

Appendix J New *in vitro* genotoxicity studies

Table of contents

Table 1: <i>In vitro</i> Gene mutation assays	1
Table 2: <i>In vitro</i> Chromosomal aberrations/ mammalian cell micronucleus test	7
Table 3: <i>In vitro</i> DNA damage (Comet assay)	20
Table 4a: Other <i>in vitro</i> assays - Genotoxicity endpoints investigated with methods not recommended for regulatory purposes	60
Table 4b: Other <i>in vitro</i> assays - Not genotoxicity endpoint but to be considered in the overall assessment	72

The evaluation of the studies has been performed according to the approach set out 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 K)**

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 authors_year

<p>Mammalian cell gene mutation test in V79 (hypoxanthine-guanine phosphoribosyl transferase (HPRT or HGPRT) gene);</p> <p>Comet assay (Table 3)*</p>	<p>Particle uptake: 0, 10, 50, 100 µg/ml</p> <p>MTT assay: 1, 10, 25, 50, 100 µg/ml</p> <p>ROS production: 1, 10, 25, 50, 100 µg/ml</p> <p>Exposure:</p> <p>HGPRT gene mutation in V-79 cells: 6 hours</p> <p>Particle uptake: up to 24 hours</p> <p>MTT assay: up to 24 hours</p> <p>ROS production: 6 hours</p>	<p>TiO₂NPs, anatase, 12-25nm (TEM)</p>	<p>NSC: 1</p> <p>Dispersion measured in the exposure media, presence of small agglomerates confirmed. Cellular uptake measured, internalisation of NPs and agglomerates confirmed.</p>	<p>Positive</p> <p>Statistically significant increase in the frequency of HPRT gene at 50 and 100 µg/ml.</p> <p>Particle uptake: Cellular uptake detected by flow cytometry and confirmed by TEM.</p> <p>ROS production (DCFH-DA): statistically significant increase of % of ROS production at all concentrations except at 1 µg/ml.</p>	<p>Reliability: 2</p> <p>Main deviations from OECD TG 476: the cytotoxicity induced by the treatment (relative survival) was not evaluated in the gene mutation experiment. The number of treated cells is not reported.</p>	<p>Limited</p>	<p>Jain et al.,2017*</p>
<p>Mammalian cell gene mutation test (Thymidine kinase (Tk) locus) in mouse lymphoma</p>	<p>Particle uptake TEM:</p> <p>0, 1, 100 µg/mL of TiO₂-NPs < 25 nm TiO₂-NPs (50 nm)</p>	<p>1) TiO₂NPs, anatase 24.2 nm (TEM)</p>	<p>NSC: 1</p> <p>Dispersion measured according to the</p>	<p>Equivocal</p> <p>No statistically significant increase compared to the</p>	<p>Reliability: 2</p> <p>Methods not reported in details, only reference to 6</p>	<p>Limited</p>	<p>Demir et al.,2017</p>

<p>L5178Y cells (OECD TG 490)</p>	<p>Mouse lymphoma assay: 0, 1, 10, 100 µg/mL of micro-particulated form of titanium dioxide (TiO₂), TiO₂ NPs (24.23 nm) and TiO₂ NPs (50 nm)</p> <p>Exp: Particle uptake TEM: 24 hours</p>	<p>2) TiO₂NPs, anatase 50.2 nm (TEM)</p> <p>3) micro-TiO₂, (no further information available)</p>	<p>Nanogenotox protocol and cellular internalisation confirmed by TEM. Both NPs and agglomerates observed in the exposed cells.</p>	<p>negative control. The Global Evaluation Factor (GEF) was not exceeded. However, a statistically significant concentration/effect trend was observed in 6 separate experiments.</p>	<p>publications provided.</p>		
<p>CD59 gene loci mutation assay, Target cells: human-hamster hybrid AL cells (CHO-K1 cells with a single copy of the human chromosome 11). Intracellular ROS production. Micronucleus assay (Table 2)*</p>	<p>CD59 gene loci mutation assay: 1 or 10 µg/mL for 24 h; MTT cytotoxicity assay; Intracellular ROS: 1 µg/mL TiO₂ NPs for 24 h</p>	<p>TiO₂NPs, anatase, 15 nm</p>	<p>NSC: 1 Level of dispersion measured and reported. Cellular internalisation of NPs and agglomerates confirmed by TEM.</p>	<p>CD59 gene loci mutation assay: TiO₂NPs alone (up to 10 µg/mL) had no effect on the mutation induction. Concurrent treatment of cells with 1 µg/mL TiO₂ NPs and 2 µg/mL As (III) induced a slight but statistically significant increase in the mutagenicity of As (III).</p>	<p>Reliability: 5 Study focused on interactions between TiO₂ NPs and As(III). No mutagenic effect reported with TiO₂NPs alone, but low concentrations used and no positive control.</p>	<p>Low</p>	<p>Wang et al., 2017*</p>

				<p>MTT cytotoxicity assay: TiO₂ NPs at 1 and 10 µg/mL had slight effects on the cell viability.</p> <p>No increase in ROS production</p>			
<p>Gene mutation assay in CD59 locus (human chromosome 11) Target cells: AL human-hamster hybrid cells (CHO-K1 cells with a single copy of the human chromosome 11). Phospho-histone H2AX (γH2AX) detected by immunofluorescence staining and quantified by western blotting.</p>	<p>CD59 gene loci mutation: 10 µg/mL (fresh and aged TiO₂ NPs) for 72 h.</p> <p>Immunofluorescence staining for γ-H2AX detection: 10 µg/mL (fresh or aged TiO₂ NPs different sizes).</p> <p>Mitochondrial dysfunction: 50 µg/mL (fresh and aged TiO₂ NPs).</p> <p>Intracellular ROS levels (DCFDA analysis): 10 µg/mL (fresh and aged TiO₂ NPs) for 72 h.</p> <p>Particle uptake and mitochondria ultrastructure analysis: 50 µg/mL three sizes of fresh and aged TiO₂ NPs.</p> <p>Apoptosis: 50 µg/mL (fresh and aged TiO₂ NPs)</p> <p>Exposure:</p>	<p>1)TiO₂NPs, anatase, 5 nm 2)TiO₂NPs, anatase, 15 nm, 3) TiO₂NPs, mixture anatase-rutile, <100 nm,</p>	<p>NSC:1</p> <p>Dispersion measured at a concentration below those used for the exposure but cellular internalisation of NPs and agglomerates is confirmed by TEM.</p>	<p>CD59 mutagenicity test: Positive but significant increases (max 1.41 x control) in mutations of CD59 gene.</p> <p>γH2AX was less increased in (p0)ALcells (95% depleted for mitochondrial DNA), indicating a possible role of mitochondria in DNA damage.</p> <p>TiO₂ NPs increased the intracellular ROS level (DCFDA analysis).</p>	<p>Reliability: 3</p> <p>Genotoxic and mutagenic effects were detected by testing methods not validated for regulatory purposes.</p> <p>Only one treatment concentration was used and no positive control.</p> <p>The same authors did not observe a CD59 effect at this concentration (10</p>	<p>Low</p>	<p>Wang et al.,2019</p>

	Immunofluorescence staining for γ -H2AX detection + Western blot for protein expression: 72 hours				$\mu\text{g/mL}$) two years before (Wang et al., 2017). This puts the reproducibility of these results into question.		
Gene mutation assay in L5178Y mouse lymphoma cells; Ames test (Table 1)*	2, 0.5, 0.125, and 0.0312 mg/mL Exposure: 4 h (+/- S9) and 24 h (-S9)	TiO ₂ NPs, 40 nm (SEM) (crystalline form unknown)	NSC: 3 No information provided on dispersion, test design not appropriate for poorly soluble substances.	Inconclusive No increase in mutation was observed. Although the treatment induced cytotoxicity, internalisation was not demonstrated	Reliability: 3	Low	Du et al.,2019*
Mammalian Cell Gene Mutation Test (Hprt) OECD TG 476 Chinese hamster lung (V79-4) fibroblasts	3, 15 and 75 $\mu\text{g/cm}^2$ 24h Positive control: MMS	TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC:1 Two dispersion protocols, good dispersion for protocol 1, and larger agglomeration for protocol 2, giving different size distribution	Negative No mutagenic effects independently of the dispersion protocol used.	Reliability: 1	High	Kazimirova et al.,2020

			and dispersion stability after 48 h were used				
Bacterial assays							
Umu assay Salmonella typhimurium	8.35-667 µg/mL Exposure: up to 1h	1)TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm 2)TiO ₂ NPs, anatase, 15 nm	NSC:1 Dispersion and stability measured under the exposure conditions for the different NPs sizes and representative concentrations.	TiO ₂ -NPs in presence of UV light induced ROS, however, UV light itself caused cytotoxic and genotoxic damage to S. typhimurium. No genotoxicity was observed under visible light (at concentrations up to 100 µg/mL) or under dark conditions (up to 667 µg/mL).	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials. In addition the test system does not belong to the methods recommended for regulatory purposes.	Low	Cupi and Baun, 2016
Bacterial reverse mutation assay (Ames test) Salmonella typhimurium TA98 and TA100 E. coli (only for cytotoxicity tests)	0, 100, 200, 300, 400, 500; Positive controls: 2-nitrofluorene, sodium azide	TiO ₂ NPs, 28 nm (crystalline form unknown)	NSC: 3 No information on level of dispersion under the	Ames test negative in both strains. The authors attribute the negative results to the antibacterial	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials	Low	Ranjan and Ramalingam, 2016

			exposure conditions.	activity of TiO ₂ NPs.			
Bacterial reverse mutation assay (Ames test) Salmonella typhimurium TA97a, TA98, TA100, TA102 and TA1535	78, 156, 312, 625, 1250 µg/plate +/- S9; at higher concentrations the precipitate interfered with the scoring.	TiO ₂ NPs, 40 nm (SEM) (crystalline form unknow)	NSC: 3 No information provided on dispersion, test design not appropriate for poorly soluble substances. Some concentrations are very high.	Negative	Reliability: 5 Bacterial systems are not suitable for testing nanomaterials.	Low	Du et al., 2019*

DCFH-DA or DCFDA: 2', 7'-dichlorofluorescein diacetate; GEF: Global Evaluation Factor; HPRT: hypoxanthine-guanine phosphoribosyl transferase; MMS: methyl methanesulfonate; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NSC: nanoscale considerations; ROS: reactive oxygen species; 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 authors_year
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<p>Micronucleus (MN) test</p> <p>human colon adenocarcinoma cell line (HCT116)</p> <p>Comet assay (Table 3)*</p>	<p>5, 10, 50 and 100 µg/cm² (50, 100, 500 and 1000 µg/mL, respectively)</p> <p>In triplicate</p> <p>Exposure for 24 h, wash out, CytoB added for 24 h</p>	<p>E171, anatase, 39% nanoparticles</p>	<p>NSC: 2</p> <p>Dispersion and stability measured, some level of agglomeration confirmed. Agglomeration specifically relevant at high concentrations, but reduced by the use of BSA or FBS. Some information on cellular exposure to particles presented for MN test but difficult to verify.</p>	<p>Positive</p> <p>concentration-dependent (5-50 µg/ cm²)</p> <p>100 µg/cm² could not be assessed due to the presence of agglomerates.</p> <p>No decrease in cell viability (Trypan blue assay)</p> <p>E171 interacts with the centromere region of kinetochore poles during mitosis.</p>	<p>Reliability: 2</p> <p>no positive controls, no independent replicates</p>	<p>Limited</p>	<p>Proquin et al., 2017 *</p>
<p>Micronucleus test</p> <p>Human PBMCs</p> <p>Comet assay (Table 3)*</p>	<p>0, 10, 50, 100, 200 µg/ml</p> <p>1.Delayed co-treatment: Treatment 24h after lymphocytes stimulation, 20h later CytoB, 72 h harvesting</p> <p>2.co-treatment protocol:</p>	<p>TiO₂ NPs, anatase, sphere, 20-60 nm (TEM)</p> <p>TiO₂ NPs, rutile: rod-like, 30x 100 nm (TEM)</p> <p>TiO₂, anatase/rutile, 45-262 nm (TEM), 3 morphologies - sphere, irregular and rod-like.</p>	<p>NSC: 1 for the five materials.</p> <p>Dispersion with sufficient energy and stability measured in the media, confirmation of presence of both constituent particles and agglomerates</p>	<p>Negative</p> <p>Significant increase of binucleate lymphocytes bearing micronuclei (MNBN= 12.0 ± 4.24, vs 6.0 ± 1.41 in controls) only in a single donor after treatment with 50 µg/ml TiO₂ rutile using the co-treatment protocol (not biologically relevant)</p> <p>No reduction of the CBPI for any treatment</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Andreoli et al., 2018 *</p>

	treatment 43.5 h after lymphocytes stimulation, 30 min later CytoB, 72 h harvesting	TiO ₂ , anatase, 50-270 nm (TEM). TiO ₂ , rutile, 50-3,000 nm (TEM), irregular					
Micronucleus test Human colon carcinoma Caco-2 cells Comet assay (Table 3)*	1, 2, 5, 10 and 20 µg/cm ² NPs (corresponding to 6.4–128.0 µg/ml) Three independent experiments Exposure: 6 h and 24 h CytoB added after the treatment for 24 h	TiO ₂ NPs, anatase, 20-60 nm (TEM)	NSC: 2 Dispersion and stability measured and some level of agglomeration confirmed.	Negative No significant reduction of the RI. After treatment with TiO ₂ NPs, the presence of particles over the cells surface at highest concentration (20 µg/cm ²) compromised a reliable identification of micronuclei and reduced the number of analysable binucleated cells. In this condition a consistent number of binucleate cells were scored only on slides from cultures treated for 24 h.	Reliability: 1	High	Zijno et al., 2015 *
Micronucleus test Peripheral Blood Lymphocytes	whole blood: 0, 45, 90, and 180 µg/mL for 72 h (three replicates)	TiO ₂ NPs, anatase, 40-110 nm (TEM)	NSC: 3 No information reported on dispersion or stability	Not interpretable Peripheral blood lymphocytes: concentration-related increase of total	Reliability: 3 The description of the methods and the results are not	none	Haleem et al., 2019

				chromosomal aberration (p≤0.05), decrease in mitotic index (MI) and blastogenic index (BI)	sufficient for the assessment. no information on the type of aberrations		
Micronucleus test TK-6 cells	Microscope-based MN test: 0, 100 and 200 µg/ml Flow cytometry MN assay: 0, 10, 50, 100, 200, 400 and 800 µg/ml Exposure: 28h (1.5 – 2 cell cycle lengths), without CytoB added	TiO ₂ NPs, anatase 8.9–15.3 nm,	NSC: 2 Dispersion measured for the highest concentration used in some cases in conditions slightly different from the exposure condition. Agglomeration confirmed, good stability for all the duration of the assay.	Microscope-based MN assay: Positive . Statistically significant and concentration-related increase of micronuclei, and decrease of RPD, at the two highest concentrations. Flow cytometry-based MN assay: contrasting and inconclusive results for cytotoxicity and MN induction, due to the interference of TiO ₂ NP with fluorescence detection with the cytometry equipment.	Reliability: 1 (microscope-based results)	High	Li et al., 2017a
Micronucleus test Micronucleus Assay with Fluorescence <i>In Situ</i> Hybridisation (FISH)	32, 64 and 128 µg/ml, Exposure: 48 h FISH: single concentration: 64 µg/ml for 48 h CytoB added after 44h;	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm.	NSC: 1 Dispersion is reported and cellular uptake confirmed by EM. A level of agglomeration is observed and also the internalisation of agglomerates in the exposed cells.	Positive TiO ₂ NPs induced MN formation at all concentrations tested. Significant increase of NPBs, NBUDs, necrotic and apoptotic index. TiO ₂ NPs induced the formation of NPB (nucleoplasmic bridges) at	Reliability: 1	High	Stocco et al., 2017*

