

Appendix K New *in vivo* genotoxicity studies

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The evaluation of the studies has been performed according the approach set in Appendix D

*** means that in this paper more than one assay is investigated/indicates when papers belong to more than one table**

**** means that in this paper both *in vitro* and *in vivo* assays are investigated (Appendix J)**

Table 1: *In vivo* Gene mutation assay

Test system/Test object	Dose/Route	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>gpt and Spi-mutation in liver</p> <p>Male C57BL/6J gpt delta mice, 6 animals/group</p>	<p>0, 2, 10, and 50 mg/kg i.v.</p> <p>Exposure: weekly for 4 consecutive weeks. Mice were euthanized on day 90 after the final injection of TiO₂NPs</p> <p>The route of administration is not relevant to dietary intake, but relevant for ADME/(geno)tox</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>Dispersion, stability and cellular internalisation measured and reported.</p>	<p>Negative</p> <p>gpt and Spi- mutation assay: Neither gpt nor Spi- mutation frequencies were significantly higher when compared with the vehicle control group at any dose. These results suggest that TiO₂NPs has no mutagenic effect on hepatocytes in mice 90 days after the last administration.</p>	<p>Reliability: 2 neither a positive control nor DNA from previous positive control was included, (although it is noted that in a previous study by the same authors (Suzuki et al., 2016) the positive controls performed as expected).</p>	<p>Limited</p>	<p>Suzuki et al., 2020</p>

Test system/Test object	Dose/Route	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>Pig-a mutation in erythrocytes gpt and Spi⁻ mutation in liver</p> <p>Male gpt Delta transgenic C57BL/6J</p> <p>5 mice/group</p> <p>Micronucleus test (Table 2), comet assay (Table 3)*</p>	<p>2, 10 and 50 mg/kg bw, intravenously, once a week for 4 consecutive weeks</p> <p>positive control: ENU or DEN</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm .</p>	<p>NSC:1</p> <p>Level of dispersion measured for each concentration and cellular internalisation confirmed by EM with Ti detection</p>	<p>Negative</p> <p>No significant increase in the frequency of Pig-a mutant frequency in erythrocytes nor of gpt and Spi⁻ mutants in liver.</p> <p>TiO₂NPs accumulated in liver and localised mainly in Kupffer cells.</p>	<p>Reliability: 1</p>	<p>Limited Route of administration not relevant for oral exposure</p>	<p>Suzuki et al.,2016*</p>

Test system/ Test object	Dose/Route	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
Pig-a Male Sprague-Dawley rats Micronucleus test (Table 2), comet assay (Table 3)*	Exposure: 3 endotracheal instillation over 8 days: 0.5, 2.5, and 10 mg/kg bw (a total particle surface area lung deposition of 87, 437, and 1700 cm ² /lung); Six rats were injected with MNU (N-methyl-N-nitrosourea) in one <i>i.p.</i> injection 35 days before kill as positive control for the mutation assay administered at a dose of 60 mg/kg	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 level of agglomeration reported for each dose and exposure confirmed by measurements in tissues	Negative No increase the frequency of mutant red blood cells and reticulocytes (target tissue exposure was demonstrated based on the positive outcome of the comet assay in blood)	Reliability: 2 Only three male rats per dose group, historical control data not reported.	Limited (limitations of the study and non-oral route of exposure)	Relier et al., 2017*

ENU: N-ethyl-N-nitrosourea; DEN: diethylnitrosamine; MNU: N-methyl-N-nitrosourea;

Table 2: *In vivo* chromosome aberrations/ micronucleus test

Test system/Test object	Dose/Route	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>Micronucleus test in bone marrow erythrocytes</p> <p>Male swiss mice, 6-8 weeks old</p> <p>Oxidative stress response</p>	<p>9.38, 18.75, 37.50, 75, 150 mg/kg bw administered by i.p. for five consecutive days</p>	<p>TiO₂NPs, anatase, < 25 nm</p>	<p>NSC: 2</p> <p>Dispersion and stability measured and some level of agglomeration confirmed.</p>	<p>Positive – after 5 days treatment statistically significant and dose-dependent increase in micronucleated polychromatic erythrocytes (MNPCEs) and decrease of the PCE:NCE ratio;</p> <p>After a 5 days recovery period since last treatment (sacrifice at day 10), the increase of MNPCEs was still statistically significant at the higher dose.</p> <p>All the animals appeared healthy during the exposure duration. No significant changes in the body weights of the animals.</p> <p>Significant changes in the activities of superoxide dismutase, catalase, and levels of reduced glutathione and malondialdehyde were observed in</p>	<p>Reliability: 1</p>	<p>Limited</p> <p>The relevance of the test system is considered limited as the route of administration is not relevant to dietary intake</p>	<p>Fadoju et al., 2019</p>

				liver and kidney of TiO ₂ NPs treated mice compared to untreated controls			
				The i.p. administration of TiO ₂ NPs induced ROS production and altered oxidative stress parameters in liver and kidney			
Micronucleus test in mouse bone marrow Male albino mice	0, 150, 250, and 500 mg/kg bw to 20-25 animals/dose and group Route of administration: i.p. Treatment: 7, 15, and 45 daily administrations	TiO ₂ NPs, 83.4 nm (SEM) (crystalline form unknown)	NSC: 2 Dispersion and stability measured and some level of agglomeration confirmed.	The Method section describes a protocol for micronucleus test, but data reported in Results concern chromosomal aberrations in an exceedingly low number of metaphases. No evaluation of the significance of the results reported in this study is possible	Reliability: 4 The description of the methods and the results is insufficient for the assessment	None The methods are inconsistent with the results reported The route of administration is not relevant to dietary intake	Rizk et al., 2020
Mammalian bone marrow chromosomal aberration test Male Swiss albino mice	Five consecutive daily oral administrations by gavage of TiO ₂ NPs in saline solution Group 1: vehicle control (0.5 ml saline solution), Groups 2-4: 50, 250 and 500 mg/kg bw	TiO ₂ NPs, 21 nm (crystalline form and shape unknown) TiO ₂ NPs, 80 nm (crystalline form and shape unknown)	NSC: 4 High doses. No information provided on dispersion.	Positive. Dose-related increase in the percentage of chromosomal aberrations. Positive. Dose-related increase in the percentage of chromosomal aberrations.	Reliability: 3 The number of scored metaphases are not consistent with the study protocol; gaps were included	Low	Ali et al., 2019

	<p>TiO₂NPs (21 nm): Groups 5-7: 50, 250 and 500 mg/kg bw TiO₂NPs (80 nm) 15 animals per group; 100 metaphases per animal analysed for chromosomal aberrations (gaps, breaks, fragments, deletions)</p>				<p>in the computation of aberrations;</p> <p>no positive control</p>		
<p>Bone Marrow Chromosomal Aberration Test</p> <p>Male Swiss albino mice, 5 animals/dose</p>	<p>50, 250 and 500 mg/kg bw i.p. injection daily for 7, 14 and 45 days</p> <p>Bone marrow collected 24 h after dosing</p>	<p>TiO₂NPs, 21 nm (crystalline form and shape unknow)</p>	<p>NSC: 3</p> <p>No information on dispersion but the use of hydroxyl propyl methyl cellulose (HPMC) as vehicle may have contributed to reduce agglomeration.</p>	<p>Inconclusive</p> <p>Dose-dependent increase of chromosomal aberration after 45 days treatment</p> <p>No significant increase in chromosomal damage after 7 and 14 days of injection (data not shown)</p>	<p>Reliability: 3</p> <p>No positive control</p> <p>Low number of metaphases analysed :300/group</p> <p>Results reported as % of total chromos</p>	<p>Low</p> <p>Study limitations and the route of administration is not relevant to dietary intake</p>	<p>Rizk et al., 2017</p>

					omal aberrations including gaps		
Micronucleus test in bone marrow Balb/c male mice 4 animals/group/dose	0.1, 1, 3 g/kg bw single administration by i.p. Bone marrow collected at 24 h 1 g/kg bw single administration by i.p. Bone marrow collected 24, 48, 72 and 96 h after dosing	TiO ₂ NPs, rutile, 28.88 nm (XRD), 5-45 nm (TEM)	NSC: 2 Ultrasonication for 15 min before use	Positive Dose-dependent increase of MNPCE. Time-dependent decrease of MNPCE for treatment at 1 g/kg bw. The percentage of MN frequencies in treated groups after 24, 48 and 72 h were higher than the control groups (p< 0.05). No significant difference in the treated group with respect to the control at 96 h.	Reliability: 2 exceedingly low baseline incidence of MNPCEs, inconsistency of tabular and graphical data	Limited Study limitations and route of administration is not relevant to dietary intake	Lotfi et al., 2016
Micronucleus test in bone marrow Balb/c male mice, 4 animals/group	10, 100, 500 mg/kg bw single administration by i.p. Bone marrow collected 24 h after dosing	TiO ₂ NPs, anatase, 20.17 nm (XRD), 1-25 nm (TEM),	NSC: 2 Suspensions dispersed in water and ultrasonicated	Equivocal Significant increase (p< 0.05) in MN frequency only at the highest dose. Inconsistent results at the lower doses	Reliability: 2 Single sampling was performed exceedingly low baseline incidence of MNPCEs,	Limited Study limitations and the route of administration is not relevant to dietary intake	Zirak et al., 2016

