± 0.54	0,21	0,4					
in vitro	Comet in vitro	Ferrante et al., 2023	SCCS	HCT-116 Human colorectal carcinoma cells	**nanoparticles:** titanium dioxide food additive E171 **type of assay:** standard alkaline comet assay **guideline: no** **treatment time**: 72h **concentrations**: 0.1, 1,10, 100, 500 ug/L **FBS serum:** 10% **S9 mix: no** **biological medium for NPs dispersion** : RPMI-1640 medium **cytotoxicity assessment:** MTT, clonogenic assays **positive control**: none **methodology:** **statistics:** One-way ANOVA and student's t-test **Additional measurements**: Western blot analysis for apoptosis markers, and tubulin and Histone H3 housekeeping proteins ; quantitative real-time PCR (qRT-PCR) for expression of apoptotic regulators	E171 equivalent	E171 equivalent, anatase, from an Italian supplier of food coloring for bakeries	**Suspension stability**: **Cell internalisation** : **Sonication**: 300 W for 15 min	E171: the percentage of nanoparticles <100 nm was 32.1%, and the percentage of microparticles was 67.9%.	not reported	**any statistical increase at any concentration** : Yes **result:** positive **result for positive control:** not done **cytotoxicity:** significantly reduced cancer cell viability in a dose-dependent manner. **ROS:** **GSH:** **internalisation:** **pro-inflammatory response:**		Positive	2	High	anatase			150.0 ±20.5 (mean) 40–360 (range) most frequent size of 176.3 ± 24.4		anatase												
in vitro	Comet in vitro	Ferrante et al., 2023	SCCS	Caco-2 Human colorectal carcinoma cells	**nanoparticles:** titanium dioxide food additive E171 **type of assay:** standard alkaline comet assay **guideline: no** **treatment time**: 72h **concentrations**: 0.1, 1,10, 100, 500 ug/L **FBS serum:** 10% **S9 mix: no** **biological medium for NPs dispersion** : RPMI-1640 medium **cytotoxicity assessment:** MTT, clonogenic assays **positive control**: none **methodology:** **statistics:** One-way ANOVA and student's t-test **Additional measurements**: Western blot analysis for apoptosis markers, and tubulin and Histone H3 housekeeping proteins ; quantitative real-time PCR (qRT-PCR) for expression of apoptotic regulators	E171 equivalent	E171 equivalent, anatase, from an Italian supplier of food coloring for bakeries	**Suspension stability**: **Cell internalisation** : **Sonication**: 300 W for 15 min	E171: the percentage of nanoparticles <100 nm was 32.1%, and the percentage of microparticles was 67.9%.	not reported	**any statistical increase at any concentration** : Yes **result:** positive, lower level of genotoxicity in Caco-2 than HCT-116 **result for positive control:** not done **cytotoxicity:** significantly reduced cancer cell viability in a dose-dependent manner. **ROS:** **GSH:** **internalisation:** **pro-inflammatory response:**		Positive	2	High	anatase			150.0 ± 20.5 (mean) 40–360 (range) most frequent size of 176.3 ± 24.4		anatase												
In vitro	Micronucleus test in vitro	Proquin et al., 2017	EFSA			E171 equivalent	E171 equivalent, anatase						Positive	EFSA	EFSA	E171, anatase					E171, anatase												
in vitro	Comet in vitro	Proquin et al., 2017	EFSA			E171 equivalent	E171 equivalent, anatase (39% nano)						Positive	EFSA	EFSA	anatase					E171, anatase (39% nano)												
in vitro	Comet in vitro	Brown et al., 2019	EFSA			E171 equivalent	E171 equivalent, anatase (0.2% rutile)						Positive	EFSA	EFSA	anatase					E171, anatase (0.2% rutile)												
in vitro	Comet in vitro	Dorier et al., 2019	EFSA			E171 equivalent	E171 equivalent, anatase						Negative	EFSA	EFSA	anatase		118 ± 53			E171, anatase												
in vitro	Other genotoxicity endpoints in vitro - ToxTracker	Brown et al., 2019	EFSA			E171 equivalent	E171 equivalent, anatase (0.2% rutile)						Negative	EFSA	EFSA	anatase					E171, anatase (0.2% rutile)												
in vivo	Chromosomal aberration test in vivo	Shelby and Witt, 1995	EFSA			E171 similar	E171 equivalent, anatase (Unitane 0-220)					intraperitoneal	Negative	EFSA	EFSA	E171 similar, anatase (Unitane 0-220)		The TiO2 material used in the study was sourced from the NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation). Therefore, it is likely that this test article was the same material used in the NCI two-year cancer bioassay (i.e., Unitane® 0-220; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number), which can be considered comparable to the current form of TiO2 added to food.			E171, anatase (Unitane 0-220)												
in vivo	Micronucleus test in vivo	Shelby and Witt, 1995	EFSA			E171 similar	E171 equivalent, anatase (Unitane 0-220)					intraperitoneal	Equivocal (EFSA)	EFSA	EFSA	E171 similar, anatase (Unitane 0-220)		The TiO2 material used in the study was sourced from the NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation). Therefore, it is likely that this test article was the same material used in the NCI two-year cancer bioassay (i.e., Unitane® 0-220; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number), which can be considered comparable to the current form of TiO2 added to food.			E171, anatase (Unitane 0-220)												
in vivo	Comet in vivo	Bettini et al., 2017	EFSA			E171 equivalent	E171 equivalent, anatase					oral	Negative	EFSA	EFSA	E171, anatase		20-340 (118) (TEM); 44.7% (<100)			anatase												
in vivo	Comet in vivo	Jensen et al., 2019	EFSA			E171 equivalent	E171 equivalent, anatase (0.2% rutile)					oral	Negative	EFSA	EFSA	E171, anatase (0.2% rutile)		3 size groups of particles: 135 ± 46 nm, 305 ± 61, 900 ± 247 nm (TEM image)															
in vivo	Comet in vivo	Sycheva et al., 2011	EFSA			E171 similar	E171 equivalent, TiO2 pigmentary, anatase (EFSA)					oral	Positive (bone marrow), negative (liver and brain)	EFSA	EFSA	anatase		160 ± 59.4			anatase												