<p>human lung carcinoma cell line (A549)</p> <p>Comet assay (Table 3), other in vitro studies (Table 4b)*</p>	<p>Harvesting 72 h</p>			<p>all the concentrations tested, but no significant induction of NBUDs.</p> <p>FISH: results showed that all TiO₂ NPs tested induced mainly clastogenic damage</p> <p>Cytotoxicity: TiO₂ NPs induced a weak, although statistically significant, decrease of CBPI and replication index at all times of exposure.</p>			
<p>Micronucleus test</p> <p>Micronucleus Assay with Fluorescence <i>In Situ</i> Hybridisation (FISH)</p> <p>Human peripheral blood lymphocytes isolated from 20 Healthy individuals (HI); 19 Polyposis coli patients (PLP); 20 Colon cancer patients (CRC).</p>	<p>10, 40 and 80 µg/ml Treatment:24 hours from the start of cell culture. Harvesting:72 hours</p>	<p>TiO₂ NPs, anatase, 34 nm (20-64 nm) (SEM)</p>	<p>NSC: 2</p> <p>Dispersion measured and agglomeration confirmed, increasing with concentration and time.</p>	<p>Positive</p> <p>Concentration-dependent increase of MN, NPB and BUD frequency in binucleated and of MN in mononucleated lymphocytes from all the groups of subjects. Weak decrease of the percentage of binucleated cells significant only in the PLP and CRC patients group and of CBPI significant for all the groups.</p> <p>FISH results showed that all TiO₂ NPs tested induced mainly clastogenic damage. MN with a signal was</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Kurza wa-Zegot a et al.,2017*</p>

Comet assay (Table 3)*				increased in a concentration dependent manner in all groups.			
<p>Micronucleus test</p> <p>Peripheral blood lymphocytes (PBL) and TK-6 lymphoblastoid human cells</p> <p>Comet assay (Table 3)*</p>	<p>3, 15 and 75 µg/cm², equivalent to 5.4, 27 and 135 µg/ml (for both TK-6 and PBL)</p> <p>Treatment TK6 cells: 4 or 24 h. CytoB (6 µg/ml) was added to the cell cultures for the next 24 h</p> <p>Blood lymphocytes (from 13 donors): treatment in G0 for 24 h, followed by phytohemagglutinin stimulation and 72 h of culture, with addition of cytoB for the last 28 h</p>	<p>TiO₂ NPs (NM-105), anatase/rutile, 15-24 nm</p>	<p>NSC: 1 for <i>in vitro</i> MN test.</p> <p>Dispersion protocol 1 (DP1) was developed in the NanoTEST project</p>	<p>In vitro MN assay in TK-6 cells: negative. No increase in the number of MNBN cells and no change in CBPI.</p> <p>In vitro MN assay on PBL: negative. TiO₂ NPs did not increase the number of MN in binucleated and mononucleated cells, and did not affect CBPI.</p>	<p>Reliability: 1 (MN in TK-6 cells)</p> <p>Reliability: 3 (MN in PBL)</p> <p>The protocol used for treatment of human lymphocytes (G0 exposure) is not in line with OECD TG 487 nor with EFSA SC recommendations on the assessment of nanomaterials (EFSA Scientific Committee, 2018a), see Appendix D</p>	<p>High (MN in TK6 cells)</p> <p>Low (MN in PBL)</p>	<p>Kazimirova et al., 2019*,*</p> <p>*</p>
<p>Micronucleus test</p> <p>Human embryonic</p>	<p>10, 100 and 1000 µg/mL for 48 hours using duplicated cultures</p>	<p>1) TiO₂ NPs, anatase, 21 nm (TEM)</p>	<p>NSC: 1</p> <p>Dispersion protocol according to Nanogenotox project,</p>	<p>Positive. Significant increases in the frequency of MNBN were observed in the two cell lines (HEK293 and</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Demiret al., 2015*</p>

<p>kidney cells (HEK293) and mouse embryonic fibroblasts (NIH/3T3)</p> <p>Comet assay (Table 3)*</p>	<p>CytoB was added 24 h before cell harvesting</p>	<p>2) TiO₂ NPs, anatase, 50 nm (TEM). 3) TiO₂ microparticulated (no further information)</p>	<p>dispersion measured in the media, some agglomerates confirmed. BSA is used as dispersion agent</p>	<p>NIH/3T3) at the highest tested concentration, which was associated with a significant decrease of CBPI) No increase in frequency of MNBN was observed for the microparticulated TiO₂ form.</p>			
<p>Micronucleus test</p> <p>immortalised human bronchial epithelial cell lines (BEAS-2B cells)</p> <p>Comet assay (Table 3)*</p>	<p>Stock solution of TiO₂ NPs (0.1 mg/ml) were prepared by dilution in 0.05% BSA and added at the final concentration of 1, 5, 15 µg/ml (CBMN) and 1, 5, 15, 30 µg/ml (flow cytometry).</p> <p>Treatments: CBMN assay: 48 treatment, CytoB added after 20 h; Flow cytometry: 48 h treatment</p>	<p>TiO₂ (NM-100), anatase, 50–150 nm</p>	<p>NSC: 1</p> <p>Dispersion and stability measured in the exposure media according to the NanoReg project. Some level of agglomeration confirmed. Cellular uptake confirmed by an indirect method demonstrating the cellular internalisation of the agglomerates.</p>	<p>Negative</p> <p>MN test: NM100 negative with CBMN assay and flow cytometry; no increase of hypodiploidy, determined by flow cytometry, with any sample.</p> <p>The flow cytometric analysis of particle uptake showed a clear increase in side scatter of the nuclei from TiO₂ NPs-exposed cells, indicating a concentration-dependent particles uptake by BEAS-2B cells.</p> <p>Cytotoxicity:</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Di Bucchianico et al., 2017 *</p>

				No effect on cell survival or RI in the concentration range tested with any TiO ₂ NPs sample.			
<p>Micronucleus test</p> <p>human umbilical vein endothelial cells (HUVECs)</p> <p>Comet assay (Table 3)*</p>	<p>1, 5, and 25 µg/mL</p> <p>MN assay: 24 hours treatment, followed by addition of CytoB and further 24 h of incubation</p> <p>Measurement of intracellular ROS and anti-oxidant production: 24 hours</p> <p>Measurement of GSH: 24 hours</p>	<p>1)TiO₂ NPs, anatase, 100 ± 14.3 nm</p> <p>2)TiO₂ NPs, anatase, 50 ± 7.6 nm</p> <p>3) TiO₂ NPs, anatase, 30 ± 5.1 nm</p> <p>4)TiO₂ NPs, anatase, 10 ± 2.3 nm</p>	<p>NSC: 2</p> <p>Ultrasonication performed in the cell medium for all the concentrations. Actual measurements for dispersion and stability not reported.</p>	<p>Positive</p> <p>Dose dependent and statistically significant increase of % MN at all concentrations with all TiO₂ NPs sizes, except TiO₂ NPs 100 nm at 1 µg/ml. Induced MN% were higher with decreasing sizes of TiO₂-NPs (10 > 30 > 50 > 100 nm)</p> <p>Measurement of intracellular ROS: statistically significant increase (% frequency of MN) at all concentrations of all TiO₂ NPs sizes (concentration-dependence and NPs size dependence).</p> <p>Measurement of GSH: the three smaller sizes of TiO₂-NPs (10, 30, and 50 nm) induced a significant decrease of reduced GSH levels. But for the TiO₂NPs (100± 14.3 nm) at the tested concentration of</p>	<p>Reliability: 2</p> <p>however, cytotoxicity elicited by treatments was not concurrently assessed, as recommended in OECD TG 487</p>	<p>Limited</p>	<p>Liao et al., 2019*</p>

				<p>1 µg/mL, the level of reduced GSH was lower, but not significantly.</p> <p>Anti-oxidant marker (Nrf2 protein) level: the levels of Nrf2 protein expression in all the TiO₂NPs-exposed groups were significantly higher than control.</p>			
<p>Micronucleus test</p> <p>Human peripheral blood lymphocytes</p> <p>Comet assay (Table 3)*</p>	<p>Blood lymphocytes were collected from: 36 respiratory disease patients (16 patients with lung cancer, 11 with chronic obstructive pulmonary diseases (COPD) and 9 with asthma) and 15 healthy controls</p> <p>CBMN: lymphocytes cultures were treated with 5 and 10 µg/ml TiO₂ 24 h after stimulation; CytoB was added at 44 h and</p>	<p>TiO₂ NPs, anatase, 40-70 nm (SEM)</p>	<p>NSC: 2</p> <p>Dispersion considered, including sonication after dilution and microfiltration but insufficient information reported on the actual measurements.</p>	<p>Negative</p> <p>CBMN assay: In all study groups, the incidence of micronuclei increased in TiO₂ NPs treated cultures, but the differences were not statistically significant; Nuclear division index (NDI) and the percentage of binucleated cells were not affected by TiO₂ NPs treatment in any group.</p>	<p>Reliability: 2</p> <p>This study used a limited protocol, with only two low concentrations tested. No data on NPs stability provided. No data on internalization provided. The purpose of the study was not to assess TiO₂ genotoxicity, but to evaluate the differential sensitivity to TiO₂ NPs ex vivo in peripheral blood lymphocytes of subject with different respiratory diseases.</p>	<p>Limited</p>	<p>Osman et al., 2018*</p>

	incubation continued until 72h.						
Micronucleus test BEAS-2B normal bronchial lung cells Comet assay (Table 3)*	1, 10 and 20 µg/mL; Exposure: 48 h, 1 and 3 weeks ROS production (DCFH-DA assay); Exposure: 24h and 1 week	TiO ₂ NPs (NM-102), anatase, 21-22 nm	NSC: 1 Dispersion verified according to the Nanogenotox protocol and cellular internalisation confirmed by TEM. Individual particles and some level of agglomeration observed as well as NPs and agglomerates within the cells.	Negative Neither acute nor chronic treatments induced significant increases in the frequency of binucleated cells with micronuclei. No induction of intracellular ROS, evaluated by flow cytometry Reduced transcription of anti-oxidant HO-1 marker at 3 weeks Cellular uptake by TEM: concentration - and time-dependent cellular uptake was observed in BEAS-2B cells. TiO ₂ NPs both as nanoparticles and nano-aggregates were mainly confined to vacuoles, although they were also present on the surface of the nuclear membrane.	Reliability: 1	Hgh	Vales et al., 2015 *
Micronucleus test	10 µg/ml for each TiO ₂ NPs 72h exposure	1) TiO ₂ NPs, anatase, 11-18 nm (TEM) 2) TiO ₂ , anatase, 60-400 nm (TEM),	NSC: 1 Dispersion measured under the exposure conditions. Good	TiO ₂ NPs rutile: Positive. Other test materials: negative	Reliability: 2 A single concentration was tested	Limited	Uboldi et al., 2016*

<p>Balb/c 3T3 (Mouse embryo fibroblasts)</p> <p>Other in vitro assays (Table 4b)*</p>	<p>uptake of TiO₂: 0.5 mg/ml TiO₂ NPs</p>	<p>different geometry (TEM)</p> <p>3) TiO₂NPs, rutile, 10-35 nm (TEM), elongated particles (TEM)</p> <p>4) TiO₂, rutile, 250-600 nm (TEM), different geometry (TEM)</p>	<p>stability confirmed up to 72h for the four materials.</p>	<p>Uptake of TiO₂ (ICP -MS): TiO₂NPs anatase and rutile were internalized more significantly (P < 0.001) compared to their bulk counterparts.</p>			
<p>Micronucleus test</p> <p>Human hepatocytes L-02 (cat# GNHu 6), hepatocellular carcinoma cell QGY (cat# TCHu 43)</p> <p>Other in vitro assays (Table 4a)*</p>	<p>0, 40, 80 µg/mL</p> <p>Time of exposure: 72h</p> <p>Cytochalasin added after the treatment harvesting 26h after treatment</p>	<p>TiO₂NPs, 21 nm (crystalline form unknown)</p>	<p>NSC: 3</p> <p>No information provided on the level of dispersion or stability.</p>	<p>Positive</p> <p>Statistically significant increase of MN, NPB and BUDs in human hepatocyte L-02 cells. No increase in hepatocellular carcinoma cells QGY</p>	<p>Reliability: 2</p> <p>No positive control</p> <p>The results are reported in a graph. No figures and number of repetitions are reported</p>	<p>Limited</p>	<p>Wang et al., 2018 *</p>
<p>Micronucleus test</p> <p>L-929 murine fibroblast cells</p>	<p>15, 30, 60 µg/mL</p> <p>24 hours incubation</p>	<p>TiO₂NPs, rutile, 90 nm (TEM)</p>	<p>NSC: 3</p> <p>No information on dispersion provided, high tendency for agglomeration mentioned.</p>	<p>Negative</p> <p>No cytotoxicity by MTT and CBPI assays</p>	<p>Reliability: 2</p> <p>Lack of information on dispersion and on the uptake</p>	<p>Limited</p>	<p>Pittol et al., 2018</p>