					inconsistency of tabular and graphical data		
<p>Micronucleus test and Mammalian bone marrow chromosomal aberration test</p> <p>Swiss-Albino mice</p> <p>Comet assay (Table 3)*</p>	<p>200 and 500 mg/kg bw per day</p> <p>90 daily oral administrations by gavage to 5 animals/sex/dose</p>	<p>TiO₂NPs, 58.25 8.11 nm (SEM) (crystalline form unknown)</p>	<p>NSC: 4 for in vivo assay.</p> <p>Insufficient information provided on dispersion and only high doses used.</p>	<p><i>In vivo</i> MN test: Positive. Significant (P<0.01) increase in the mean percentages of MNPCEs at the highest dose.</p> <p>The ratio PCE/total erythrocytes not affected by treatments, at any dose.</p> <p>Chromosome aberration test: Positive. Statistically significant increase (P<0.01) in the incidence of chromosomal aberrations at the highest dose.</p>	<p>Reliability: 2</p> <p>Data show some inconsistencies in the comparison of total MNPCEs or aberrant cells and their frequencies.</p>	<p>Limited</p>	<p>Chakrabarti et al., 2019 *,**</p>
<p>Micronucleus test in bone marrow cells</p> <p>Male Wistar rats</p> <p>Comet assay (Table 3)*</p>	<p>Sub-chronic (60 days) oral administration by gavage of TiO₂ NPs suspended in distilled water at 50, 100 and 200 mg/kg</p>	<p>TiO₂NPs, anatase, 5-12 nm</p>	<p>NSC: 2</p> <p>Sonication performed with no additional information.</p>	<p>Positive.</p> <p>Statistically significant and dose-related increase on MNPCEs at the two highest doses. Significant decrease of PCE/total erythrocyte ratio at top dose.</p>	<p>Reliability: 2</p> <p>Treatment determined distinct hematotoxicity,</p>	<p>Limited</p>	<p>Grissa et al., 2015 *</p>

	bw/day to 6 animals/dose				with formation of abnormally shaped red blood cells with Heinz bodies. It is not clear whether this could have biased the scoring of MNPCEs in bone marrow.		
Micronucleus test in bone marrow Female Wistar rats 6-8 animals/group Comet assay (Table 3)*	single dose 0.59 mg/kg bw equal to 1% of LD50 intravenous injection with sacrifice after 1 day, 1 week, 2 weeks, 4 weeks, MN determination in bone marrow cells (immature erythrocytes)	TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 for <i>in vivo</i> , as results for the bimodal distribution of particles are reported and 61% are at 84 nm, suggesting little agglomeration.	Inconclusive No increase in the incidence of MN in immature erythrocytes (PCEs) and no evidence of treatment related cytotoxicity (decrease in the proportion of immature erythrocytes)	Reliability: 3 Single low dose in combination with the inappropriate sampling schedule; no positive control	Low Study limitations and the route of administration not relevant to dietary intake	Kazimirova et al., 2019*,* *

<p>Micronucleus test in peripheral blood reticulocytes (RETs)</p> <p>Male Wistar rats</p> <p>6 animals/dose</p> <p>Comet assay (Table 3)*</p>	<p>5, 25, and 50 mg/kg/bw intravenously for 30 days at weekly intervals</p> <p>Blood collected after the last administration.</p>	<p>TiO₂NPs, anatase, 10-26 nm (TEM)</p> <p>(the Panel noted that 18% copper was reported for the particle surface chemical composition)</p>	<p>NSC: 1</p> <p>Good level of dispersion confirmed for the dispersion protocol but for a concentration 100 to 1000 times lower than those used for i.v. administration, thus possible agglomeration at the used doses cannot be excluded. Ti is measured in tissues but there is no information on cell internalisation.</p>	<p>Positive</p> <p>Dose-dependent increase of MN-RETs (p<0.05)</p>	<p>Reliability: 3</p> <p>No positive control was included</p> <p>Poor reporting of the study design</p>	<p>Low</p> <p>Study limitations and the route of administration not relevant to dietary intake, chemical composition of the test material</p>	<p>Kumar et al.,2016 *</p>
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<p>Micronucleus test in peripheral blood reticulocytes (RETs)</p> <p>Male gpt Delta transgenic C57BL/6J,</p> <p>5 mice/group</p>	<p>2, 10 and 50 mg/kg bw/week for 4 consecutive weeks</p> <p>intravenously</p> <p>The frequency of MN was determined in the blood specimens collected on day 2 and day 9 after final injection.</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>Level of dispersion measured for each concentration and cellular internalisation confirmed by EM with Ti detection.</p>	<p>Negative</p> <p>No decrease in % reticulocytes: % RETs in the 50 mg/kg TiO₂NP-treated group was significantly higher than that in the control on day 2</p>	<p>Reliability: 2</p> <p>No positive control</p>	<p>Limited administration route is not relevant to dietary intake</p>	<p>Suzuki et al., 2016*</p>
<p>Micronucleus test in bone marrow</p> <p>Male albino mice</p> <p>Comet assay (Table 3), other <i>in vivo</i> assays (Table 4)*</p>	<p>100, 200, and 400 mg/kg bw;</p> <p>i.p, once a week for one month</p> <p>10 animals/group</p> <p>Positive control: cyclophosphamide (CP)</p>	<p>TiO₂, anatase (no further information available)</p>	<p>NSC: 3</p> <p>No information provided on dispersion or stability.</p>	<p>Positive</p> <p>Statistically significant increase in MNPCEs at the highest dose only.</p>	<p>Reliability: 3</p> <p>Sampling time not reported</p> <p>Poorly reported results</p>	<p>Low characterisation of the test material. Study limitations; The route of administration is not relevant to dietary intake</p>	<p>El-Bassyouni et al., 2017*</p>

<p>Chromosome aberrations</p> <p>Swiss albino male mice</p> <p>5 animals/dose</p> <p>Comet assay (Table 3)*</p>	<p>0.2, 0.4, and 0.8 mg/kg/day by gavage for 28 days</p> <p>Positive control: MMC (by i.p.)</p> <p>Bone marrow sampled 18 h after the last treatment</p> <p>Analysed 150 metaphases per animal</p>	<p>TiO₂NPs, rutile, 21-31 nm (TEM), spherical and rod-shaped particles (TEM), 21-31 nm (TEM)</p>	<p>NSC: 2</p> <p>Dispersion measured and high level of agglomeration confirmed.</p>	<p>Positive</p> <p>Dose-related increase of cells with structural chromosomal aberrations (excluding gaps), statistically significant at the two highest doses, where mitotic index was reduced by 40 and 65 %, respectively</p>	<p>Reliability: 2</p> <p>Data in Table 1 are not consistent with the scoring of 750 metaphases (150 x 5 animals), as stated in Methods</p>	<p>Limited</p>	<p>Manivan et al., 2020*</p>
<p>Micronucleus test in bone marrow cells</p> <p>male Swiss albino mice</p> <p>5 animals/group</p>	<p>gavage 10, 50, 100 mg/kg bw /day for 14 days</p> <p>positive control EMS, single ip 100 mg/kg b.w.</p>	<p>TiO₂NPs, anatase, 20-50 nm</p>	<p>NSC: 1</p> <p>Sonication and level of agglomeration reported for each dose, a level of agglomeration observed</p>	<p>Positive</p> <p>Dose-related increase on MNPCEs statistically significant only at the highest dose.</p> <p>Data on bone marrow toxicity not reported.</p>	<p>Reliability: 1</p>	<p>high</p>	<p>Shukla et al., 2014*</p>
<p>Micronucleus test in peripheral blood</p> <p>Male Sprague-Dawley rats (4 rats/group)</p>	<p>Three endotracheal instillations over 8 days resulting in 0.5, 2.5 and 10 mg/kg bw</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>level of agglomeration reported for each dose and exposure confirmed by</p>	<p>Equivocal</p> <p>2 h after treatment: No increase in MN frequency</p> <p>35 days after treatment:</p>	<p>Reliability: 3</p> <p>No controls</p> <p>No adequate</p>	<p>Low</p>	<p>Relier et al., 2017*</p>