<p>Chromosomal aberration test</p> <p>Human peripheral lymphocytes (human donor)</p> <p>Comet assay (Table 3); other <i>in vitro</i> assays (Table 4a)*</p>	<p>25, 75 and 125 µM (2, 6 and 10 µg/ml) TiO₂ NPs</p> <p>Exp: 24h</p>	<p>TiO₂NPs, anatase/rutile, 21 nm</p>	<p>NSC: 2</p> <p>Dispersion measured under the exposure conditions (FBS used in the medium). Presence of agglomerates confirmed, good stability up to 24h.</p>	<p>Inconclusive</p> <p>Significant concentration dependent increase in the percentage of structural aberration only including gaps</p>	<p>Reliability: 3</p> <p>Insufficient reliability</p> <p>Due to the data analysis and presentation of the results</p>	<p>Low</p>	<p>Patel et al., 2017 *</p>
<p>Micronucleus test</p> <p>Target cells: human-hamster hybrid A_L cells (CHO-K1).</p>	<p>cytokinesis-block micronucleus (CBMN) assay test: 1 or 10 µg/mL for 24 h</p> <p>Intracellular reactive oxygen species (ROS): 1 µg/mL TiO₂ NPs for 24 h</p>	<p>TiO₂NPs, anatase, 15 nm</p>	<p>NSC: 1</p> <p>Level of dispersion measured and reported. Cellular internalisation of NPs and agglomerates confirmed by TEM.</p>	<p>Negative</p> <p>No increase of MN in TiO₂ NPs treated cells compared to untreated controls</p>	<p>Reliability: 5</p> <p>Study designed to investigate the interactions between TiO₂ NPs and As(III). Treatments with TiO₂ NPs alone served as controls. Only low TiO₂ NPs concentrations used and no positive control.</p>	<p>Low</p>	<p>Wang et al. 2017</p>
<p>Micronucleus test</p> <p>Four cell lines: Lung epithelial A549; human glioblastoma A172; human neuroblastoma SH-SY5Y; human hepatocellular</p>	<p>10, 50, 100 and 200 µg/mL</p> <p>3-24 h treatment plus 24 h of culture in fresh medium</p> <p>Micronuclei were evaluated by flow cytometry</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>Cellular Uptake was confirmed by Flow Cytometry</p>	<p>Negative</p> <p>TiO₂ NPs were internalized by all cell lines with concentration- and time-dependent trend.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Brandao et al., 2020</p>

carcinoma HepG2 cells	Positive control: MMC						
Micronucleus test Human bronchial epithelial BEAS-2B cells Comet assay (Table 3)*	10 and 100 µg/ml for 48 hours; cytochalasin B was added 6h after beginning of treatment Positive control: MMC MN scored in 2000 binucleated cells per concentration in two independent experiments	TiO ₂ (NM-100), anatase, 50-150 nm	NSC: 1 NANOGENOTOX project dispersion protocol and confirmation of cellular internalisation	Negative NM-100 failed to induce a significant increase of micronuclei at any tested concentration. Viability and cell replication, as measured by Proliferation Index, did not decrease after treatment at any tested concentration.	Reliability: 1	High	Zijno et al. 2020*
Micronucleus test in the colon cancer cell line HT29-MTX-E12 Comet assay (Table 3)*	0.5, 5 and 50 µg/ml MN scoring by flow cytometry	1) E171, anatase, 170 nm 2) TiO ₂ NPs, anatase, < 25 nm	NSC: 2 The protocol intentionally accepted, large agglomeration. No information provided on agglomeration in the exposure media.	Negative No MN induction was observed upon engineered nanomaterials exposure under the experimental conditions chosen with both E171 and TiO ₂ NPs E171 had a strong impact on the flow cytometry MN detection, reducing MN incidence by more than	Reliability: 3 The flow cytometry approach applied is considered not sufficiently reliable for TiO ₂ (nano)particles	Low	Franz et al., 2020*

				<p>90% when present in the sample analysed.</p> <p>A lower interference, which according to the study authors did not interfere with MN quantification, was observed with TiO₂ NPs.</p> <p>No increase of hypodiploid cells, proposed by the study authors as a marker of aneugenicity, was observed after treatment with E171 and TiO₂ NPs</p>			
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BI: blastogenic index; BSA: bovine serum albumin; CBPI: cytokinesis-block proliferation index; CytoB: cytochalasin B; DCFH-DA or DCFDA: 2', 7'-dichlorofluorescein diacetate; FBS: Fetal Bovine Serum; FISH: Fluorescence *In Situ* Hybridisation; GSH: reduced glutathione; MI: mitotic index; MNBN: micronucleated binucleated (cells); MPs: microparticles; NPBs: nucleoplasmic bridges; NBUDs: nuclear buds; NPs: nanoparticles; PBL: Peripheral blood lymphocytes; PBMC: peripheral blood mononuclear cells;

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 authors_year
Comet assay Human colon carcinoma	0.143 µg/cm ² (corresponding to 1µg/mL) for 24h	E171 (39% nano)	NSC: 2 Dispersion and stability measured, some level of agglomeration confirmed.	Positive Statistically significant increase (median comet tail and intensity)	Reliability: 2 single concentration	Limited	Proquin et al. 2017*

Caco-2 cells	<p>Positive control: H₂O₂</p> <p>4 replicates, duplicate experiments</p> <p>50 cells analysed/slide/experiment</p>		<p>Agglomeration specifically relevant at high concentrations, but reduced by the use of BSA or FBS.</p>	<p>Selection of concentrations with viability (Trypan blue) > 80%</p> <p>Decreased viability: 27% at 14.3 µg/cm² and 73% at 143 µg/cm²</p> <p>ROS: no significant increase at 0.143 or 1.43 µg/cm²</p>			
<p>Comet assay</p> <p>Human colon carcinoma</p> <p>Caco-2 cells</p>	<p>0.143, 1.43 µg/cm² (corresponding to 1 µg/mL, 10 µg/mL) for 24h</p> <p>Positive control: H₂O₂</p> <p>4 replicates, duplicate experiments</p> <p>50 cells analysed/slide/experiment</p>	TiO ₂ NPs, anatase, 10-30 nm (SEM)		<p>Positive</p> <p>Statistically significant increase (median comet tail and intensity)</p> <p>Decreased viability: 48.4% at 143 µg/cm²</p> <p>ROS: no significant increase at 0.143 or 1.43 µg/cm²</p>	<p>Reliability: 1</p> <p>only 2 concentrations</p>	High	
<p>Comet assay + ROS measurement</p> <p>Human colon carcinoma</p>	<p>0.143 µg/cm² (corresponding to 1µg/mL) for 24h</p> <p>Positive control: H₂O₂</p>	TiO ₂ , 535 nm, > 100 nm (SEM) (crystalline form unknown)		<p>Positive</p> <p>Statistically significant increase (median comet tail and intensity)</p> <p>Decreased viability: 33% at 143 µg/cm²</p>	<p>Reliability: 2</p> <p>single concentration</p>	Limited	

Caco-2 cells	4 replicates, duplicate experiments 50 cells analysed/slide/experiment			ROS: significant. increase at 1.43 $\mu\text{g}/\text{cm}^2$			
Comet assay Human peripheral blood mononuclear cells (PBMCs) (2 donors)	0, 10, 50, 100, 200 $\mu\text{g}/\text{mL}$ for 24h Positive control: H2O2 Viability (Trypan blue) 2 experiments Duplicate slides/concentration Analysis of 100 cells/concentration Steady-state levels of DNA 8-oxodG: by HPLC:ECD	TiO ₂ NPs, anatase, 20-60 (TEM)	NSC: 1 for the five materials. Dispersion with sufficient energy and stability confirmed in the medium, presence of particles and agglomerates confirmed.	Positive Statistically significant increase SSB (% DNA in tail) for NPs from 50 $\mu\text{g}/\text{mL}$ for anatase. No cytotoxicity observed. Significant increases of DNA 8-oxodG at 100 $\mu\text{g}/\text{mL}$ after 6h and 24h	Reliability: 1	High	Andreoli et al., 2018* Identification of a subpopulation of cells more sensitive to DNA damage (monocytes)
		TiO ₂ NPs, rutile: rod-like, 30-100 (TEM)		Positive Statistically significant increase in SSB for rutile NPs from 100 $\mu\text{g}/\text{mL}$. No cytotoxicity observed. Significant increases of DNA 8-oxodG at 100 $\mu\text{g}/\text{mL}$ after 6h and 24h	Reliability: 1	High	
		TiO ₂ , anatase/rutile, with 3 morphologies (spheres, irregular and rod-like), 45-262 nm (TEM)		Positive Statistically significant increase in SSB for mixture NP from 50 $\mu\text{g}/\text{mL}$. No cytotoxicity observed. Significant increases of DNA 8-oxodG at 100 $\mu\text{g}/\text{mL}$ after 6h and 24h	Reliability: 1	High	
		TiO ₂ , anatase, 50-270 nm (TEM),		Positive Statistically significant increase in SSB for MP	Reliability: 1	High	

		morphology by TEM different from the NPs		from 200 µg/mL for anatase No cytotoxicity observed. Significant increases of DNA 8-oxodG at 100 µg/ml after 6h and 24h			
		TiO ₂ , rutile, 50-3000 nm (TEM), morphology by TEM different from the NPs		Positive Significant increase in SSB for rutile MP from 200 µg/mL. No cytotoxicity observed. Significant increases of DNA 8-oxodG at 100 µg/ml after 6h and 24h	Reliability: 1	High	
Comet assay +/- Fpg and Endo III Human colon carcinoma Caco-2 cells Oxidative stress (OGG1 expression)	1, 2.5 µg/cm ² (corresponding to 6.4 and 16.0 µg/mL) 2, 4, 6 and 24 hours Positive control: H ₂ O ₂ Both concentrations were sub-toxic (data not shown) Steady-state levels of DNA 8-oxodG measured by HPLC/ECD OGG1 expression determined by western blot: 2.5 µg/cm ² NPs	TiO ₂ NPs, anatase, 20-60 nm (TEM)	NSC: 2 Dispersion and stability measured and some level of agglomeration confirmed.	Positive In few experimental points with no concentration or time related effects. 3-fold increase in SSBs after 2h exposure to 2.5 µg/cm ² with Fpg. 2-fold increase in SSBs after 24h exposure to 1 µg/cm ² with Endo III. Significant increase in basal levels of DNA 8-oxodG compared to control at 6 (mainly) and 24 h of treatment. Increase comparable to oxidation level produced by the positive control. Statistically significant	Reliability: 2 Only two concentrations Cytotoxicity data not provided	Limited (more a mechanistic study)	Zijno et al. 2015*

	(corresponding to 16.0 µg/ml) for 6 h			over-expression of OGG1 protein.			
Comet assay Human dermal microvascular endothelial	10 mg/L NPs for 24 h Cytotoxicity: Lactate dehydrogenase (LDH) release measurement	1) TiO ₂ NPs, rutile, 1-3 nm,	NSC: 1 Cellular internalisation measured, agglomeration well-considered and accounted in the results, different levels	Positive Statistically significant increase in OTM with both TiO ₂ NPs. Cytotoxicity: 13% at 40 mg/mL	Reliability: 3 High level of damage in negative control	Low	Bayat et al.,2015

<p>cell line (HMEC-1)</p> <p>+ ROS measurement</p>	<p>Measurements of Olive tail moment (OTM)</p>	<p>2) TiO₂NPs, anatase/rutile, 30 nm</p>	<p>mentioned for the different types of NPs.</p>	<p>ROS:</p> <p>TiO₂-NPs anatase/rutile (30 nm) produced some ROS in the cell culture media.</p> <p>TiO₂-NPs rutile (1-3 nm) did not have significant oxidative capability</p>	<p>cells (50%).</p> <p>Single concentration used.</p> <p>Insufficient description of experimental details.</p>		
<p>Comet assay</p> <p>Human peripheral blood lymphocytes (whole blood cells)</p> <p>(3 healthy donors)</p>	<p>0, 50, 100 and 150 mM (0, 3995, 7990, and 11985 µg/mL)</p> <p>Exposure: 1h</p> <p>Negative control: phosphate buffer saline (PBS) (solvent)</p> <p>100 nuclei /concentration</p> <p>Visual scoring of comet shaped nucleoids</p> <p>Mean number of cells with DNA damage</p>	<p>TiO₂NPs, anatase, 45 angström (4.5 nm),</p>	<p>NSC: 4</p> <p>No information provided on dispersion and high concentrations administered.</p>	<p>Inconclusive</p> <p>No statistically significant increase of DNA damage compared to PBS.</p>	<p>Reliability: 3</p> <p>Inappropriate study design.</p> <p>Exposure only for 1h.</p> <p>No positive control used.</p> <p>Type of scoring of low sensitivity.</p>	<p>Low</p>	<p>Bajic et al., 2017</p>

					No information provided on cytotoxicity, and on uptake. High concentrations administered.		
Comet assay BEAS-2B normal human bronchial lung cells A549 human alveolar carcinoma lung cells + 8-oxo-dGuo and 5-methyl deoxycytosine (5-Me-dC)	0, 10 and 100 µg/mL of TiO ₂ NPs Exposure: 4, 24 or 48 h FBS free culture medium Measurements of comet tail intensity Negative control: FBS free culture medium Cytotoxicity: MTT assay (50, 100, 150 and 200 µg/mL)	TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 2 Dispersion and stability evaluated before and under the exposure conditions. Hydrodynamic diameter shifted to 720 ± 20 nm and the PDI to 0.50 ± 0.02, suggesting agglomeration, particularly after 48h of exposure. 48 h after dilution in culture medium hydrodynamic diameter >1 µm by DLS.	Positive Induction of SSB and/or alkali labile sites at 100 µg/mL at all time points in both cell lines. After 4h: significantly higher levels of DNA damage in A549 cells than in BEAS-2B. After 48h: more damage in BEAS-2B cells than in A549 cells. Increase of 8-oxo-dGuo lesions at 100 µg/mL at all time points in both cell lines.	Reliability: 2 No positive control used. No cytotoxicity observed after 4h.	Limited	Biola-Clier et al., 2017

<p>quantification + analysis of DNA repair gene expression + DNA-methylation</p>	<p>Quantification by HPLC-MS/MS By RT-qPCR</p>			<p>Cytotoxicity: observed only after 24 or 48h, never at 4h. More than 70% of viable cells after 48 h indicating moderate cytotoxicity.</p> <p>DNA repair:</p> <p>A549 cells: slight increase in DNA repair activity at 100 µg/mL after 24h (base excision repair mechanisms and nucleotide excision repair), followed by a considerable reduction in DNA repair activity at 48h post-exposure.</p> <p>BEAS-2B cells: at 100 µg/mL (3 time points) considerable reduction in cells ability to repair DNA lesions.</p>			
<p>Comet assay Human peripheral blood lymphocytes (1 donor)</p>	<p>25, 75 and 125 µM TiO₂ NPs (2, 6, 10 µg/mL) Exposure: 24h Negative control: untreated cells Positive control: mitomycin C (MMC)</p>	<p>TiO₂NPs, anatase/rutile, 21 nm</p>	<p>NSC: 2</p> <p>Dispersion measured under the exposure conditions (FBS used in the medium). Presence of agglomerates confirmed, good stability up to 24h.</p>	<p>Positive</p> <p>Statistically significant increase in % Tail DNA and Olive Tail Moment at 75, and 125 µM</p> <p>Statistically significant increase % Tail DNA only at 25 µM</p>	<p>Reliability: 3 Cells only from a single donor were analysed</p>	<p>Low</p>	<p>Patel et al., 2017*</p>

	<p>Measurement of Olive tail moment (μm) and % of DNA in the tail</p> <p>50 cells analysed/concentration</p>				<p>No information on cytotoxicity provided</p> <p>Protocol insufficiently described</p> <p>Only 50 cells/concentration were analysed</p> <p>MMC not relevant for comet assay as a positive control substance</p>		
<p>Comet assay</p> <p>human lung carcinoma</p>	<p>10, 20 and 40 $\mu\text{g}/\text{cm}^2 = 32, 64$ and 128 $\mu\text{g}/\text{mL}$</p> <p>Exposure: 48 h</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm.</p>	<p>NSC: 1</p> <p>Dispersion is reported and cellular uptake confirmed by electron microscopy. A level of</p>	<p>Positive</p> <p>statistically significant and concentration-related increase of % tail DNA. Fpg: positive at 128 $\mu\text{g}/\text{mL}$.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Stocco et al., 2017*</p>

<p>cell line (A549)</p> <p>+/- Fpg</p> <p>and Endo III</p>	<p>Trevigen protocol used; Measurement of % of DNA in the tail</p> <p>Negative control: untreated cells</p> <p>Positive control: H2O2</p> <p>100 cells analysed/concentration in 2 experiments.</p> <p>Cytotoxicity: colony forming efficiency (CFE): (1.25-80 µg/cm² = 8.3-266.6 µg/mL, 24, 48, 72 h exposure)</p>		<p>agglomeration is observed and also the internalisation of agglomerates in the exposed cells.</p>	<p>Endo III: positive at all concentrations.</p> <p>Weak but statistically significant decrease in cell viability (highest decrease %CFE around 60% after 24h with 80 µg/cm² TiO₂NPs).</p>			
<p>Comet assay</p> <p>Human peripheral blood mononuclear cells PBMC</p> <p>Healthy individuals (HI); Polyposis</p>	<p>10, 40 and 80 µg/mL</p> <p>Exposure: 30 min.</p> <p>Negative control: untreated cells</p> <p>Positive control: H2O2</p> <p>Measurement of olive tail moment</p>	<p>TiO₂NPs, anatase, 34 nm (20-64 nm) (SEM)</p>	<p>NSC: 2</p> <p>Dispersion measured and agglomeration confirmed, increasing with concentration and time.</p>	<p>Positive</p> <p>HI: non statistically significant concentration dependent induction of DNA damage for OTM and increase in % tail DNA.</p> <p>PLP: statistically significant increase of DNA damage</p>	<p>Reliability: 2</p> <p>Short term exposure: 30 min.</p>	<p>Limited</p> <p>Relevance of the increased sensitivity of the diseased patients is not certain, but interesting information.</p>	<p>Kurzawa-Zegota et al., 2017*</p>

<p>coli patients (PLP); Colon cancer patients (CRC)</p> <p>Several donors (20 samples/donors/patients)</p>	<p>(OTM) and % tail DNA</p> <p>Viability assay (trypan blue): generally >92%, but always >80%</p> <p>2 replicate slides/concentration</p>			<p>for OTM and increase in % Tail DNA at 80 µg/mL.</p> <p>CRC: concentration dependent induction of DNA damage for OTM and % Tail DNA.</p> <p>CRC showed increased sensitivity to the treatment compared to HI and PLP cells.</p>			
<p>Comet assay</p> <p>+/-Fpg test</p> <p>Human colon adenocarcinoma Caco-2 cell line;</p> <p>Human hepatoblastoma HepG2 cell line;</p> <p>mES cells (primary mouse</p>	<p>0; 0.98; 1.95; 3.9; 7.8; 15.6 µg/cm²</p> <p>(corresponding to 3.13, 6.25, 12.5, 25, 50 µg/ml)</p> <p>Exposure: 4 h</p> <p>Negative control: untreated cells</p> <p>Positive controls: H₂O₂ and KBrO₃</p> <p>Cytotoxicity: WST-1 (cell proliferation reagent), 24h exposure, expressed as LC20 (concentration of particles which</p>	<p>E 171, anatase (0.2% rutile), 390 nm (DLS)</p>	<p>NSC: 2</p> <p>Dispersion and stability measured in the exposure media. Some level of agglomeration confirmed. Good stability up to 24h confirmed for the high concentration (50 µg/mL, equivalent to 15.6 µg/cm²).</p>	<p>Positive</p> <p>Positive for mouse stem cells for the highest concentration.</p> <p>For HepG2 and Caco-2: statistical significance - linear regression (both cell lines) and ANOVA on pooled exposure groups (HepG2).</p> <p>+Fpg:</p> <p>Positive: statistical significant increase in Fpg sensitive sites in HepG2 cell line</p> <p>Negative in Caco-2 and mouse stem cell lines</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Brown et al., 2019*</p>

<p>embryonic fibroblasts</p> <p>+ Measure of depletion of intracellular GSH</p> <p>+ ToxTracker assay (see table 4a - other in vitro tests)</p>	<p>caused the death of 20% of the cells)</p> <p>Measurement of % tail DNA and fpg sensitive sites</p> <p>2 experiments with 3 independent replications</p> <p>Depletion of intracellular GSH: 24h exposure</p>			<p>Slight cytotoxicity: HepG2 = 15.6 µg/cm²; Caco-2 cells = 31.25 µg/cm²</p> <p>Measurement of total glutathione in HepG2 and Caco-2 cells: concentration dependent decrease of total GSH in both cell types (more pronounced in HepG2).</p>			
<p>Comet assay</p> <p>THP-1 cell line (human monocytic leukemia cells)</p> <p>+/- Fpg test</p> <p>+ ROS measurements</p>	<p>1, 10 and 25 µg/mL</p> <p>Exposure: 24h</p> <p>Cytotoxicity measured by MTS assay</p> <p>Measurement of %DNA in the tail and sites sensitive to Fpg</p> <p>Negative control: untreated cells</p> <p>Positive control: CdSO₄</p>	<p>TiO₂ (NM-100), anatase, 50-150 nm</p>	<p>NSC:1</p> <p>Dispersion and stability measured in the exposure media for all the duration of the assay according to the Nanogenotox protocol. The presence of small and stable agglomerates confirmed. Cellular internalisation measured, presence of agglomerates observed in endosomes.</p>	<p>Equivocal</p> <p>Weak induction of DNA damage (non-significant concentration-dependent increase of DNA strand breaks).</p> <p>+Fpg: Negative</p> <p>No significant effect on formation of Fpg-sensitive sites.</p> <p>No effect on cell viability observed up to 100 µg/mL.</p> <p>Weak effects on ROS levels, no significant increase even after 24h incubation</p>	<p>Reliability: 1</p>	<p>Limited</p>	<p>Brzicova et al., 2019</p>

	Evaluation of uptake						
Comet assay +/- Fpg Human peripheral blood mononuclear cells (PBMCs) from 13 donors	<p>3, 15 and 75 µg/cm² corresponding to 6.75, 33.75, 168.75 µg/mL)</p> <p>Exposure: 4h or 24h</p> <p>Negative control: untreated cells</p> <p>Positive control: H₂O₂</p> <p>Measure of % DNA in the tail and Fpg sensitive sites</p> <p>Duplicate slides/sample</p> <p>100 cells analysed/sample</p>	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	<p>NSC: 1</p> <p>Two dispersion protocols (DP1 and DP2) used for comet assay. The intention was to assess the effect of agglomeration.</p> <p>Dispersion protocol 1 (DP1) ==> NSC:1</p> <p>Although only incomplete information on dispersion is presented, specific confirmation of sufficient level of dispersion (Bimodal distribution, 102 (±15) nm and 285 (±67) nm and of the stability of the dispersion (Size stability Stable ~ 2 days) is presented using DLS.</p> <p>(Dispersion protocol 1 (DP1) was developed in the NanoTEST project)</p> <p>Dispersion protocol 2 (DP2) is specifically</p>	<p>Positive in 9 out of 13 donors using DP1</p> <p>DP1 pooled analysis: significant increases in DNA strand breaks at 75 µg/cm² after 4 h exposure and at 15 and 75 µg/cm² after 24 h exposure. No increase in Fpg sensitive sites after 4 or 24 h exposure.</p> <p>DP2: no significant increase in DNA damage observed.</p>	Reliability: 1	High	Kazimirova et al., 2019*,**

			<p>designed for getting a level of agglomeration</p> <p>DLS demonstrated agglomeration (779 ±382 nm) of the dispersion, and the dispersion was described as not stable (" agglomerated").</p>				
<p>Comet assay</p> <p>Human hepatoblastoma HepG2 cell line, not pre-treated and pre-treated for 16 h with tert-butylhydroquinone (tBHQ cells)</p> <p>+ Nrf2(-/-) knock-out HepG2 cells</p>	<p>Wild type cell line: 0.1, 1 and 10 µg/mL</p> <p>Knock out cell line: 1 µg/mL</p> <p>Exposure: 24h or 6-24h</p> <p>Triplicate experiments</p> <p>100 cells analysed/sample</p> <p>Calculation of Olive tail moment (OTM)</p> <p>Positive control: no</p> <p>Viability test: details not reported</p>	<p>TiO₂NPs, anatase, 10-25 nm (SEM, TEM)</p>	<p>NSC: 2</p> <p>Dispersion and zeta potential measured although not under the exposure conditions, the high absolute zeta potential and the use of very low concentrations suggest that a good level of dispersion is expected even if not directly measured.</p>	<p>Positive</p> <p>Comet assay:</p> <p>Increase in OTM in HepG2 cells at 1 µg/mL in function of exposure time (6-24h).</p> <p>Increases of 3.4 fold of OTM in Nrf2 (-/-) HepG2 cells, 1.1 fold in tBHQ-HepG2 cells after exposure to 1 µg/mL.</p> <p>ROS production in HepG2 cells: concentration and time dependent increase.</p> <p>Malonaldehyde (MDA) content: statistically significant and concentration-related increase.</p> <p>superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)</p>	<p>Reliability: 2</p> <p>OTM used for assessment</p> <p>Insufficient experimental details</p> <p>No positive control</p>	<p>Limited</p>	<p>Shi et al., 2015**</p>

+ ROS production				activities: statistically significant and concentration-related decrease. Viability: not more than 10% reduction after 24h (information included but details not shown).			
Comet assay +/- Fpg PMBC and lymphoblastoid TK6 cells (blood cells) human cerebral endothelial cells (HCEC) (vascular/central nervous system) rat hepatocytes and	0.12, 0.6, 3, 15 and 75 µg/cm ² , which correspond to 0.57, 2.9, 14.4, 72.0 and 360.2 µg/mL Exposure: 30 min, for 1, 2, 4 h and for 24 h Only 4h for Kupffer cells. Negative control: untreated cells and Endorem® Positive control: H ₂ O ₂ and for liver cells: 7000 µW/cm ² UV radiation Scoring of 50 comets/slide (100 comets/sample)	TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC:2 Dispersion considered with two dispersion protocols (Vedisp and UPdisp) with different levels of dispersion from the EU project NanoTest. Additional information extracted from Magdolenova et al., 2012 (DOI: 10.1039/c2em10746e) Vedisp dispersion with lower level of agglomeration, confirmed stability and a bimodal size distribution with one peak at around 100 nm and a second below 300 nm. Dose-dependent effects were observed.	Positive Vedisp: TK6, lymphocytes, BeWo, Cos-1 and Kupffer cells UPdisp: 16HBE14o, HCEC, TK6, Cos-1, HEK293 and hepatocytes Induction of DNA damage is associated with NP type, time of exposure and concentration. Weak evidence of positive concentration-response association. No significant differences in concentration-response associations among cell lines. Induction of significant level of SSBs in absence of cytotoxicity with TiO ₂ NPs Vedisp.	Reliability: 1 The results were not reported in details in this publication but they can be verified in other publications.	High	Cowie et al., 2015

<p>Kupffer cells (liver)</p> <p>monkey Cos-1 and human HEK293 cells (kidney)</p> <p>human bronchial 16HBE14o cells (lung)</p> <p>human BeWo b30 (placenta)</p>	<p>Measurement of % DNA tail + Fpg sensitive sites</p> <p>Visual scoring for comets from BeWo b30 and HCEC cells (score attributed)</p> <p>Cytotoxicity:</p> <ul style="list-style-type: none"> - TK6 cells and Cos-1: relative growth activity and trypan blue exclusion test (TBE) - lymphocytes: TBE - Kupffer cells, hepatocytes and HCEC: MTT assay - 16HBE14o: WST-1 assay - BeWo b30 cells: WST-1 assay and TBE 		<p>UPdisp corresponds to dispersion with large agglomerates, mean value around 700 nm.</p>				
<p>Comet assay</p> <p>Caco-2 human colon carcinoma cells</p>	<p>50 µg/mL</p> <p>Exposure: 6, 24 or 48 h</p> <p>Measurement of % tail DNA</p>	<p>1) TiO₂NPs, anatase/rutile (95/5%), 12±3 nm (TEM) [A12]</p> <p>2) TiO₂NPs rutile/anatase</p>	<p>NSC: 1</p> <p>Dispersion and stability measured under the exposure conditions. Presence of unstable agglomerates confirmed, especially</p>	<p>Negative</p> <p>No induction of SB (strand breaks) or alkali labile sites</p> <p>No overt cell cytotoxicity.</p>	<p>Reliability: 3</p> <p>Only one concentration</p>	<p>Low</p>	<p>Dorier et al., 2015</p>

	<p>Negative control: untreated cells</p> <p>Positive control: no</p> <p>Cell viability: MTT assay (0-200 µg/mL) after 24h exposure</p>	(90/10%), 22±4 (TEM) [R20]	<p>for R20. Cellular uptake measured.</p> <p>R20 high agglomeration in water and exposure medium >1000 nm, PdI >0.8</p>		<p>was used.</p> <p>No positive control was used.</p> <p>R20 high agglomeration was present what influenced the reliability of the results.</p>		
<p>Comet assay</p> <p>+/- Fpg</p> <p>Caco-2 human colon carcinoma cells</p> <p>Co-culture of Caco-2 with HT29-MTX</p>	<p>Exposures:</p> <ul style="list-style-type: none"> - acute: 6, and 48h, 10 or 50 µg/mL - chronic (repeated): 3 weeks (twice/week), 10 or 50 µg/mL <p>Negative control: untreated cells</p> <p>Positive control: H2O2 Verification of</p>	<p>1) E171, >95% anatase, 118±53 nm), % of NPs 30-55% (5 batches analysed)</p> <p>2) TiO₂NPs, anatase, 12±3 nm (TEM) [A12]</p> <p>3)TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>Dispersion and stability measured under the exposure conditions. Possible presence of small agglomerates but good stability confirmed up to 48h. Cellular uptake measured.</p>	<p>Acute exposure: Negative</p> <p>Caco-2 or Caco-2-HT29-MTX exposed to A12 or E171: no significant DNA damage with or without Fpg</p> <p>Repeated exposure:</p> <p>Positive :</p> <p>Caco-2 exposed to E171: significant increase in Fpg sensitive sites.</p>	<p>Reliability: 3</p> <p>Results are insufficiently reported .</p> <p>%tail DNA was expressed as</p>	Low	Dorier et al., 2017