	Analysis 2 h and 35 days after exposure		measurements in tissues	Statistically significant increase of MN frequency at all tested doses without dose-response relationship	study design		
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CP: cyclophosphamide; EM: electron microscopy; EMS: ethyl methane sulfonate; MMC: mitomycin C; MN: micronuclei; MNPCEs: micronucleated polychromatic erythrocytes; PCE: polychromatic erythrocytes; RETs: reticulocytes

Table 3: *In vivo* DNA damage (Comet assay)

Test system/Test object	Dose/Route	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 in whole blood cells NMRI/Han mice, 7 weeks old, 5 female /group	1000, 2000 mg/kg bw, gavage, total dose in 24 hrs Comet assay: whole blood collected from the tail vein at 2 and 24 hrs from the end of treatment Acute toxicity test: evaluation 14 days after treatment	TiO ₂ NPs, anatase, 45 A (4.5 nm)	NSC: 4 No information provided on dispersion.	<i>Comet assay:</i> negative no increase in the mean number of cells with DNA damage. <i>Acute toxicity:</i> increase in body weight associated with enlarged livers and bloody abdominal ascites.	Reliability: 3 Non-standard comet assay in the parameters used for the evaluation of DNA damage No positive control	Low	Dekanski et al.,2018

				<p><i>Liver:</i> extensive histological changes; increased AST and ALT.</p> <p><i>Serum:</i> increased levels of urea and creatinine</p>			
<p>Comet assay in whole blood cells</p> <p>Wistar albino rats (4 months old) 6 males/dose</p>	<p>100 mg/kg bw by gavage, daily for 60 days</p>	<p>TiO₂NPs, anatase, 5-10 nm</p>	<p>NSC: 4</p> <p>High dose with no information on dispersion</p>	<p>Comet assay: positive (significant increase of total score for DNA damage)</p> <p>Toxicity: decreased weight gain; increased cholesterol, glucose, triglycerides and IL-6 levels; decreased SOD, total antioxidant status and catalase activity.</p>	<p>Reliability: 3</p> <p>data expressed as "DNA damage score, arbitrary units" (unusual definition)</p> <p>A single dose has been evaluated</p> <p>No positive control</p>	<p>Low</p>	<p>Grissa et al.,2017</p>
<p>Comet assay in liver</p> <p>Male albino mice (20-25 g)</p> <p>10-14 mice /group</p>	<p>Gavage</p> <p>150 mg/kg/day for 2 weeks (dose selection based on previous literature studies showing liver damage)</p>	<p>TiO₂NPs, anatase, 21nm (SEM)</p>	<p>NSC: 4</p> <p>A process for addressing dispersion is reported but results are not presented</p>	<p>Inconclusive</p> <p>3-fold increase in % tail DNA in the liver with unclear impact of liver toxicity</p> <p>Increased markers liver toxicity (ALT, AST, MDA), massive focal degeneration hepatocytes with mononuclear cell infiltration, inflammatory cascade</p>	<p>Reliability: 3</p> <p>A single dose has been evaluated</p> <p>No positive control</p> <p>Main focus on modulation of the effects induced by idebenone, carnosine and vitamin E</p>	<p>Low</p>	<p>Azim et al.,2015</p>

				(IL-6, TNF alpha, macrophage activation), apoptosis (caspase-3 activity, up-regulated expression of nuclear factor-erythroid-2-related factor 2, nuclear factor kappa B and Bax genes; down-regulated Bcl-2	severe liver injury no measures for identifying cytotoxicity in the Comet assay (ghost, halo cells)		
Comet assay +/- Fpg in Peyer' immune cells Adult male Wistar rats (175–200 g) 10-12 rats/group	gavage <i>in vivo</i> Study: TiO ₂ NPs (NM-105) and E171 (10 mg/kg bw per day for 7 days) Control group: only water	1) E 171, anatase, 20-340 nm (118 nm) (TEM); 44.7% (< 100 nm 2) TiO ₂ NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 Dispersion and stability according to the Nano genotox protocol. Cellular internalisation confirmed for the <i>in vivo</i> experiment.	Comet assay +/- Fpg in PP cells: negative both E171 and TiO ₂ NPs (NM-105)	Reliability: 2 no positive control	Limited	Bettini et al., 2017
Comet assay in heart Males albino rats, adult, 8/group	Gavage once daily (90 days) 0, 1200 mg/kg bw day Control groups untreated, saline	TiO ₂ NPs, anatase/rutile, < 100 nm	NSC: 4 Sonication x 10 min before treatment; no additional information provided.	Inconclusive Increased levels in the % of tailed nuclei, tail length, tail DNA%, and tail moment with unclear impact of heart toxicity Histopathology: disorganized, degenerated and apoptotic	Reliability: 3 A single dose was analysed (1/10 LD50) No positive control severe tissue injury	Low	El-Din et al., 2019

				cardiomyocytes + evidence of oxidative burden (increased 3-nitrotyrosine)			
Comet assay in testis Male albino rats (150-200 g) 10 rats/group	Gavage 1200 mg/kg bw/day 12 weeks Group I: negative control Group II: 5% gum acacia solution (solvent for TiO ₂ NPs) Group III: N-acetylcysteine (NAC) (100 mg/kg) Group IV: TiO ₂ NPs Group V: NAC + TiO ₂ NPs	TiO ₂ NPs, 21 nm (crystalline form and shape unknow)	NSC:4 Acacia gum used as dispersant agent (mentioned as solvent) no indication on the level of dispersion achieved. No additional information on dispersion or stability. High dose used (1200 mg/kg bw/day) by gavage.	Inconclusive Induction of DNA damage in the testes (only photographs) with unclear impact of testis toxicity Histopathological changes identified in the testis together with activation of TNF α ; <i>Oxidative stress:</i> increased MDA levels and decreased GSH and testosterone in the blood	Reliability: 4 single dose; no positive controls; no numerical data Focus on the antioxidant effect of NAC	Low	Elnagar et al., 2018
Comet assay in liver Male Wistar male albino	Gavage 600 mg/kg bw/day 5 days	TiO ₂ NPs, anatase, 60 \pm 10 nm (TEM) (47 \pm 8% of particles; the remaining	NSC:4 Dispersion considered but measured for different conditions	Inconclusive Increased levels of damage in the liver (tail length, tail moment, and relative DNA content in the	Reliability: 3 No details on the comet assay in materials and methods	Low	Fadda et al., 2019

rats (180–200 g)	<p>Group I: control (1% Tween)</p> <p>Group II: TiO₂ NPs</p> <p>Group III: TiO₂ NPs + Melatonin (50 mg/kg)</p> <p>Group IV: TiO₂ NPs + Carnosine (200 mg/kg)</p> <p>Melatonin & Carnosine administered for 3 weeks</p>	particles were agglomerates 100-500 nm)	(including solvent) than those used for the actual exposure.	<p>tail) with unclear impact of liver toxicity</p> <p><i>Liver:</i> increased levels of ALT, MDA, caspase-3 and decreased levels of GSH.</p> <p><i>Sera:</i> increased levels of TNF-α, IL-6, C-reactive protein (CRP), IgG, nitric oxide (NO), vascular endothelial growth factor (VEGF).</p> <p><i>Histology in the liver:</i> necrosis in all hepatocytes, pyknosis, kariolysis, vacuolated cytoplasm.</p> <p>Combined treatments with carnosine or melatonin decreased the observed effect.</p>	<p>Single dose and no positive controls</p> <p>Focus on antioxidants effects of carnosine and melatonin.</p> <p>severe liver injury</p>		
<p>Comet assay in liver</p> <p>Male Wistar albino rats (150-170 g)</p> <p>10 rats/group</p>	<p>Gavage</p> <p>1 g/kg bw/day</p> <p>21 days</p> <p>Group 1: control (1% CMC)</p> <p>Group 2: TiO₂-NPs</p>	TiO ₂ NPs, anatase, 60 ± 10 nm (TEM) (47 ± 8% of particles; the remaining particles were agglomerates 100-500 nm)	<p>NSC: 4</p> <p>High doses. The characterisation provided is not convincing</p>	<p>Inconclusive</p> <p>Increases in the tail length and DNA% in the tail in the liver with unclear impact of liver toxicity</p> <p><i>Liver:</i> increased levels of MDA and caspase-3</p>	<p>Reliability: 3</p> <p>Single dose and no positive controls</p> <p>Focus on antioxidants effects of Quercetin and Idebenone</p> <p>Severe toxicity and inflammation</p>	Low	Fadda et al., 2018