<p>mucus-secreting cells</p> <p>Quantification of ROS (CAT, GSR, SOD1 and 2)</p> <p>Gene expression</p>	<p>test system for Fpg: A549 cells exposed to riboflavin +UVA</p> <p>3 independent replicates</p> <p>Measurement of % tail DNA</p> <p>Cytotoxicity:</p> <ul style="list-style-type: none"> - Caco-2 cells: WST-1 assay, exposure to 0-200 µg/mL for 6 or 48h - co-culture Caco-2/HT29-MTX: propidium iodide exclusion <p>Evaluation of uptake</p>			<p>Caco-2/HT29-MTX cells exposed to E171: significant increase in Fpg sensitive sites at 50 µg/mL</p> <p>Negative: No increase in strand breaks for both mono and co-culture.</p> <p>In Caco-2 cells exposed to an acute concentration of E171 for 48 h, intracellular ROS content was significantly higher at all concentrations tested. A similar increase was observed in Caco-2/HT29-MTX cells at all three time-points (6 h, 24 h, 48 h, acute exposure), and ROS levels were found to increase in a concentration-dependent manner.</p> <p>In both cell models, intracellular ROS levels were higher in repeatedly-exposed cells than in untreated cells.</p> <p>Cells repeatedly exposed to E171 did not display higher ROS levels than acutely-exposed cells. TiO₂-NPs also caused intracellular accumulation of ROS,</p>	<p>fold-change compared to %tail DNA in control cell. No absolute values reported in the exposure groups and the spontaneous damage cannot be assessed.</p>		
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				<p>although to a lesser extent than E171.</p> <p>No effect on cell viability was observed.</p>			
<p>Comet assay</p> <p>+/- Fpg</p> <p>Caco-2 colon carcinoma cells/HT29 MTX co-culture.</p> <p>Other assays: ROS, 8-oxo-dGuo (HPLC, MS/MS), gene expression (DNA repair etc.), DSB and 53BP1 foci count</p>	<p>50 µg/mL</p> <p>Exposure: 24h (comet)</p> <p>Negative control: untreated cells</p> <p>Positive controls: MMS for SSBs</p> <p>Verification of test system for Fpg: A549 cells exposed to riboflavin +UVA</p> <p>Measure of % tail DNA</p> <p>8-oxo-dGuo level by HPLC/MS-MS.</p> <p>Viability: MTT assay 6 and 48h exposure with several concentrations, MTT interference checked after 24 h exposure at 50 µg/mL</p>	<p>1) E171, 118±53 nm</p> <p>2) TiO₂NPs, anatase, 12±3 nm [A12]</p> <p>3) TiO₂NPs (NM-105), anatase/rutile, 15-24 nm</p>	<p>NSC: 2</p> <p>Dispersion measured under the exposure conditions. The level of dispersion but not stability was demonstrated using DLS.</p>	<p>Negative</p> <p>No SSBs and no Fpg sensitive sites detected at the only one concentration tested.</p> <p>No effect on viability up to 200 µg/mL.</p> <p>No significant increase in DSB detected via 53BP1 foci immunostaining.</p> <p>No significant increase of the 8-oxodGuo level.</p> <p>Significant increases in ROS levels.</p>	<p>Reliability: 2</p> <p>Only one test concentration used.</p>	<p>Limited</p>	<p>Dorier et al., 2019</p>

<p>Comet assay</p> <p>-/+ Fpg</p> <p>A549 (human alveolar carcinoma lung cells)</p> <p>TK6 (human lymphoblastoid cell line)</p>	<p>0.01 to 75 µg/cm² (A549)</p> <p>(corresponding to 0.14–140 µg /mL)</p> <p>0.14 to 140 µg /mL (TK6)</p> <p>Exposure for 3 or 24 h (comet)</p> <p>Measurement of % DNA in tail</p> <p>Positive control: H₂O₂ (SBs) Ro19-8022 plus visible light (Fpg sensitive sites)</p> <p>Cytotoxicity: alamar Blue</p> <p>Viability: colony forming efficiency (9-12 days)</p> <p>Negative control: untreated cells</p>	<p>TiO₂ (NM-100), anatase, 50-150 nm</p>	<p>NSC: 1</p> <p>Dispersion and stability considered according to the Nanogenotox protocol and measured in the culture media (BSA used in the suspension). Good dispersion and stability confirmed up to 24h.</p>	<p>Positive</p> <p>Concentration-dependent DNA damage, both SBs and Fpg sensitive sites, at 3 h; decreased at 24 h. More damage in A549 cells than in TK6 cells.</p> <p>Cytotoxicity:</p> <ul style="list-style-type: none"> - A549 cells: no effect on cell viability - TK6: slight decrease in viability after 3h, reversed after 24h. 	<p>Reliability: 1</p>	<p>High</p>	<p>El Yamani et al., 2017</p>
<p>Comet assay, HeLa cells</p>	<p>50 and 200 µg/mL</p> <p>Exposure: 48h</p>	<p>TiO₂NPs, 20.3±2.1 nm (TEM) (crystalline form unknown)</p>	<p>NSC: 2</p> <p>Insufficient information on dispersion and stability but test</p>	<p>Positive</p> <p>Increase in DNA SBs at 50 and 200 µg/mL.</p>	<p>Reliability: 2</p> <p>No experim</p>	<p>Limited</p>	<p>Ferraro et al., 2016</p>

	<p>Whole cells compared with isolated nuclei.</p> <p>Cytotoxicity assay – MTT</p> <p>Intracellular uptake</p> <p>Visual scoring</p> <p>Positive control: H₂O₂</p>		<p>design adequate for poorly soluble material. Cellular internalisation of NPs measured but images not reported.</p>	<p>No cytotoxicity at these concentrations observed.</p>	<p>ental details given.</p> <p>only 2 concentrations</p>		
<p>Comet assay</p> <p>+/- Fpg</p> <p>Caco-2 (Colorectal adenocarcinoma cells)/ HT29 co-culture</p>	<p>0, 12.5, 50, 150, and 350 µg/mL</p> <p>Exposure: 24 and 48h</p> <p>Positive controls: MMS and KBrO₃</p> <p>Viability (proliferation assay)</p>	<p>1) TiO₂NPs, anatase, 70-80 nm (TEM)</p> <p>2) TiO₂NPs, rutile, rods, 40-70 nm (TEM)</p>	<p>NSC:1</p> <p>Dispersion measured in the cell culture medium (BSA used in the suspension) according to Nanogenotox protocol. Presence of agglomerates confirmed, good stability measured up to 48h. Cellular uptake and translocation measured.</p>	<p>Positive</p> <p>Viability >80% at 24 h, lower at 48 h but still >70%.</p> <p>Statistically significant concentration -dependent increase in DNA SBs at 24h, lower at 48h for anatase.</p> <p>Statistically significant increase at 12.5, 50 and 150 µg/mL at 24h and at all concentrations at 48h for rutile.</p> <p>No significant increase in Fpg sensitive sites observed for both test materials.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Garcia-Rodriguez et al., 2018</p>

<p>Comet assay</p> <p>PBMCs from a single donor</p> <p>Measurements of:</p> <ul style="list-style-type: none"> - antioxidant enzymes - ROS - oxidative stress markers 	<p>50, 100, 250 and 500 ppm (50, 100, 250 and 500 µg/mL)</p> <p>Exposure: 1h</p> <p>Cytotoxicity test - erythrocytes (haemolysis)</p> <p>Measure of tail length</p> <p>Positive control: none</p>	<p>TiO₂NPs, anatase/rutile, 17.8 nm (XRD), 46-60 nm (TEM)</p>	<p>NSC: 3</p> <p>Insufficient information provided on dispersion.</p>	<p>Positive</p> <p>Haemolysis: concentration-dependent from 50 ppm (52.5% at 250 ppm).</p> <p>Concentration-dependent increase in DNA damage from 50 ppm.</p> <p>DNA damage seen at cytotoxic concentrations.</p> <p>Oxidative stress markers: concentration-dependent increase in catalase (CAT), and SOD, Lipid peroxidation (LPO): concentration-dependent increase in MDA</p> <p>GST and GSH: concentration-related decreased activity</p> <p>ROS generation: TiO₂ NPs generate the three ROS: superoxide radicals, hydroxyl radicals and hydrogen peroxide.</p>	<p>Reliability: 3</p> <p>Limited experimental details.</p> <p>Inappropriate design:</p> <ul style="list-style-type: none"> - only one exposure time, too short - no positive control. 	<p>Low</p>	<p>Khan et al., 2015</p>
<p>Comet assay</p> <p>+/- Fpg</p> <p>HT-29 (human colorectal)</p>	<p>0, 2, 4, 6, 8, 10 µg/mL</p> <p>Exposure: 24h</p> <p>Cellular uptake: 24h</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>A protocol for dispersion is applied and cellular uptake quantified for all concentrations (ICP-MS). The highest</p>	<p>Positive</p> <p>Cytotoxicity: no effect in either trypan blue or MTT assay.</p>	<p>Reliability: 1</p> <p>H₂O₂ as a positive control not</p>	<p>High</p>	<p>Schneider et al., 2017</p>

adenocarcinoma cells)	<p>Cytotoxicity: trypan blue and MTT</p> <p>Positive control: H₂O₂</p>		<p>concentrations seem close to the plateau for cellular uptake.</p>	<p>No statistically significant increase in DNA SBs at 8 and 10 µg/mL.</p> <p>Increase in DNA SBs in presence of fpg at 8 and 10 µg/mL.</p> <p>Cellular uptake: significant increase of metal content.</p> <p>Apoptotic potential: significant increases in % early and late apoptotic cells observed at all concentrations at similar levels.</p>	<p>appropriate for assessment of Fpg sensitive sites.</p>		
<p>Comet assay</p> <p>NIH/3T3 mouse fibroblast cells</p> <p>SVK14 human keratinocytes</p> <p>BJ human fibroblasts (foreskin)</p> <p>ROS production</p>	<p>TiO₂ NPs at IC₅₀: 508.6-5659.8 µg/mL</p> <p>Exposure: 1h (ROS), 6 h (comet assay, mitochondrial membrane potential change, cell cycle determination)</p> <p>24/48 h (MTT, Raman spectroscopy)</p> <p>Viability (MTT)</p> <p>3 separate experiments</p>	<p>1) TiO₂NPs, 28 nm (crystalline form unknown)</p> <p>2) TiO₂NPs ("Nanorutil"), 128 nm</p>	<p>NSC: 1 for both materials.</p> <p>Particle characterisation and internalisation in cells confirmed by Raman spectroscopy microscopy, agglomerates observed within the cells.</p>	<p>Inconclusive</p> <p>IC₅₀ ranging from 500 to 5700 µg/mL depending on TiO₂ NPs sample and cell line.</p> <p>TiO₂NPs (28 nm) (with higher IC₅₀): substantial damage in NIH/3T3 cells.</p> <p>TiO₂NPs ("nanorutil"): almost no damage.</p> <p>Highest DNA SBs in TiO₂ NPs (28 nm).</p> <p>Intracellular ROS production: significant effect of TiO₂NPs at IC₅₀</p>	<p>Reliability: 3</p> <p>Few experimental details.</p> <p>Single exposure time point used.</p> <p>Testing at very high IC₅₀ cytotoxic</p>	<p>Low</p>	<p>Tomankova et al., 2015</p>

Apoptosis detection	Measure of % DNA in head and tail Negative control: not reported Positive control: not reported			concentration in all cell lines. Apoptosis: increased % apoptotic cell death after 24 h in all studied TiO ₂ NPs at IC50 concentrations and all cell lines except SVK14 cell line with "Nanorutile" sample.	concentrations. No positive control reported		
Comet assay +/- Fpg Caco-2 human colon carcinoma cell line (differentiated and undifferentiated)	10, 25, 100 µg/mL on basis of viability results Exposure: 24h Uptake Viability: cell counts after 24 h treatment. Positive controls: MMS and KBrO ₃ Measure of %tail DNA	TiO ₂ (NM-100), anatase, 50-150 nm	NSC: 1 Dispersion and stability measured according to the Nanogenotox protocol. Cellular internalisation of the NPs confirmed.	Equivocal Viability >80% over range 1-200 µg/mL Slight statistically significant increase in SBs and decrease in Fpg sensitive sites at 10 µg/mL. No increase in SBs or Fpg sensitive sites at higher concentrations.	Reliability: 1	Limited	Vila et al., 2018
Comet assay A549 human lung	0, 50, 100, and 200 µg/mL (equivalent to 0, 13, 26 and 52 µg/cm ²) Exposure: 48h	TiO ₂ NPs, anatase, 15 nm (TEM)	NSC:2 Dispersion measured under the exposure conditions. The results suggest agglomeration but in the presence of FBS the agglomerates	Positive Viability: effects seen at 50 µg/mL, 24, 48, 72 h (80% viability or less at 100 µg/mL)	Reliability: 1	Limited The relevance of the tested material is not clear	Wang et al., 2015

<p>carcinoma cell line</p> <p>+ morphological changes, cell cycle progression (flow cytometry with PI staining), analysis of mitochondrial membrane potential and apoptosis</p>	<p>Positive control: no</p> <p>Measure of Tail length, OTM, and % of tail DNA</p> <p>Viability (MTT), exposure 24, 48 and 72h (25, 50, 100 and 200 µg/mL)</p> <p>Morphological changes: fluorescence microscopy, SEM</p>		<p>are stable and the size lower than 100nm.</p>	<p>Concentration and time-dependent inhibition of cell proliferation</p> <p>Statistically significant increase in DNA SBs at 50 µg/mL and above</p> <p>Morphological changes in the cells at 50 and 100 µg/mL (typical apoptotic morphological changes of cell shrinkage)</p>			
<p>Comet assay</p> <p>Caco-2 human colon carcinoma cell line</p> <p>+ Apoptosis</p>	<p>50, 100, 200 and 500 µg/mL</p> <p>Exposure: 24h</p> <p>Viability: Calcein AM/propidium iodide staining (CCK-8 kit assay)</p> <p>Negative control: untreated cells</p> <p>Positive control: UV</p>	<p>TiO₂NPs, anatase/rutile (95/5%), 15±3 nm (SEM)</p>	<p>NSC: 2</p> <p>Dispersion measured under the exposure conditions. Agglomeration confirmed and size of agglomerates increasing with concentration. The authors indicate that the apparent non-monotonic response may be related to limited exposure at high concentrations</p>	<p>Negative</p> <p>No DNA damage (SBs)</p> <p>Viability – no effect at 24 or 48 h.</p> <p>Apoptosis: slight increase in cells exposed to TiO₂-NPs at 50 and 100 µg/mL</p>	<p>Reliability: 3</p> <p>Few experimental details given.</p> <p>Inappropriate control for comet</p>	<p>Low</p>	<p>Yang et al., 2018</p>

	OTM as descriptor Three experiments.		due to formation of larger agglomerates.		assay (UV)		
Comet assay Whole blood leukocytes from 6 donors.	0.4, 0.8, 1.2, 4, 8 mg/mL (i.e. up to 100 mM). Cells embedded in agarose and then treated with NPs for 1 h. Positive control: H ₂ O ₂ 6 experiments Viability; trypan blue Antigenotoxicity: first exposed to 50 μM H ₂ O ₂ and then to NPs (not in gel, so different exposure from above)	TiO ₂ NPs, anatase, 45 A (4.5 nm)	NSC:3 Insufficient information provided on dispersion and agglomeration under the exposure conditions.	Inconclusive Viability: >90% though very high concentrations were used. Concentration -dependent increase in SBs, substantial but not significant, probably because of crude scoring method.	Reliability: 3 Insufficient details of description of the experiments. Short and only one exposure time used. Insensitive visual scoring system used (apparently only nucleoid	Low	Lazic et al., 2019

					s with any level of damage were recorded)		
Comet assay +/- Fpg HEK293 (human embryonic kidney) NIH/3T3 (mouse embryonic fibroblast) + cell-transformation assay on NIH/3T3 cells	10, 100 and 1000 µg/mL Exposure: 1h Negative control: water Positive control: EMS 2 independent exp. 200 cells scored Measure of %tail DNA Soft-agar colony assay: after 3 weeks Viability: determination of living cells by counting with fluorescence staining	1) TiO ₂ NPs, anatase, 21 nm (TEM) 2) TiO ₂ NPs, anatase, 50 nm (TEM). 3) TiO ₂ microparticulated (no further information)	NSC:1 Dispersion protocol according to Nanogenotox project, dispersion measured in the media, some agglomerates confirmed. BSA is used as dispersion agent	Inconclusive Viability: 82% for HEK293 and 85% for NIH/3T3 at 1000 µg/mL. TiO ₂ NPs: positive, induction of SB in a very high concentration only (1000 µg/mL) TiO ₂ microparticulated: negative Results are similar in both cell lines used. No increase of Fpg sensitive sites with nano- or microforms. Soft-agar colony assay: significant increases in colony number at the highest concentration of TiO ₂ NPs. No effects with TiO ₂ microparticulated.	Reliability: 2 Short and only one exposure time used.	Low Inconclusive results	Demir et al.,2015*

<p>Comet assay</p> <p>+/- Fpg, ± light. Minigels.</p> <p>immortalised human bronchial epithelial cell lines (BEAS-2B cells)</p>	<p>1, 5, 15 µg/mL</p> <p>Exposure: 3 h, 24 h</p> <p>Light exposure after lysis</p> <p>50 cells scored/sample</p> <p>Measure of % DNA tail</p> <p>Positive control: Ro 19-8022 photosensitiser +light irradiation</p> <p>Viability: alamar Blue</p> <p>Cytotoxicity: various methods used (MI, % apoptosis/necrosis, cell cycle perturbations)</p> <p>Uptake measured</p>	<p>TiO₂ (NM-100), anatase, 50-150 nm</p>	<p>NSC:1</p> <p>Dispersion and stability measured in the exposure media according to the NanoREG project. Some level of agglomeration confirmed. Cellular uptake confirmed by an indirect method (flow cytometry) demonstrating the cellular internalisation of the agglomerates.</p>	<p>Induction of SB: negative</p> <p>Induction of Fpg sensitive sites: positive.</p> <p>Viability – only slight effects; always >80%. No significant effect at 48 h.</p> <p>No increase in SB at 3 or 24 h. Increase in Fpg sensitive sites at 2 higher concentrations at 3 h.</p> <p>Additional SB seen after light exposure.</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Di Bucchianico et al., 2017*</p>
<p>Comet assay</p> <p>human umbilical vein endothelial</p>	<p>1, 5, and 25 µg/mL</p> <p>Exposure time: 4 h</p> <p>Each concentration prepared in triplicate</p>	<p>1)TiO₂ NPs, anatase, 100 ± 14.3 nm 2)TiO₂ NPs, anatase, 50 ± 7.6 nm</p>	<p>NSC: 2</p> <p>Ultrasonication performed in the cell medium. Actual measurements for</p>	<p>Positive</p> <p>Comet assay: statistically significant effects (OTM) at all concentrations of all TiO₂ sizes, except TiO₂ NPs (100 nm) at 1 µg/mL (a positive concentration-</p>	<p>Reliability: 2</p> <p>No positive control.</p>	<p>Limited</p>	<p>Liao et al., 2019*</p>

<p>cells (HUVECs) + ROS measures</p>	<p>Measurement of intracellular ROS (DCFDA probe) and anti-oxidant production: 24 hours</p> <p>Measurement of GSH (Sigma kit): 24 hours</p> <p>Cytotoxicity (CCK-8 assay): 24 h – results described in Zeng C, Feng Y, Wang W, et al Environ Toxicol. 2018;33(12):1221-1228</p> <p>Positive control: none</p>	<p>3) TiO₂ NPs, anatase, 30 ± 5.1 nm 4) TiO₂ NPs, anatase, 10 ± 2.3 nm</p>	<p>dispersion and stability not reported</p>	<p>dependent and negative size-dependent effect relationship (T100 < T50 < T30 < T10)).</p> <p>Cytotoxicity: significant from the lowest tested concentration (1 µg/mL).</p> <p>Significant production of ROS: concentration-dependent and size-dependent.</p>	<p>DNA damage reported as OTM.</p> <p>No data on dispersion provided.</p> <p>No data on cell internalization provided.</p>		
<p>Comet assay PBMCs I (healthy and lung diseases donors: lung cancer w/o chemotherapy, chronic obstructive</p>	<p>Comet: DNA damage and repair - treatment for 30 minutes with subsequent incubation for 30 minutes.</p> <p>Concentrations: 10, 30 or 50 µg/mL.</p> <p>Negative control: medium</p>	<p>TiO₂ NPs, anatase, 40-70 nm (SEM)</p>	<p>NSC:2</p> <p>DLS measurements mentioned but results not included, sonication in DMSO and microfiltration (0.2 µm) but insufficient information reported on the actual measurements in culture medium.</p>	<p>Healthy donors: Inconclusive</p> <p>No statistically significant increase in % tail DNA, but significant using OTM at 30 and 50 µg/mL.</p> <p>Patient donors groups: positive</p> <p>Significant increase in % tail DNA at 30 or 50 µg/mL</p>	<p>Reliability: 2</p> <p>Short term exposure for 30 min. used.</p> <p>No data on NPs stability</p>	<p>Low</p> <p>The evaluation was focused on the cells from healthy donors</p> <p>Short term exposure for 30 min. used.</p>	<p>Osman et al., 2018*</p>

<p>pulmonary diseases (COPD), asthma)</p>	<p>Positive control: H2O2</p> <p>Information of comet methodology in Gopalan, 2009.</p> <p>50 cells scored per slide. The main experiments were repeated three times.</p>			<p>Using OTM: significant DNA SBs in respiratory disease patients compared to healthy individuals.</p>	<p>provided .</p> <p>No data on internalization provided .</p>		
<p>Comet assay +/- Fpg BEAS-2B normal bronchial lung cells</p>	<p>1, 10 and 20 µg/mL</p> <p>Exposure time: 24 h, 1 and 3 weeks</p> <p>Positive control: EMS (0.5 mM).</p> <p>Soft-agar anchorage-independent colony formation assay (carcinogenesis): exposure for 4 weeks.</p> <p>Measurement of % DNA in tail</p>	<p>TiO₂NPs (NM-102), anatase, 21-22 nm</p>	<p>NSC: 1</p> <p>Dispersion verified according to the Nanogenotox protocol and cellular internalisation confirmed by TEM. Individual particles and some level of agglomeration observed as well as NPs and agglomerates within the cells.</p>	<p>Negative</p> <p>No DNA SBs detected after 24 h also with Fpg enzyme.</p> <p>After 1 and 3 weeks of exposure no significant increases in the levels of primary and oxidative DNA damage.</p> <p>Colony formation assay indicated no cytotoxicity.</p> <p>Cellular uptake by TEM: Dose- and time-dependent cellular uptake was observed in BEAS-2B cells.</p> <p>Nanoparticles and nano-aggregates were mainly confined to vacuoles, although they were also</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Vales et al.,2015*</p>

				<p>present on the surface of the nuclear membrane.</p> <p>A direct concentration–effect relationship was observed when the total number of colonies, as well as the number of medium-large size colonies, was determined.</p> <p>Only the results obtained after evaluating the number of medium-large colonies exposure to 20 µg/mL attain statistical significance.</p>			
<p>Comet assay</p> <p>Chinese hamster lung fibroblasts V-79</p>	<p>0, 1, 10, 25, 50, 100 µg/mL</p> <p>Exposure time: 6h</p> <p>Positive control: none</p> <p>Particle uptake: 0, 10, 50, 100 µg/mL</p> <p>Cytotoxicity:</p> <p>MTT assay (3, 6, and 24 h): 1, 10, 25, 50, 100 µg/mL</p>	<p>TiO₂NPs, anatase, 12-25nm (TEM)</p>	<p>NSC: 1</p> <p>Dispersion measured in the cDMEM exposure media after suspension of NPs and sonication, however the time of the measurement not given (0 or 24 h).</p> <p>Presence of small agglomerates confirmed. Cellular uptake measured, internalisation of NPs and agglomerates confirmed.</p>	<p>Positive</p> <p>Comet assay: statistically significant increase of % DNA tail and OTM observed at 25, 50, 100 µg/mL.</p> <p>Cellular internalization study by flow cytometry revealed a statistically significant concentration dependent uptake.</p> <p>Cellular internalization and localization of TiO₂NPs in cells assessed by TEM clearly demonstrated appearance and sub-cellular localization TiO₂</p>	<p>Reliability: 2</p> <p>Exposure only for 6 h was used.</p> <p>Scoring of only 50 cells per concentration (25 cells/slides).</p>	<p>Limited</p>	<p>Jain et al., 2017*</p>

	<p>Flow cytometry with PI staining (6 and 24 h)</p> <p>Particle uptake: FACS – 6 h; TEM/EDX - 2, 4, 6 and 24 hours.</p> <p>ROS production (6 h): 1, 10, 25, 50, 100 µg/ml</p>			<p>NPs, in time dependent fashion. Reported translocation to the nucleus and mitochondria not clear. SEM/EDX analysis confirmed the internalization.</p> <p>Significant (from 10 µg/mL) concentration dependent increase in the production of ROS</p> <p>Cytotoxicity: MTT assay statistically significant reduction of % MTT at 10 µg/mL after 24 h, at 25 and 50 µg/mL after 6 and 24 h, at 100 µg/mL after 3, 6 and 24 h of exposure.</p> <p>Dead cells increased (PI uptake) stat. significantly at all concentrations after 24 h and only at 100 µg/mL after 6 h of exposure.</p>	No positive control.		
<p>Comet assay</p> <p>Murine macrophage cell line RAW 264.7</p>	<p>10, 25, 50, 75, and 100 µg/mL</p> <p>Exposure time: 24 h</p> <p>Cytotoxicity (24 h): resazurin 10, 25, 50, 75, and 100 µg/mL</p>	<p>TiO₂ NPs, 58.25 ± 8.11 nm, (SEM) (crystalline form unknown)</p>	<p>NSC: 3</p> <p>Inadequate and insufficient information provided on dispersion.</p>	<p>Positive</p> <p>Comet: significant concentration-dependent increase in all parameters.</p> <p>Cytotoxicity: % cell viability decreased significantly in a concentration-dependent manner (37% at 100</p>	<p>Reliability: 2</p> <p>No positive control used.</p> <p>No data on</p>	Limited	<p>Chakrabarti et al., 2019**</p>

	<p>Positive control: none</p> <p>100 Comets scored/concentration</p> <p>Parameters studied: Tail length, % DNA in tail, Olive Tail moment</p>			µg/mL and 78% at 10 µg/mL)	internalization provided		
Comet assay	<p>10 µg/L TiO₂NPs</p> <p>Exposure time: 48 and 72 hrs</p> <p>Positive control: H₂O₂</p> <p>Including diffusion assay (i.e. comet assay w/o electrophoresis)</p> <p>Cytotoxicity by Trypan blue exclusion assay.</p>	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	<p>NSC: 2</p> <p>Ultrasonication performed in medium (3h) and UV spectra used for measuring agglomeration</p>	<p>Inconclusive (due to the high cytotoxicity)</p> <p>Comet assay: statistically significant increase of DNA damage after 48 and 72 h exposure.</p> <p>Diffusion assay: after 48 and 72 h a statistically significant high increase of apoptotic amniotic cells.</p> <p>Cell viability: 48 and 72 h of exposure led to statistically significant reduced amniotic cell viability (ca by half).</p>	<p>Reliability: 3</p> <p>Only one, concentration used.</p> <p>No information on number of cells scored.</p> <p>High cytotoxicity</p>	Low	Mottola et al., 2019*
Comet assay	<p>1 µg/L and 10 µg/L</p> <p>Exposure time: 15, 30, 45, and 90 min</p>	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	<p>NSC: 1</p> <p>Specific protocols for verifying dispersion and stability, and confirmation of</p>	<p>Positive</p> <p>Comet assay: concentration dependent statistically significant increase in %</p>	<p>Reliability: 3</p> <p>No information on</p>	Low	Santonastaso et al., 2019*