	<p>Group 3: TiO₂ NPs + Quercetine (200 mg/kg bw)</p> <p>Group 4: TiO₂ NPs + Idebenone (200 mg/kg bw)</p>			<p><i>Sera</i>: increased in ALT, glucose, TNF-α, IL-6, CRP, IgG, NO and VEGF.</p> <p><i>Histopathology</i>: TiO₂ NPs induced severe degeneration of most hepatocytes (nuclear pyknosis, karyolysis, cytoplasmic vacuolation)</p> <p>Combined treatments with Quercetin and Idebenone decreased the observed effect.</p>	concurrent with increase in DNA damage		
<p>Comet assay in liver</p> <p>Male Sprague-Dawley rats, (160–200 g)</p> <p>10 rats/group</p>	<p>Gavage</p> <p>150 mg/kg bw</p> <p>6 weeks</p> <p>Group 1: TiO₂ NPs</p> <p>Group 2: TiO₂ NPs + thymoquinone (20 mg/kg bw)</p> <p>Group 3: TiO₂ NPs + avenanthramides (20 mg/kg bw)</p> <p>Group 4: thymoquinone</p>	<p>TiO₂ NPs, 21 nm (crystalline form and shape unknow)</p>	<p>NSC: 4</p> <p>High dose. Insufficient information on dispersion, Tween 80 used for the administration by gavage.</p>	<p>Inconclusive</p> <p>Increased DNA damage (tail length and tail moment) with unclear impact of liver toxicity</p> <p><i>Histopathological</i> alterations observed in the liver, brain, lung, kidney, heart and testes.</p> <p><i>Hematology</i>: increased total leukocytic count, lymphocytes and neutrophils</p>	<p>Reliability: 3</p> <p>Single dose and no positive controls</p> <p>severe toxicity and inflammation concurrent with increase in DNA damage</p>	<p>Low</p>	<p>Hassanein et al., 2017</p>

	<p>Group 5: avenanthramides</p> <p>Group 6: control (1% Tween)</p>			<p><i>Sera</i>: increased levels of AST, ALT, LPO, and TNF-alpha and decreased levels of GSH and testosterone</p>			
<p>Comet assay lung +/- Fpg and Ogg1 enzymes in liver and lung</p> <p>Lean Zucker rats, female, 8 -13 weeks of age, 10 animals per group</p>	<p>Gavage</p> <p>50 and 500 mg/kg bw once a week for 10 weeks; sacrifice 24 hours after last administration</p> <p>positive controls were KBrO₃ treated THP-1 cells</p>	<p>E171, anatase (0.2% rutile), three size groups of particles: 135 ± 46 nm, 305 ± 61, 900 ± 247 nm (TEM image)</p>	<p>NSC: 2</p> <p>A dispersion protocol from ENPRA project mentioned, TEM indicates all three groups larger than 100nm and the hydrodynamic size in the media is reported as 270nm, Z potential of -37.2mV (Ref 14). However, all these measurements have been done at a much lower concentration, and signs of agglomeration were observed.</p>	<p><i>Comet assay in liver and lung</i> +/- Fpg or Ogg1 enzymes: negative;</p> <p>Repair capacity of oxidatively damaged DNA in lung (modified Comet assay): negative results</p> <p><i>Telomere length</i> in liver, lung, spleen: shortening in lung (p<0.05)</p> <p>Expression tight junction proteins (TJP1) in colon mucosa: TJP1 down-regulated (p<0.01)</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Jensen et al.,2019</p>

<p>Comet assay +/- Ogg1 and EndoIII enzymes in liver and lung</p> <p>B6C3F1, male, 6- 7 week old, 5 animals per group</p>	<p>50 mg/kg bw/day for 3 days, i.p.</p> <p>sacrifice 4 hours after last treatment</p> <p>MMS positive control</p>	<p>TiO₂ NPs, anatase, 8.9–15.3 nm (TEM)</p>	<p>NSC:1</p> <p>Dispersion considered under the exposure conditions for the only dose used. Some level of agglomeration confirmed. Quantification of tissue accumulation (presence in liver and lungs following i.p. administration) confirms systemic exposure of tissues.</p>	<p><i>Comet assay +/-</i> Ogg1 and EndoIII enzymes: positive in liver without enzymes; positive in liver and lung with enzymes. Analysis of %tail DNA. MMS positive control</p> <p>TiO₂ NPs accumulation in liver and lung</p> <p><i>gene expression:</i> metabolic homeostasis altered in liver; induced ox stress, inflammatory response, apoptosis in lung.</p>	<p>Reliability: 2</p> <p>Single dose</p> <p>No histopathological assessment of the toxicity</p>	<p>Limited</p> <p>Due to study limitation and the route of administration is not relevant to dietary intake</p>	<p>Li et al., 2017b</p>
<p>Comet assay in blood and liver</p> <p>Male Wistar rats (220 g), 6 animals per group</p>	<p>0.5 mg/kg bw/day for 45 days by gavage</p>	<p>TiO₂ NPs, 42 nm (TEM) (crystalline form unknow)</p>	<p>NSC:1</p> <p>Insufficient information on dispersion and stability, possible (large) agglomeration, but low dosage administered by gavage.</p>	<p>Negative results (% tail DNA)</p> <p><i>Redox</i> parameters in blood: no changes in GSH levels, GPx activity and CAT activity</p> <p><i>ADME:</i> Ti concentration in blood, kidney and liver were statistical significantly</p>	<p>Reliability: 2</p> <p>Single low dose</p> <p>No positive control</p>	<p>Limited</p>	<p>Martins et al., 2017</p>

			Quantification of tissue accumulation (liver, blood and kidney), confirms systemic exposure of organs following oral administration.	increased compared to control			
Comet assay in sperm Males Wistar rats, 8-week old, 6 rats/dose	i.v. 0, 5, 25, and 50 mg/kg bw 30 days (weekly interval administration)	TiO ₂ NPs, anatase, 10-20 nm (TEM)	NSC: 1 Insufficient information provided on dispersion and stability for the i.v. administration, but dose-dependent accumulation in testis	Positive: increased DNA breaks (tail length, tail movement, tail migration) at 25 and 50 mg/kg Bioaccumulation in testicular cells. Reduced sperm count, increase in apoptosis (DNA fragmentation, caspase-3), creatine kinase. <i>Antioxidant enzymes:</i> decrease in CAT, GSH-Px, and SOD; increase in lipid peroxidase.	Reliability: 3 limited number of cells, 50 cells/slide) unusual parameters to measure DNA damage by Comet assay (tail movement). No positive controls	Low Due to the study limitations and the route of administration is not relevant to dietary intake	Meena et al., 2015a
Comet assay in liver and lung C57BL/6 (B6JBOM-F)	Intratracheal instillation, i.v. and gavage 162 µg/animal TiO ₂ NPs	TiO ₂ NPs, 10.5 nm (crystalline form and shape unknow)	NSC: 1 Dispersion measured under the exposure conditions.	<i>Comet assay:</i> liver negative: no increase in DNA strand breaks (% tail DNA and tail length) in all exposure conditions;	Reliability: 3 No positive controls	Low	Modrzynska et al., 2018

female mice, 9/group 6 weeks,	Sacrifice 1, 28, 180 days after single administration		Possible small agglomerates present.	<p>lung equivocal: small increase statistically significant by intratracheal instillation at a single time point (180 days)</p> <p>ROS measured in an acellular system</p> <p>TiO₂ NPs detected in liver at 180 days</p>	unknown no. of cells counted		
Comet assay in heart, liver, kidney Kun Ming, 6-8 weeks old, 8/sex/group	<p>Gavage 2 g/kg bw, 7 days</p> <p>control group</p> <p>group 1: TiO₂NPs (2 g/kg bw),</p> <p>group 2: TiO₂NPs + GSPE (167 mg/kg bw)</p> <p>group 3: TiO₂NP + GSPE (500 mg/kg bw).</p>	TiO ₂ NPs, anatase, 10-25 nm (TEM, SEM)	<p>NSC: 2</p> <p>Sonication of the suspension performed with PBS. Dispersion measured in a much lower concentration than the actual dose administered. A level of agglomeration confirmed even at lower concentrations than the high dose used (2g/kg).</p>	<p>Inconclusive; increase in tail DNA% and tail moment in heart, liver, kidney with unclear impact of tissue toxicity</p> <p>Effects decreased by Grape seed procyanidin extracts (GSPE).</p> <p><i>Histopathology:</i> <i>heart:</i> slight changes such as deranged architecture and slight vacuolar degeneration of myocardial cells, interstitial hyperemia and edema <i>liver:</i> variable degrees of dilatation of sinusoids accompanied by deranged and</p>	<p>Reliability: 3</p> <p>Single dose</p> <p>No positive control</p> <p>Mild (heart) to moderate (liver, kidney) target tissue injury</p> <p>Focus on Grape seed procyanidin extracts</p>	Low	Niu et al., 2017