Human sperm cells	Cytotoxicity (15, 30, 45, and 90 min): sperm vitality and motility Positive control: benzene		exposure to particles by EM. Stability is confirmed up to 30min, some agglomeration observed at longer exposure periods. EM confirms the presence of nanoparticles and small agglomerates within the cells	DNA tail from 15 min of exposure. Sperm motility: exposure to 1 µg/L and 10 µg/L for 15, 30, 45, and 90 min did not induce statistically significant changes in vitality, whereas motility (progressive and non-progressive) was statistically significantly reduced after 45 and 90 min.	number of cells scored. Benzene as a positive control used for comet is not appropriate.		
Comet assay +/- Fpg and Endo III Balb/c 3T3 (Mouse embryo fibroblasts) Cell transformation assay (CTA, Table 4b)*	Comet assay (2, 24, 48 and 72 h of exposure): 10, 20 and 40 µg/cm ² (32, 64 and 128 µg/mL); with EndoIII and Fpg enzymes (2 and 24 h) Cytotoxicity: colony forming efficiency (24, 48 or 72 h of exposure) – 10, 20 and 40 µg/cm ² Positive control/ H ₂ O ₂ Uptake measure	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Dispersion (DLS) is reported for deionised water and cMEM medium (0-72 h); cellular uptake confirmed by EM. A high level of agglomeration is observed in cMEM for uncoated TiO ₂ NPs already after 0 h. Internalisation of agglomerates in the exposed cells after 24h.	Positive Comet assay: after 2 and 24h treatments TiO ₂ NPs (P25) induced a significant increase of primary DNA damage. After 48 and 72 h the level of DNA damage was very low, it was at the control level. Statistical significant increases of Fpg sensitive sites at all tested concentrations for TiO ₂ NPs (P25) at 2h and at low concentration at 24h Statistical significant increases of Endo III sensitive sites at all tested	Reliability: 1	High	Stocco et al., 2016*

				concentrations for TiO ₂ NPs (P25) at 2h. Cytotoxicity: cytotoxic effect starting from 24h exposure.			
Comet assay +/- Fpg TH1 (human renal proximal tubule epithelial cell line)	Comet assay (3 and 24 h): 2.2, 22, and 165 µg/mL (corresponding to 1, 10 and 75 µg/cm ²); static and dynamic conditions. Positive control for strand breaks: none Oxidative damage with Fpg (positive control RO 19-8022). Cytotoxicity (3 and 24 h): alamar Blue.	TiO ₂ NPs, 5- 10 nm (TEM) (crystalline form and shape unknown)	NSC: 1 Dispersion and stability measured and reported. Confirmation of cellular internalisation of NPs and agglomerates with quantitative estimation (ICP-MS).	Negative Comet assay: no statistically significant increase in DNA strand breaks and oxidative DNA damage under both static and dynamic conditions. Cytotoxicity: no effect observed after 3 or 24 h. Uptake efficiency for TiO ₂ NPs (2.2 µg/mL, 24 h): 0.389% from total applied concentration.	Reliability: 2 Not clear how many repeat experiments and replicates were used.	Limited	Sramkova et al., 2019
Comet assay +/- OGG1 (measure of oxidative DNA 8-OH guanine damage)	0.01, 0.1, 1 and 10 µg/mL Cytotoxicity was measured up to 100 µg/mL Exp: 24h harvesting times (0, 24, 48 and 72 h)	TiO ₂ NPs, 28-49 nm (SEM) (crystalline form unknown)	NSC: 1 Dispersion measured under the exposure conditions (suspension with FBS). Possible presence of agglomerates, especially after 24h. Cellular uptake measured, concentration-	Negative Genotoxicity: Comet assay +/-OGG1: negative. Cytotoxicity: No significant decrease in cell viability up to 10 µg/mL, Significant reductions at 100 µg/mL.	Reliability: 1	High	Franchi et al., 2015*

<p>human fibroblast cells (GM07492)</p>	<p>Negative control: medium</p> <p>Positive controls: methyl methanesulfonate (MMS) and potassium bromide (KBrO3)</p> <p>Measure of % DNA in tail.</p> <p>Mitochondrial activity of the fibroblasts was assessed by an XTT assay, as an indirect measurement of cell viability. The results of the XTT assay were further confirmed by the ViaCount assay.</p>		<p>dependent internalisation of NPs and agglomerates confirmed.</p>	<p>agglomerates were observed freely in the cytoplasm</p> <p>Flow cytometry (SSC): Clear concentration-dependent effects of the TiO₂NPs on uptake levels in GM07492 cells</p>			
<p>Comet assay</p> <p>+/- Fpg</p> <p>Human bronchial epithelial BEAS-2B cells</p>	<p>0, 0.1, 1, 10 and 100 µg/mL</p> <p>Exposure: 24h</p> <p>Negative control: untreated</p> <p>Positive control: H₂O₂ for SB</p>	<p>TiO₂ (NM-100), anatase, 50-150 nm</p>	<p>NSC: 1</p> <p>NANOGENOTOX project dispersion protocol and confirmation of cellular internalisation</p>	<p>Positive</p> <p>Statistically significant increase of % tail DNA starting from 1 µg/mL and statistically significant increase of Fpg positive sites at 1 and 10 µg/mL.</p>	<p>Reliability: 1</p> <p>Positive control for Fpg is missing</p>	<p>High</p>	<p>Zijno et al., 2020*</p>

	Analyse: 100 randomly selected Comets Measure of %-tail DNA Cell viability: trypan blue			Cytotoxicity: no reduction of cell viability			
Comet assay Human sperm cells (125 healthy donors = pooled sample)	1 µg/L Exposure: 15, 30, 45, 90 minutes Negative control: untreated Positive control: benzene Measure of % tail DNA TUNEL: % sperm with fragmented DNA	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 Dispersion considered and measured in the media but the none of the criteria fully meet	Positive Statistical significant increase % tail DNA after 30, 45 and 90 minutes. TUNEL: Statistical significant increase % DNA fragmentation index	Reliability: 3 No information on number of cells scored. Benzene as a positive control used for comet is not appropriate. Only one concentration used. Short exposure time,	Low	Santonastaso et al., 2020

					No uptake demonstrated only agglomerates outside of cells		
Comet assay Human mammary carcinoma cells (MCF-7 cells)	Comet assay at 1, 10 and 100 µg/mL of nano-TiO ₂ Exposure Comet: 12h Exposure cytotoxicity: 6, 12, 24, and 48 h at 1, 2, 5, 10, 20, 50, and 100 µg/mL of nano-TiO ₂ Negative control: untreated Positive control: none Cytotoxicity measurement by Water-soluble tetrazolium (WST) assay Apoptosis/necrosis by microscopical	1) TiO ₂ NPs, anatase/rutile, 56 nm (TEM) 2) TiO ₂ , anatase, 325 mesh.	NSC: 2 Incomplete information on dispersion is presented. SEM provided but does not address internalisation. DLS results provided but not clear if they were performed in PBS or culture medium.	Positive TiO ₂ NPs: statistically significant increase % tail DNA at 10 (ca 30% cytotoxicity) and 100 µg/mL (50% cytotoxicity) and comet area at 100 µg/mL. TiO ₂ : statistically significant increase % tail DNA and comet area at 100 µg/mL. Cytotoxicity: significant decreases in viabilities upon exposure to TiO ₂ NPs from 20 µg/mL at 6 h, 5 µg/mL at 12 h, 5 µg/mL at 24 h and 2 µg/mL at 48 h. Increased level of apoptosis (10, 50, 100 and 200 µg/mL) and necrosis (50, 100 and 200 µg/mL)	Reliability: 2 No positive control; No information on comet procedure (just referring to Tice et al, 2000)	Limited	Kumar et al., 2020

	analysis of PI and FITC-annexin fluorescence intensity						
Comet assay +/- Fpg 2D monolayer and 3D spheroid cultures of HepG2 human liver cells	0, 1, 10, 30, 75 µg/cm ² (Corresponding to 0, 2.82, 28.2, 84.8, 212 µg/mL) Exposure: 24h Negative control: untreated Positive control: H ₂ O ₂ for SB and Ro 19-8022 plus light for Fpg positive sites Measure of % tail DNA Cytotoxicity: alamar Blue assay and confocal microscopy	TiO ₂ NPs, anatase, 5.45 nm (TEM)	NSC:2 Dispersion considered and partial information provided.	Negative No cytotoxicity and no increase in DNA SB or in Fpg positive sites	Reliability: 1	High	Elje et al., 2020
Comet assay Human bronchial epithelial (HBE),	0, 5, 25, 50 and 100 µg/mL Exposure: 24h	1) TiO ₂ NPs (JRCNM10202a), anatase, 17 nm 2) TiO ₂ (JRCNM102200a), anatase, 117 nm	NSC:1 NANOREG protocol with detailed dispersion assessment	Positive in the different cell lines with TiO ₂ NPs (17 nm) and TiO ₂ (117 nm) HBE and Caco-2 cells: no difference between TiO ₂ NPs 17 nm-LA and 17 nm-SA as	Reliability: 1	High	Murugadoss et al., 2020**

<p>colon epithelial (Caco-2) and monocytic (THP-1) cell lines</p> <p>+ROS formation</p>	<p>Negative control: BSA</p> <p>Positive control: MMS</p> <p>Analysis of 50 comets per well</p> <p>3 experiments</p> <p>Measure of % tail DNA</p> <p>ROS: 4, 64, 256 µg/mL in serum-free exposure medium 24 hours exposure</p> <p>Cell viability was assessed by cellular leakage of LDH using a kinetic assay</p>			<p>well as TiO₂ 117 nm-LA and 117 nm-SA</p> <p>THP-1 cells: TiO₂NPs 17 nm-LA more potent than TiO₂NPs 17 nm-SA; no differences between TiO₂ 117 nm-LA and 117 nm-SA</p> <p>No cytotoxicity</p> <p>Agglomeration state of TiO₂ influences the biological responses not only depending on the cell type but also depending on the primary particle size.</p> <p>Total glutathione (GSH): statistically significant reduction at the highest concentration in the three cell lines, both TiO₂NPs and TiO₂</p> <p>Markers of pro-inflammatory response: statistically significant increase IL-6, IL-1β, TNF-α, mid and high concentrations, HBE cells; I IL-1β, TNF-α, high concentration, HTP-1 cells both TiO₂NPs and TiO₂</p>			
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Comet assay	0, 0.5, 5 and 50 µg/mL	1) E171, anatase, 170 nm	NSC: 2 The protocol intentionally accept large agglomeration. No information provided on agglomeration in the exposure media.	Inconclusive (Negative without proof of internalisation) Low cytotoxicity of both E171 and TiO ₂ NPs after 24 or 48 h (not less than 88% comparing to the control values)	Reliability: 3	Low	Franz et al., 2020*
Colon cancer cell line HT29-MTX-E12	Exposure: 48 h Negative control: untreated Positive control: EMS and MMS Measure of tail intensity Cell viability: MTS assay	2) TiO ₂ NPs, anatase, < 25 nm					

CFE: Colony forming efficiency; EMS: ethyl methane sulfonate; FBS: Fetal Bovine Serum; GSH: reduced glutathione; GSH-Px: glutathione peroxidase; LDH: Lactate dehydrogenase; MDA: Malonaldehyde; MMC: mitomycin C; MMS: methylmethanesulfonate; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OTM: Olive Tail Moment; PBMC: peripheral blood mononuclear cells; PBS: phosphate buffer saline; PI: propidium iodide; SB: strand breaks; SOD: superoxide dismutase; SSB: single strand breaks; 8-oxo-dGuo: 8-oxo-7,8-dihydro-2'-deoxyguanosine; WST: Water-soluble tetrazolium

Table 4a: Other *in vitro* assays - Genotoxicity endpoints investigated with methods not recommended for regulatory purposes

DNA damage response (incl. Gadd 45 α, ATM, γH2AX, DDIT3, 53BP1 and ToxTracker)

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 authors_year
<p>Up-regulation of Growth Arrest and DNA damage-inducible 45a protein (Gadd45a) Mouse embryo fibroblasts (PW)</p> <p>HIF-1a (+/+) cells</p> <p>HIF-1a (-/-) cells</p>	<p>0, 1.25, 2.5, 5, 7.5, 10, 20 µg/ml</p>	<p>TiO₂NPs, anatase/rutile (90/10), 28 nm, 10-60 nm (TEM)</p>	<p>NSC: 3</p> <p>Dispersion by ultrasonication (30min) in physiological saline with no additional information.</p>	<p>TiO₂NPs did not induce any significant Gadd45a up-regulation.</p> <p>No significant cytotoxicity was observed at any experimental concentration and time points.</p>	<p>Reliability: 5</p> <p>The study was focused on Cobalt nanomaterial while TiO₂NPs was used as negative control. The endpoint is not a univocal marker of genotoxicity (Gadd45a is not induced only by DNA damage). The top concentration used (20 µg/ml) is too low to rule out relevant effects.</p> <p>Insufficient information on the target cells is reported. No</p>	<p>Low</p>	<p>Feng et al., 2015</p>

					positive control was used.		
ATM phosphorylation and γ H2AX (flow cytometry). Human fibroblast (GM07492) cells (untransformed cells) Comet assay (Table 3)*	0.01, 0.1, 1 and 10 μ g/mL Exp: 24h +recovery times (0, 24, 48 and 72 h)	TiO ₂ NPs, 28-49 nm (SEM) (crystalline form unknown)	NSC: 1 Dispersion measured under the exposure conditions (suspension with FBS). Possible presence of agglomerates, especially after 24h. Cellular uptake measured, concentration-dependent internalisation of NPs and agglomerates confirmed.	Inconclusive analysis only at 10 μ g/mL. At this concentration no alterations in phosphorylated ATM and γ H2AX. No significant decrease in mitochondrial enzyme activity (XTT assay) as indicator for cell viability was found up to 10 μ g/mL, while the enzyme activity was decreased to a level of about 65 % at 100 μ g/mL (calculated IC50: 168.9 μ g/mL).	Reliability: 3 Too low test concentrations	Low	Franc hi et al., 2015 *
Target cells: human primary epidermal keratinocytes Phospho-histone H2AX (γ H2AX)	γ -H2AX assay: 1, 100 μ g/ml Internalization and localization of particles: 100 μ g/ml	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Dispersion measured and agglomeration observed. Cellular internalisation confirmed by TEM.	γ -H2AX assay, % of cells with γ -H2AX foci: Positive no significant increase at 1 μ g/ml; slight but statistically	Reliability: 2 Study indicative of DNA damage although γ -H2AX assay is	Limited	Katha wala et al., 2015

immunofluorescence assay	<p>ROS production: 50 µg/ml, 100 µg/ml</p> <p>Exposure:</p> <p>γH2AX assay: 24 hours</p> <p>ROS production: 4, 8 hours</p>			<p>significant increases at 100 µg/ml. ROS production (CellROX dye, fluorogenic reagent): concentration and time dependent induction of ROS at sub-lethal concentrations, in particular localised in mitochondria. NPs not detected in the nucleus, but in the perinuclear region.</p>	not a standardised test.		
<p>ToxTracker assay</p> <p>ToxTracker reporter cell lines Bsc12-GFP and Rtkn-GFP (DNA replication stress), Btg2-GFP (p53-associated cellular stress), Srxn1-GFP and BlvrB-GFP</p>	<p>ToxTracker assay: observed by flow cytometry</p> <p>ToxTracker reporter cells (based on embryonic stem cells) in presence of primary mouse embryonic fibroblasts</p> <p>0, 0.98, 1.95, 3.9, 7.8, 15.6 µg/cm²</p> <p>(corresponding to 0, 3.13, 6.25, 12.5, 25, 50 µg/ml)</p>	E 171, anatase (0.2% rutile), 390 nm (DLS)	<p>NSC:2</p> <p>Dispersion and stability measured in the exposure media but dilution to obtain the different concentration-ranges performed later. Some level of agglomeration confirmed. Good stability up to 24h confirmed for the high concentration (50 µg/ml, equivalent to 15.6 µg/cm²).</p>	<p>ToxTracker assay:</p> <p>negative</p> <p>no statistically significant difference in any reporter activation at any of the concentration.</p> <p>Cytotoxicity (>50%) at concentrations above 7.8 µg/cm²</p>	<p>Reliability: 2</p> <p>(In the same study positive results were reported in a comet assay in 3 cell lines and in a FPG-comet in one cell line. This discrepancy highlights lower sensitivity of ToxTracker in detecting DNA breakage and</p>	Limited	<p>Brown et al., 2019 *</p>