				<p>swollen hepatocytes with some of the cells showing necrosis. <i>kidney:</i> the lumens of renal tubule were swollen and filled with the proteinic liquids, serious swelling in the renal glomerulus and infiltration of inflammatory cells emerged.</p> <p><i>enzymatic activities:</i> increased LDH, CK and CKMB. Increased ALT, AST, BUN and Cr levels.</p> <p><i>Oxidative stress markers:</i> increased ROS and MDA levels in heart, liver and kidney. Decreased SOD and GSH-Px in the liver and kidney tissues. No changes in heart. Decreased Nrf2, NQO1, HO-1 and GCLC protein expression in heart and liver NQO1 and GCLC protein expression in kidney.</p>			
Comet assay in liver and kidney	Wild type (wt) mice: 500, 1000 and 2000 mg/kg bw Knock-out (ko) mice: 1000 mg/kg bw	TiO ₂ NPs, anatase, 10-25 nm (SEM, TEM)	NSC: 4 for the <i>in vivo</i> assay Very high doses no	<i>Comet assay in liver and kidney: positive;</i> measured Olive tail moment: dose-dependent increase in	Reliability: 2 No positive control	Limited	Shi et al., 2015**

<p>ICR and ICR Nrf2^(-/-) knock out mice, 6–8 weeks old, 20 g, 12 wt/sex/group ; 8 knock-out/sex/group</p>	<p>gavage once a day for 7 days</p> <p>overnight fasting before treatment</p> <p>sacrifice one day after last treatment</p>		<p>indications for ensuring proper dispersion.</p>	<p>wt and ko mice (up to 11x increase in kidney ko mice vs 7x wt, high dose)</p> <p><i>ROS</i> levels in liver and kidney: dose-dependent increase stronger in ko than wt</p> <p><i>Oxidative stress</i>: MDA level in liver and kidney dose-dependent increase; SOD and GSH-Px activities reduced; stronger changes in ko than wt mice</p>	<p>No information on histopathology reported</p> <p>Insufficient parameter measured (Olive tail moment)</p>		
<p>Comet assay in bronchiolar lavage (BAL) cells, lung and liver</p> <p>Female C57BL/6J BomTac mice, 6–7 weeks old, 8 animals/group</p>	<p>Pulmonary exposure by intratracheal instillation</p> <p>Single treatment with 18, 54 or 162 µg/mouse (corresponding to 1.5, 5, 15 working days at the Danish occupational exposure level for TiO₂ (6 mg Ti/m³-10mg TiO₂/m³))</p> <p>Sacrifice 1, 3 and 28 days after treatment</p> <p>H₂O₂ positive control</p>	<p>TiO₂ NPs, rutile, 10 nm,</p>	<p>NSC:1</p> <p>Dispersion measured for all exposure conditions and no differences observed among doses. Good level of dispersion confirmed for TiO₂ NPs</p>	<p><i>Comet assay</i>: positive;</p> <p>i) BAL cells: negative results for %tail DNA; increased tail length at day 3 (low and intermediate doses; p<0.001);</p> <p>ii) lung: increased %tail DNA and tail length all doses (no dose response) at day 28 (p<0.001)</p> <p>iii) liver: increased %tail DNA at day 3 (only high dose) and increased tail length at</p>	<p>Reliability: 1</p>	<p>Limited</p> <p>Due to the route of exposure</p>	<p>Wallin et al., 2017</p>

				<p>day 28 (lowest and highest doses)</p> <p><i>Pulmonary inflammation level:</i> increased inflammatory cells (neutrophils, macrophages, eosinophils, lymphocytes) in BAL cells at day 1 and 3 ($p < 0.001$), day 28</p>			
<p>Comet assay in brain</p> <p>Male wistar rats (8 week old)</p> <p>6 rats/group</p>	<p>i.v. (caudal vein)</p> <p>0, 5, 25, 50 mg/kg bw once a week for 4 weeks</p>	<p>TiO₂ NPs, anatase, 10–20 nm (TEM)</p>	<p>NSC:1</p> <p>Dispersion measured under the exposure conditions (PBS used for the IV injection). Dose-dependent Ti accumulation measured in the brain.</p>	<p><i>Comet assay:</i> positive; increased tail length, tail movement and tail migration in brain cells (25 and 50 mg/kg).</p> <p><i>oxidative stress:</i> increased ROS, NO, MDA, IFN-gamma, TNF-alpha and activation of NF-KB. Decreased levels of SOD and GSH-Px. Expression of apoptosis markers (p53, Bax, Bcl-2, and cyto c).</p>	<p>Reliability: 2</p> <p>No positive control to measure DNA damage by comet assay (tail movement)</p> <p>No information on histopathology reported</p>	<p>Limited</p> <p>route of the administration is not relevant to dietary exposure</p>	<p>Meena et al., 2015b</p>

<p>Comet assay in blood</p> <p>Male rats Long-Evans, (10-12 weeks)</p> <p>3/males/group</p>	<p>i.p.</p> <p>5 mg/kg bw of TiO₂ dissolved in 1 mL xylocaine for 3 days (daily i.p. injection)</p> <p>group G1: sacrificed after 48 hrs</p> <p>group G2: sacrificed after 72 hrs</p>	<p>TiO₂ NPs, 21 nm (crystalline form and shape unknow)</p>	<p>NSC: 3</p> <p>No information provided on the dispersion conditions and the dispersion level achieved in the i.p.suspensions .</p>	<p><i>Comet assay</i> (lymphocytes): no cytotoxicity or induction of DNA breaks in group G1 or G2.</p> <p><i>Histopathology:</i> <i>liver in group G1:</i> vacuoles in the hepatocyte cytoplasm. <i>Liver in group G2:</i> hepatocytes with foamy cytoplasm and nucleus with granular chromatin. <i>Kidney:</i> slight glomerular retraction and moderate vascular congestion.</p>	<p>Reliability: 4</p> <p>No data provided (only photographs of cells) No positive controls</p>	<p>Low</p>	<p>Moran-Martinez et al., 2018</p>
<p>Comet assay +/- Fpg in liver, kidney, testis</p> <p>Wild-type and Ogg1^{-/-} mice</p>	<p>i.v.</p> <p>single dose 5 mg/kg bw; sacrifice at day 1 and day 7 after dosing</p>	<p>TiO₂NPs, 21 nm [the Panel noted that from the description of the test material, it can be P25/NM-105 (anatase/rutile, 15-24 nm)]</p>	<p>NSC: 1</p> <p>Dispersion and stability measured for the final solution (BSA and PBS used in the suspension),</p>	<p><i>Comet assay +/- Fpg</i> in liver, kidney, testis; Day 1: no DNA damage Day 7: positive; increased % tail DNA only + Fpg in testis (only in wt mice)</p> <p>positive control: X-ray treated mice</p> <p><i>Sperm DNA fragmentation assay:</i> no changes</p>	<p>Reliability: 2</p> <p>Single dose</p> <p>difficult interpretation of the results (i.e. positive only in testis and at a late harvest time)</p>	<p>Low</p> <p>the route of administration is not relevant to dietary intake</p>	<p>Asare et al., 2016</p>

				<p><i>Gene expression</i></p> <p>Ogg1-/-testis, day 7: increased expression genes involved in DNA damage response (Atr, Rad51, Ddb), oxidative stress response (Sod1)</p>			
<p>Comet assay in liver and kidney</p> <p>Swiss-Albino mice, 7–8 weeks old, 5/sex/dose</p>	<p>Gavage</p> <p>200 and 500 mg/kg bw/day for 90 days (OECD TG 408, 1998 compliance)</p> <p>Cyclophosphamide (CP) positive control</p>	<p>TiO₂ NPs, 58.25 ± 8.11 nm (SEM) (crystalline form unknow)</p>	<p>NSC: 4 for in vivo assay.</p> <p>Insufficient information provided on dispersion and only high doses used.</p>	<p>Inconclusive; increase in % tail DNA at the high dose</p> <p>liver and kidney (200 and 500 mg/kg bw) with unclear impact of tissue toxicity: decreased S phase cells, increased G2/M cells, decreased cell viability</p> <p><i>Sub-chronic oral toxicity:</i> severe toxicity at 500 mg/kg; bleeding in the cranial and peritoneal cavities</p> <p><i>Biochemistry & haematological</i> High-dose group: increased levels of liver enzymes, triacylglycerol and total cholesterol; decreased red blood cell count and haemoglobin</p>	<p>Reliability: 3</p> <p>No positive control</p> <p>Severe injury in the target tissues, liver and kidney</p>	<p>Low</p>	<p>Chakrabarti et al., 2019 *,**</p>

				<p>concentration, decreased platelet count, and increased white blood cell count.</p> <p><i>Histopathology:</i> high dose group: Liver: abnormal cellular architecture with signs of bleeding in hepatic cells. Kidney: tubule nephrosis with acute tubular necrosis, disrupted glomerulus, loosely packed epithelial cells in the tubular lumen.</p> <p><i>Behavioural observations:</i> high-dose group: abnormality in autonomic, CNS, somato-motor activity.</p>			
<p>Comet assay in leukocytes</p> <p>Wistar, 4 months, 6/males/dose</p>	<p>Gavage</p> <p>50, 100, and 200 mg/kg bw per day for 60 days</p>	<p>TiO₂ NPs, anatase, 5-12 nm</p>	<p>NSC:2</p> <p>Sonication performed with no additional information.</p>	<p><i>Comet assay in leucocytes: positive;</i> increased DNA damage (as Tail moment) in 100 and 200 mg/kg bw treated animals</p> <p><i>Hematological parameters :</i> TiO₂ NPs reduced RBC and HCT, while increased MCV,</p>	<p>Reliability: 2</p> <p>No positive control</p> <p>Insufficiently reported parameter (tail moment);</p> <p>no information on cytotoxicity in the comet assay</p>	<p>Limited</p>	<p>Grissa et al., 2015*</p>

				<p>PLT, MPV and WBC in a dose-related manner.</p> <p><i>Blood smears analysis:</i> 50 mg/kg bw group: abnormally shaped red cells; 100 and 200 mg/kg bw groups: same as above + lymphocytes and neutrophils with abnormally shaped nuclei and hypersegmented nuclei.</p>			
<p>Comet assay in PBMC</p> <p>Wistar, rat female, 8 weeks old, 6-8 animals per group</p>	<p>intravenous injection 0.59 mg/kg bw</p> <p>single exposure, different time-points of sacrifice (1 day, 1, 2 and 4 weeks)</p> <p>H₂O₂ positive control (ex vivo exposure)</p>	<p>TiO₂NPs (NM-105), anatase/rutile, 15-24 nm</p>	<p>NSC: 1 for <i>in vivo</i>, as results for the bimodal distribution are reported and 61% are at 84 nm, suggesting little agglomeration.</p>	<p><i>Comet assay</i> +/- Fpg in isolated PBMCs: positive; % tail DNA increased only at day 1 w/o Fpg (dispersion with low agglomeration)</p>	<p>Reliability: 2</p> <p>a single dose tested</p>	<p>Limited the route of administration is not relevant to dietary intake</p>	<p>Kazimirova et al., 2019 *,**</p>
<p>Comet assay in splenocytes</p> <p>male Wistar rats, 8 weeks old (220 g),</p>	<p>5, 25, and 50 mg/kg bw of TiO₂NPs, i.v., once a week for 30 days</p>	<p>TiO₂NPs, anatase, 10-26 nm (TEM)</p> <p>(the Panel noted that 18% copper was reported</p>	<p>NSC: 1</p> <p>Good level of dispersion confirmed for the dispersion protocol but for a</p>	<p><i>Comet assay</i> in splenocytes: inconclusive; increase of comet parameters (tail moment, length, migration) at 25 and 50 mg/kg b.w. with</p>	<p>Reliability: 3</p> <p>No positive control</p> <p>Moderate to severe target tissue injury at the two highest dose levels.</p>	<p>Low</p> <p>Due to the study limitations and the route of administration</p>	<p>Kumar et al., 2016*</p>

<p>6 animals per group</p>		<p>for the particle surface chemical composition)</p>	<p>concentration 100 to 1000 times lower than those used for i.v. administration, thus possible agglomeration at the used doses cannot be excluded . Ti is measured in tissues but there is no information on cell internalisation.</p>	<p>unclear impact of tissue toxicity</p> <p>Ti dose-dependent accumulation in spleen</p> <p><i>Histopathology</i> in spleen: dose-related proportion of dead cells and reduction in white matter, apoptosis/necrosis, infiltration of the megakaryocytes.</p> <p>ROS increased</p> <p><i>Hematological</i> Parameters: increase in neutrophils, IFN-γ, and IL-4-secreting CD4+ cells at 50 mg/kg b.w.</p> <p>M1 macrophage (from spleen) activation: dose-dependent increase of nitric oxide (NO), expression NF-κB, COX-2, IL-1β, IL-6, and TNF-α. Inflammatory cytokines increased also in blood. M2 macrophages activation: sign</p>		<p>not relevant to dietary intake</p>	
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				increase expression arg-1, Ym-1 at 5 mg/kg bw, not confirmed at the highest dose.			
Comet assay in liver Male gpt Delta transgenic C57BL/6J, 8 weeks old, 5 animals per group	i.v. 2, 10 or 50 mg/kg bw/week for 4 weeks (maximum dose selected stably dispersed for a long period) Comet assay: day 3 after the last administration; no positive control	TiO ₂ NPs (P25), anatase/rutile, 15-24 nm	NSC: 1 Level of dispersion measured for each concentration and cellular internalisation confirmed by EM with Ti confirmation.	<i>Comet assay</i> in liver: negative ; no increased % tail intensity. Localization of TiO ₂ NPs: Ti detected in parenchymal hepatocytes and Kupffer cells (also in the nucleus)	Reliability: 2 no positive control timing of sacrifice not in line with OECD TG 489 however it is considered in this case a minor limitation because the presence of particles was demonstrated	Limited	Suzuki et al., 2016*
Comet assay in liver Male albino mice, 10 animals/group	400 mg/kg bw TiO ₂ anatase i.p single dose once per week for one month positive control: cyclophosphamide	TiO ₂ , anatase (no further information available)	NSC: 3 No information provided on dispersion or stability.	<i>Comet assay</i> in liver: positive ; measured % cells with DNA damage; a score 0-3 of damage visually assigned to cells. Increased at the highest dose (19%) vs control (9%) Oxidative stress: significant increase at the highest dose of glutathione peroxidase activity, albumin, globulin, and A/G	Reliability: 3 Not standard method to analyse Comet assay parameters Sampling time not reported	Low Due to the study limitations and the route of administration is not relevant to dietary intake	EI-Bassyouni et al., 2017*

				ratio, ALT and AST, B-cell population			
Comet assay in brain Male Swiss Webster, 10–12 weeks, 3/group	Gavage 500 mg/kg bw/day 5 days exposure; sacrificed after 24 h, 7 days, and 14 days	TiO ₂ NPs, anatase/rutile, 46.23 ± 3.45 nm (TEM)	NSC: 4 High dose only, insufficient information on dispersion and stability	<i>Comet assay:</i> inconclusive; significant increase in tail length and tail moment at 14 days; not increased %tail DNA with unclear impact of tissue toxicity <i>Histopathology:</i> extracellular and intracellular brain edema (after 24 h and 7 days), vacuolation of nerve cells (after 14 days)	Reliability: 3 Single dose tested No positive control Moderate to severe target tissue injury	Low	Mohamed and Hussein, 2016*
Comet assay in liver, bone marrow, spleen, thymus, lymph nodes Swiss albino male mice (20–25 g) 5 mice/group	Gavage 0.2, 0.4 and 0.8 mg/kg bw; 28 days	TiO ₂ NPs, rutile, 21-31 nm (TEM), spherical and rod-shaped particles (TEM), 21-31 nm (TEM)	NSC: 2 Dispersion measured and high level of agglomeration confirmed.	Positive increased % tail DNA in spleen (all doses), liver, thymus and lymph nodes (0.4 and 0.8 mg/kg bw) and bone marrow (only the highest dose).	Reliability: 2 No positive control for the comet assay No information on organ toxicity Poorly reported, unclear protocol (number of scored cells/animal)	Limited	Manivanan et al., 2020*