<p>(oxidative stress) and Ddit-GFP (protein damage)</p> <p>mES cells (primary mouse embryonic fibroblasts)</p>	<p>Exposure: 24h</p> <p>Positive controls carcinogenic quartz DQ12 (for oxidative stress and inflammation), Cisplatin, diethyl maleate, tunicamycin, KBrO₃</p> <p>Measure of GFP expression. Activation of Bsc12-GFP and Rtkn-GFP reporters indicate DNA damage; Srxn1-GFP and BlvrB-GFP indicate cellular oxidative stress; Ddit-GFP associated with unfolded protein response.</p> <p>Cell survival measured by flow cytometry.</p> <p>Only GFP inductions at compound concentrations that showed >25% cell survival are used in the analysis.</p>				<p>DNA oxidative damage compared to Comet assay)</p>		
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Analysis of the expression of GADD45A and DDTI3 as markers of DNA damage	up to 25.8 µg/cm ²	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Information on dispersion available. Cellular Particle Uptake was verified using Flow Cytometry.	Negative No impact by TiO ₂ NPs on gene expression patterns of GADD45A and DDIT3 genes.	Reliability: 5 The induction of GADD45A and/or DDIT3 may reflect oxidative DNA damage but is not an unambiguous marker of DNA damage	Low	Hufnagel et al., 2020
Genomic instability							
Induction of genomic instability (RAPD PCR) in human amniotic fluid cells	-10 µg/L TiO ₂ NPs -10 µg/L of TiO ₂ NPs plus 100 mg/L of lincomycin Exp: 48 and 72 hours	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 Ultrasonication performed and UV spectra used for measuring agglomeration but the information is insufficient to conclude which level of agglomeration is needed for getting absorbance at this range.	RAPD-PCR test: exposure to TiO ₂ NPs induced a statistically significant effect. Cytotoxicity: reduction of cells viability (blue trypan) and induction of apoptosis (diffusion assay).	Reliability: 5 The effects are evaluated by a non-standard method It is unclear if the effect is related to genomic instability	Low	Motto la et al., 2019 *
Induction of genomic instability (RAPD PCR)	1 µg/L and 10 µg/L Exp: 15, 30, 45, and 90 min	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Specific protocols for verifying dispersion	RAPD-PCR test: exposure to TiO ₂ NPs induced	Reliability: 5 The effects are evaluated by a	Low	Santonastaso et

in human sperm cells			and stability, and confirmation of exposure to particles by EM. Stability is confirmed up to 30 min, some agglomeration observed at longer exposure periods. EM confirms the presence of NPs and small agglomerates within the cells.	a statistically significant effect. ROS induction was detected by DCF assay.	non-standard method It is unclear if the effect is related to genomic instability		al.,2019*
Telomere length (TL) Human hepatocytes L-02 (cat# GNHu 6), hepatocellular carcinoma cell QGY (cat# TCHu 43)	0, 40, 80 µg/mL Exp: 24 and 72h	TiO ₂ NPs, 21 nm (crystalline form unknow)	NSC: 3 No information provided on the level of dispersion or stability.	Decrease of TL in L-02 cells but not in QGY cells. Down-regulation of the expression of Nrf-2 and core shelterin components in L-02 cells. Upregulation of the expression of Nrf-2 and core shelterin components in QGY cells	Reliability: 5 Each sample was analysed in triplicate but it is unclear how many samples were analysed. Thus, the meaning of the statistical significance reported for effects on telomere length is unclear.	Low (when strictly regarding genotoxicity because the study does not address genotoxicity <i>stricto sensu</i>) Since effects on telomere length are regarded as indicator for ageing processes and were considered to be associated with, e.g., degenerative diseases, such effects might be considered relevant for the overall evaluation of TiO ₂ . However,	Wang et al.,2018*

						due to the unclear meaning of the statistical significance reported for effects on TL in this study, these study results are of low relevance also in this respect.	
DNA binding							
DNA binding to human genomic DNA (<i>in vitro</i>)	Methods: UV-visible spectroscopy and quenching of the ethidium bromide (EtBr) fluorescence emission spectrum	TiO ₂ NPs, anatase/rutile, <100 nm	NSC:2 a set of related properties are reported for water and culture media at different concentrations but without direct measurement of the level of agglomeration	Positive UV-visible study: TiO ₂ NPs increased UV absorbance. This might be due to electrostatic interactions with the phosphate backbone leading to a conformational change in DNA Quenching study: decreased EtBr fluorescence compatible with TiO ₂ NPs	Reliability: 2	Limited	Patel et al., 2016

				intercalating between DNA bases			
<p>DNA binding to DNA isolated from lymphocytes (human donor)</p> <p>DNA binding to human genomic DNA (in vitro)</p>	<p>Exposure:</p> <p>extent of DNA binding by fluorescence measurements: 2-3 minutes</p> <p>Method: DNA binding measured by quenching of the ethidium bromide (EtBr) fluorescence emission spectrum</p>	TiO ₂ NPs, anatase/rutile, 21 nm	<p>NSC: 2</p> <p>Dispersion measured under the exposure conditions (FBS used in the medium). Presence of agglomerates confirmed, good stability up to 24h.</p>	<p>Positive</p> <p>Displacement of EtBr intercalated between the planar bases of DNA by TiO₂ NPs suggest an intercalative mode of binding.</p> <p>Strong binding affinity of TiO₂ NPs and DNA. (binding constant (K_b) = 4.158 x 10⁶ M⁻¹)</p>	<p>Reliability: 2</p> <p>Binding with isolated DNA in an acellular system.</p> <p>Binding measured only by a fluorescence quenching method</p>	Limited	Patel et al., 2017*
DNA binding to calf thymus DNA	<p>Methods: 1) UV-visible spectroscopy; 2) fluorescence quenching; 3) circular dichroism (CD); 4) docking analysis</p>	TiO ₂ NPs, rutile, 14 nm (XRD)	<p>NSC: 2</p> <p>Solutions used for titration were dispersed in Milli Q water and subjected to sonication</p>	<p>Positive by UV-visible:</p> <p>Hyperchromicity due to unwinding of double stranded DNA; strong binding affinity (binding constant (K_b) = 5.4 x 10³ M⁻¹).</p> <p>Docking analysis: binding to the</p>	<p>Reliability: 2</p> <p>Test item poorly described</p>	Limited	Ali et al., 2018

				minor groove, specifically at A:T base pairs			
Binding of TiO ₂ -NPs to single stranded (ssDNA) and double stranded DNA (dsDNA) (from salmon testes)	Method: Capillary electrophoresis coupled to UV detection and Infrared spectroscopy;	TiO ₂ NPs, 21 nm (no further information)	NSC: 2 sonication and partial information provided, agglomeration confirmed by measurements of the mean hydrodynamic diameter, no information on distribution	Positive: 1) Binding to both dsDNA and ssDNA 2) Infrared spectroscopy indicate that the binding interaction occurs via the phosphate backbone of DNA. 3) ssDNA is more easily absorbed on DNA than dsDNA. 4) Binding of TiO ₂ NPs with DNA produce a decrease in hydrodynamic diameter as measured by DLS.	Reliability: 2	Limited	Alsudi rand Lai, 2017

<p>DNA Binding of TiO₂NPs alone and in combination with Doxorubicin (DOX) to calf thymus DNA <i>in vitro</i>; <i>In vivo</i> antiproliferative role of the combination TiO₂NPs+ DOX in T47D and MCF7 breast cancer cell lines</p>	<p>Methods: UV–Vis absorption Spectroscopy and circular dichroism (CD); DNA thermal denaturation studies; flow cytometry and fluorescence microscopy for <i>in vitro</i> experiments</p>	<p>TiO₂NPs, anatase, nominal size <10 nm</p>	<p>NSC: 1 for the binding experiments. NSC: 3 for the cell studies (Sonication but no information on stability or dispersion in the cell media)</p>	<p>Positive 1) Binding of TiO₂NPs to DNA demonstrated by increased UV absorbance; 2) TiO₂NPs and DOX form a thermally stable complex with DNA; 3) CD spectra confirmed changes in DNA conformation induced by TiO₂ alone and in combination; 4) treatment of cells with a combination of TiO₂NPs and DOX shows more uptake of DOX and increased the toxicity of the single compounds</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Hekmat et al., 2013</p>
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<p>DNA Binding of TiO₂NPs alone and in combination with Paclitaxel (PTX) to calf thymus DNA <i>in vitro</i>; <i>In vivo</i> antiproliferative role of the combination TiO₂ NPs+ PTX in MB-231 breast cancer cells</p>	<p>Methods: UV–Vis absorption Spectroscopy and circular dichroism (CD); fluorescence spectroscopy in the presence of ethidium bromide (EtBr)</p>	<p>TiO₂NPs, anatase, < 10 nm</p>	<p>NSC:1 for the binding experiments. NSC:3 for the cell studies (Sonication but no information on stability or dispersion in the cell media</p>	<p>Positive 1) Binding of TiO₂NPs to DNA demonstrated by increased UV absorbance; 2) TiO₂NPs and PTX form a thermally stable complex with DNA; 3) CD spectra indicate changes in DNA conformation induced by TiO₂ alone and in combination; 4) fluorescence results suggest that PTX+ TiO₂ NPs form a complex via a non-intercalative mechanism; 4) a combination of TiO₂ NPs and PTX increased the toxicity of the single compounds in MB-231 cells</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Hekmat et al., 2020</p>
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EtBr: ethidium bromide; GFP: green fluorescent protein; FBS: Fetal Bovine Serum; TL: Telomere length

Table 4b: Other *in vitro* assays - Not genotoxicity endpoint but to be considered in the overall assessment

DNA methylation							
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 authors_year
Genomic DNA methylation	100 µg/mL TiO ₂ Exposure: 24 and 72 hours	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Dispersion and stability measured and reported, cellular internalisation confirmed.	TiO ₂ NPs affected the DNA methylation pattern in several mammalian cell lines <i>in vitro</i> : global DNA methylation was decreased; the promoter methylation of several specific genes was increased; the expression levels of several genes involved in the regulation of DNA methylation was altered.	Reliability: 5 The study does not address a genotoxicity endpoint, but it should be considered in the overall assessment of TiO ₂	Low (regarding genotoxicity, however it could be worth to address also epigenetic endpoints).	Pogribna et al., 2020
DNA methylation: ELISA analysis of the methylation	40 µg/cm ² Exp: 48 and 72 hrs	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Dispersion is reported and cellular uptake confirmed by EM. A level of agglomeration	48 h after exposure, TiO ₂ NPs did not affect methylation level. At 72 h of exposure, TiO ₂ NPs	Reliability: 5 This part of the study does not address a genotoxicity endpoint, but it	Low (regarding genotoxicity, however it could be worth to address also	Stocco et al., 2017*

level of LINE1 human transposon in human lung carcinoma cell line A549			is observed and also the internalisation of agglomerates in the exposed cells.	induced statistically significant demethylation. In a modified comet assay (see Table 3) oxidative DNA damage was reported in the same experimental conditions inducing demethylation	a should be considered in the overall assessment of TiO ₂	epigenetic endpoints).	
Genomic DNA methylation, intracellular ROS production human lung carcinoma cell line A549, human bronchial epithelial cell line 16HBE	concentrations of 0.1, 1, 10, 100 µg/mL Exp: 48h	TiO ₂ NPs, anatase 60 nm	NSC: 2 Dispersion measured in the culture medium but at a single concentration, some level of agglomeration confirmed. The presence of FBS in the test system may facilitate the dispersion. Two clusters of particles are reported, one around 100nm and another larger but the information is not reported for the tested concentrations. No information on stability. Low	Significant reduction of genomic DNA methylation levels after treatment with TiO ₂ NPs in both cell lines in the presence of a significant increase of the intracellular ROS content (DCFH-DA assay)	Reliability: 5 The study does not address a genotoxicity endpoint, but it should be considered in the overall assessment of TiO ₂	Low (regarding genotoxicity, however it could be worth to address also epigenetic endpoints).	Ma et al., 2017

			concentrations also tested.				
Transcriptomic and epigenomic changes in phagocytic murine cells line J774	10 µg/well 24h later, RNA isolation and spectrophotometric analysis	TiO ₂ , 1µm (no further information available)	NSC: 3 Sonication but no information on agglomeration/stability in the test media	Overall reduction of DNA methylation; bidirectional changes in methylation of some specific loci. In a few cases (loci involved in immune and inflammatory signalling) both transcriptional and DNA methylation changes occurred at the same time.	Reliability: 5 The study does not address a genotoxicity endpoint The analysis of DNA methylation pattern should be considered in the overall assessment of TiO ₂	Low (regarding genotoxicity, however it could be worth to address also epigenetic endpoints)	Emi et al., 2020
Cell transformation							
Cell transformation assay (morphological neoplastic transformation) in Balb/c 3T3 (Mouse embryo fibroblast)	10, 20 and 40 µg/cm ² Exp: 24, 48 and 72h	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Dispersion is reported and cellular uptake confirmed by EM. A level of agglomeration is observed and also the internalisation of agglomerates in the exposed cells.	Statistically significant increases in the frequency of morphologically transformed cells after treatment with TiO ₂ NPs (P25)	Reliability: 5 The testing method does not address genotoxicity.	Low (regarding genotoxicity, however it could be taken into consideration in the assessment of carcinogenicity)	Stocco et al., 2016 *

<p>Cell transformation assay (morphological neoplastic transformation) in Balb/c 3T3 (Mouse embryo fibroblasts)</p>	<p>uptake of TiO₂: 0.5 mg/ml TiO₂ NPs</p>	<p>1) TiO₂NPs, anatase, 11-18 nm (TEM)</p>	<p>NSC: 1</p> <p>Dispersion measured under the exposure conditions. Good stability confirmed up to 72h for the four materials.</p>	<p>Cell transformation assay: significant induction of transformed colonies (foci type III) with rutile, no significant effect with anatase.</p>	<p>Reliability: 5</p> <p>The testing method does not address genotoxicity.</p>	<p>Low (regarding genotoxicity, however it could be taken into consideration in the assessment of carcinogenicity)</p>	<p>Uboldi et al., 2016*</p>
	<p>Colony forming efficiency (CFE) test: 1, 5, 10 µg/ml</p>	<p>2) TiO₂, anatase, 60-400 nm (TEM), different geometry (TEM)</p>					
	<p>Exp:</p> <p>Cell transformation assay (CTA): 72 h exposure</p> <p>uptake of TiO₂: 72h exposure</p>	<p>3) TiO₂NPs, rutile, 10-35 nm (TEM), elongated particles (TEM)</p>					
	<p>Colony forming efficiency (CFE) test: 24 h and 72 h</p>	<p>4) TiO₂, rutile, 250-600 nm (TEM), different geometry (TEM)</p>					

FBS: Fetal Bovine Serum;