Comet assay in spleen Wistar rats 10 animals/group	Gavage 20 or 40 mg/kg bw per day 90 days	TiO ₂ (from Sigma, no information on constituent particle size distribution nor crystalline form)	NSC: 3 Suspension in 0.5 % hydroxypropyl methylcellulose with no indication of dispersion level or stability	Positive Dose dependent increase of 3 parameters analysed (tail length, %DNA in tail and the tail moment) Histopathology of the spleen indicated a dose-dependent increase in tissue damage as indicated by lymphoid necrosis, white and red pulp expansion, increased number of macrophages, haemorrhage and haemosiderin deposits, fibrosis. Suppression of immune response (analysis of several immunotoxic and cytotoxic parameters)	Reliability: 3 No positive controls No experimental details (including number of cells counted) Insufficient reporting Mild to moderate injury in the target tissue	Low	Hashem et al., 2020
Comet assay in white blood cells and BAL cells Female	17 and 117 nm by gavage and oropharyngeal aspiration (10, 50, 250 µg/mouse)	1) TiO ₂ NPs (JRCNM1020 2a), anatase, 17 nm	NSC: 1 Specific protocol based on NANOREG for getting	Positive Blood (TiO ₂ NPs 17 nm and TiO ₂ 117 nm) Increase in % tail DNA by TiO ₂ NPs 17 nm	Reliability: 2	Limited	Murugadoss et al., 2020 **

<p>Mice C57BL/6JRj, 8-week-old</p>	<p>Analysis in BAL and blood cells 3 days after treatment positive control for Comet assay: H₂O₂</p>	<p>2) TiO₂ (JRCNM1022 00a), anatase, 117 nm</p>	<p>different levels of agglomeration.</p>	<p>(small aggregates) and TiO₂ 117 nm (large and small aggregates) at all doses higher increase in % tail DNA by large 117 nm aggregates BAL cells: results not shown</p>	<p>No dose-response</p>		
<p>Comet assay in BAL cells, lung and liver C57BL/6j mice, 7-week-old females , 7 mice/group for BAL and tissue collection 5 mice/group for histology 2-4 mice/vehicle control</p>	<p>18, 54 and 162 µg/mouse (intratracheal) BAL cells, lung, liver: assessment at 1, 3, 28, 90, 180 days post exposure Positive control: H₂O₂-treated A549 cells Histopathology: 28, 90 and 180 days post-exposure</p>	<p>1) TiO₂NPs, anatase (11% rutile) (XRD), 12-50 nm (TEM) 2) TiO₂NPs, anatase (6% rutile) (XRD), 16-28 nm (TEM)</p>	<p>NSC: 1 Dispersion protocol and information on the level of dispersion provided. Confirmation of exposure and cellular internalisation does not include confirmation that the particles contain Ti.</p>	<p><i>Comet assay:</i> negative Scattered statistically significant decreases and few positive results, no dose related (% tail DNA and tail length) <i>ROS measurements:</i> dose-dependent increase induced by both TiO₂ NPs; visualisation of TiO₂ NPs in lung tissue; <i>histopathology:</i> cell composition in bronchoalveolar lavage fluid; acute</p>	<p>Reliability: 3 Limited reporting on Comet assay; focus on inflammatory response</p>	<p>Low due to the route of exposure and limitations of the study</p>	<p>Danielsen et al.,2020</p>

				inflammation measured as neutrophil influx into the lung; acute phase response pulmonary and hepatic.			
Comet assay in liver, kidney and lung Sprague-Dawley rats 15 rats/group (1 control and 2 experimental groups) Other in vivo assays (Table 4)*	intratracheal instillation 0.2 and 1.0 g/kg bw single administration analysis at 1, 3 and 7 days after exposure	TiO ₂ NPs, anatase, 10-25 nm (SEM, TEM)	NSC:1 Dispersion measured and exposure confirmed by measuring Ti in tissues	<i>Comet assay: positive</i> (dose-dependent increase in Olive tail moment and % DNA tail). No differences between 1, 3, 7 days post exposures TiO ₂ NPs localization in liver, kidney and lung only after exposure to 1 g/kg bw. Peak reached after 1 day post exposure. <i>Oxidative stress markers:</i> dose-dependent increased levels of MDA and decreased levels of GSH and SOD in all tissues Activation of the PI3K-AKT/FOXO3a signalling pathway. <i>Gene expression:</i> dose-dependent increases of XRCC1,	Reliability: 3 Limited reporting of the results; Inappropriate high doses for intratracheal administration of particles; moderate to severe injury in the target tissue (lung); no positive control	Low limitation of the study and route of exposure	Han et al., 2020b*

				<p>ChK2, and GADD45a (Table 4)</p> <p>Histopathology</p> <p>Lung: inflammatory changes in both groups and more severe in the high-dose group</p> <p>Histopathological findings also for liver and kidney with inadequate reporting</p>			
<p>Comet assay +/- Fpg in liver</p> <p>male Swiss albino mice</p> <p>5 animals/group</p>	<p>gavage 10, 50, 100 mg/kg bw /day for 14 days;</p> <p>analysis: 24h after exposure;</p> <p>positive control EMS, single i.p. 100 mg/kg b.w.</p> <p>positive control for oxidative stress K₂Cr₂O₇</p>	<p>TiO₂NPs, anatase, 20-50 nm</p>	<p>NSC: 1</p> <p>Sonication and level of agglomeration reported for each dose, a level of agglomeration observed</p>	<p>Comet assay: positive</p> <p>-Fpg: % tail DNA at two highest doses; OTM positive all doses</p> <p>+Fpg: increased DNA damage at the same doses as -Fpg</p> <p><i>Histopathology:</i> not significant lesions at 10 and 50 mg/kg bw; angiectasis at the highest dose.</p> <p><i>increase oxidative stress markers:</i> MDA, ROS increase at 50 and 100 mg/kg bw; GSH</p>	<p>Reliability: 1</p>	<p>High</p>	<p>Shukla et al., 2014*</p>

				<p>decrease at the highest dose.</p> <p><i>Biochemical markers of liver function: ALT, ALP (significant increase at 50 and 100 mg/kg bw)</i></p> <p><i>protein levels: dose-related increase of p53, Hsp70-60, caspase</i></p>			
<p>Comet assay</p> <p>lung, peripheral blood and liver</p> <p>Male Sprague-Dawley rats (4 rats/group)</p>	<p>three endotracheal instillation over 8 days resulting in 0.5, 2.5 and 10 mg/kg bw</p> <p>Analysis 2 hours and 35 days after exposure</p> <p>Positive control MMS (150 mg/kg)</p>	<p>TiO₂NPs (P25), anatase/rutile, 15-24 nm</p>	<p>NSC: 1</p> <p>level of agglomeration reported for each dose and exposure confirmed by measurements in tissues</p>	<p>Comet assay: positive in lung at 2 hours and 35 days, all tested doses; positive in liver at 2 hours and 35 days, mid and high doses; positive in blood at 35 days mid and high doses</p> <p>Hedgehog cells recorded: no target tissue cytotoxicity observed</p> <p>Toxicity markers: LDH in BAL cells: significant increase only at 2 hours, mid and high doses.</p> <p>Inflammation markers (interleukins, TNF-</p>	<p>Reliability: 2</p> <p>No negative control values are reported (DNA damage parameters expressed after normalization to negative controls)</p>	<p>Limited</p> <p>Biological relevance of the results evaluated in relation to historical control ranges not reported. Non-oral route of exposure.</p>	<p>Relier et al., 2017*</p>

				alpha, IFN-gamma, KC-GRO): significant increase in plasma and BAL cells only at 2 hours, highest dose			
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ADME: Absorption, distribution, metabolism, excretion. A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BAL: broncho-alveolar lavage; BUN: blood urea nitrogen; CAT: Catalase; CP: cyclophosphamide; CRP: C-reactive protein; CK: creatine kinase; CKMB: creatine kinase MBGSH: reduced glutathione; EMS: ethyl methane sulfonate; GCLC: glutamate-cysteine ligase catalytic subunit; GSH: Reduced Glutathione, GSH-Px: glutathione peroxidase; HCT: Haematocrit; HO-1: heme oxygenase 1; LDH: Lactate dehydrogenase; LPO: lipid peroxidation; MDA: Malonaldehyde; MMS: methylmethanesulfonate; MCV: Mean corpuscular volume; MPV: platelet volume; NAC: N-acetylcysteine; NO: nitric oxide; NQO1: NAD(P)H dehydrogenase[quinine] 1; OTM: Olive Tail Moment; PBMC: peripheral blood mononuclear cells; PLT: Platelets; RBC: red blood cells; ROS: reactive oxygen species; SOD: superoxide dismutase; TNF-alpha: Tumor necrosis factor alpha, VEGF: vascular endothelial growth factor; WBC: white blood cells

Table 4: Other *in vivo* assays

Test system/ Test object	Dose/Route	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 instability							
Inter Simple Sequence Repeat (ISSR)-PCR Mice, 10 animals / group	i.p. 100, 200, and 400 mg/kg; positive control: cyclophosphamide i.p single dose once per week for one month	TiO ₂ , anatase (no further information available)	NSC: 3 No information provided on dispersion or stability.	ISSR analysis: carried out in male mice using six anchor primers. Significant difference only in treated mice at 400 mg/kg bw	Reliability: 3 The effects on genomic instability were evaluated by a non standard method.	Low study limitations and the route of administration is not relevant to dietary intake	EI-Bassyouni et al.,2017*

Test system/ Test object	Dose/Route	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
PCR-SSCP Male Swiss Webster mice, 10–12 weeks, 3 animals/group	Gavage 500 mg/kg bw/day 5 days exposure/ sacrificed after 24 h, 7 days, and 14 days	TiO ₂ NPs, anatase/rutile, 46.23 ± 3.45 nm (TEM)	NSC: 4 High dose only, insufficient information on dispersion and stability	Mutation in Presenilin 1 (PSEN1) gene at exon 5 was detected in 1 animal/group of the 7 days and 14 days sampling time; mutations in the PSEN1 gene <i>Histopathology:</i> extracellular and intracellular brain edema (after 24 h and 7 days), vacuolation of nerve cells (after 14 days)	Reliability: 3 The SSCP technique applied is of limited value to detect mutations induced by a mutagenic treatment. Irrelevant results	Low	Mohamed and Hussein, 2016*

Test system/Test object	Dose/Route	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
Random Amplification of Polymorphic DNA (RAPD) test on blood nucleated cells male rats, min 12 animals / group	0.5 mg/ml i.p. for 18 injections (approximately 2.5 mg/kg bw) 18 injections/6 weeks DNA amplification with random primers	TiO ₂ NPs, 27 nm (SEM) (crystalline form unknow)	NSC: 3 Limited information provided on dispersion and no information on stability.	Increase in the Coefficient of Genomic DNA Fragmentation (Cfr): 0.4328 + 0.00548 (TiO ₂ NPs) versus 0.4023 + 0.0064 (control) statistically significantly (p < 0.05 by ANOVA test)	Reliability: 3 The effects on genomic instability were evaluated by a non standard method. Coefficient of fragmentation is an uncommon parameter. Only one dose. No positive control. Methods and results poorly reported.	Low limitations of the study and the route of administration is not relevant to dietary intake	Minigaliev et al., 2018
DNA binding							
DNA binding to liver DNA from intranasal administration	intranasal administration 300 µg/rat per day for 45 days	1)TiO ₂ NPs, anatase, < 25 nm	NSC: 1 sonication and confirmation of exposure	Positive (binding to DNA): TiO ₂ NPs anatase and TiO ₂ NPs anatase/ rutile mixture Negative: TiO ₂ particles.	Reliability: 1	High	Jin et al., 2013

Test system/ Test object	Dose/Route	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
n of TiO ₂ -NPs to Sprague-Dawley rats (<i>in vivo</i> study)	1) UV-VIS absorption spectra 2) micro-synchrotron radiation x-ray fluorescence (m-SRXRF) on DNA run in agarose gel 3) Atomic Force microscopy	2) TiO ₂ , rutile, < 5 µm 3) TiO ₂ NPs, anatase/rutile (95-90/5-10%), < 100 nm	by measuring Ti in DNA	DNA fragments: Presence of TiO ₂ NPs anatase, but not TiO ₂ particles Aggregates of TiO ₂ NPs in DNA molecules by AFM (networks of tangled DNA strands). No such structures with TiO ₂ rutile particles			
DNA binding to liver DNA from i.p. injected ICR mice (<i>in vivo</i> study)	i.p. 5, 10, 50, 100,150 mg/kg bw per day for 14 days Methods for DNA binding: 1) UV-Vis Absorption Spectroscopy; 2) Extended X-Ray Absorption Fine Structure (EXAFS) Spectroscopy; 3) Circular Dichroism (CD) Spectroscopy	TiO ₂ NPs, anatase, 5 nm (XRD)	NSC: 1 sonication and confirmation of exposure by measuring Ti in liver DNA	Positive: electrostatic interactions leading to changes in DNA conformation EXAFS: TiO ₂ NPs is bound to 3 oxygen/nitrogen atoms and 2 phosphorous atoms in DNA Increased liver weights (range 50-150 mg/kg) Dose-dependent increase in TiO ₂ -NPs in liver DNA by ICP-MS	Reliability: 1	High	Li et al., 2010

Test system/Test object	Dose/Route	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
	Method for in vivo measurements of TiO ₂ -NP in DNA: Inductively Coupled Plasma Mass Spectrometry (ICP-MS)						
DNA damage							
DNA Fragmentation in brain male rats	Gavage 500 mg/kg bw 30 days 20 animals per group colorimetric quantitation upon staining with diphenylamine	TiO ₂ NPs, 90 nm (40-140 nm) TEM (crystalline form unknown)	NSC:4 Dispersion considered prior the administration of the single high dose used (500 mg/kg bw, gavage). Results on achieved level of dispersion	Administration of TiO ₂ NPs significantly reduced Nrf2 concentration and NQO1 mRNA expressions with a simultaneous increase of INOS and DNA fragmentation % compared to the control group	Reliability: 3 methods and results poorly reported; method used is not standardised	Low	Kandeil et al., 2019

Test system/ Test object	Dose/Route	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
			not provided.				
<p>Expression of proteins involved in DNA damage response: GADD45α, XRCC1 and Chk2</p> <p>Oxidative stress: (DCFH-DA), MDA, SOD and GSH-peroxidase</p> <p>Lung, liver kidney,</p>	<p>Intratracheal instillation</p> <p>0.2 and 1.0 g/kg body weight</p> <p>Single dose, groups of rats were sacrificed at 1, 3 and 7 day after administration</p>	<p>TiO₂NPs, anatase, 10-25 nm (SEM, TEM)</p>	<p>NSC: 1</p> <p>Dispersion measured and exposure confirmed by measuring Ti in tissues</p>	<p>In the lung, liver and kidney of rats, TiO₂ NPs exposure resulted in dose-dependent increases of XRCC1, Chk2, and GADD45α expression</p> <p><i>Oxidative stress markers:</i> dose-dependent increased levels of MDA and decreased levels of GSH and SOD in all tissues</p> <p>Histopathology</p> <p>Lung: inflammatory changes in both groups and more severe in the high-dose group</p>	<p>Reliability: 3</p> <p>Limited reporting of the results</p> <p>Inappropriate high doses for intratracheal administration of particles</p>	<p>Low</p> <p>study limitations and route of administration not relevant to oral exposure</p>	<p>Han et al., 2020b*</p>

Test system/Test object	Dose/Route	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
Sprague-Dawley rats				Histopathological findings also for liver and kidney with inadequate reporting			
NOT a genotoxicity endpoint							
DNA methylation Thirty two male Albino rats were used in this study (average weight of 200-230 g)	Gavage Group I (Control group): saline solution 0.9% Group II: Nigella Sativa oil 2 ml/kg bw Group III: TiO ₂ NPs 100 mg/kg bw Group IV: TiO ₂ NPs (100 mg/kg bw) + Nigella Sativa oil (2 ml/kg bw) 6 weeks	TiO ₂ NPs, anatase/rutile, 21 nm	NSC: 4 No verification of dispersion level. High dose used with insufficient information provided.	DNA methylation in liver tissue samples: significantly decreased in TiO ₂ NPs treated group (1.53 ±0.35) compared to control (2.87 ±0.07) Malonaldehyde (MDA) level in blood: significantly increased in TiO ₂ treated group (14.59±2.15) compared to control (8.63 ±0.06) Glutathione (GSH) level : significantly decreased in TiO ₂ NPs treated group (0.16 ±0.04) compared to control (0.32 ±0.02)	Reliability: 3 Poorly reported (not clear if the animals were exposed on every day within six weeks or only on the first day with examination after six weeks). Only one dosage (100 mg/kg). DNA methylation is not a	Low due to insufficient reliability of the study, although the investigation of epigenetic changes has some relevance for carcinogenicity	El Dine et al., 2018

Test system/Test object	Dose/Route	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
					genotoxicity endpoint. No positive control.		

DCFH-DA or DCFDA: 2', 7'-dichlorofluorescein diacetate; MDA: Malonaldehyde; SOD: superoxide dismutase; SSCP: Single-Strand Conformation Polymorphism;