

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

**Table of contents**

**Table 1: *In vivo* gene mutation assay..... 2**

**Table 2: *In vivo* chromosome aberrations/ micronucleus assay..... 5**

**Table 3: *In vivo* DNA damage (Comet assay)..... 15**

**Table 4: Other *In vivo* assays..... 21**

Tables summarising *in vivo* studies on TiO<sub>2</sub> considered in the re-evaluation of E171 (EFSA ANS Panel, 2016). The studies have been evaluated based on the criteria set in Appendix D.

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

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

**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 (dispersions (dispersion and/or confirmation of internal exposure), assigned according to Appendix E | Result  | Reliability/Comments   | Relevance of the result | Reference                            |
|--|---|--|---|---|--|-------------------------|--------------------------------------|
| <p>gene mutation assay hypoxanthine-guanine phosphoribosyl transferase (<i>hprt</i>) gene</p> <p>lung epithelial cells isolated from the lungs of female SPF F334 Fischer rats</p> | <p>10 or 100 mg/kg Intratracheal instillation. Fifteen months after exposure, bronchoalveolar lavage (BAL) cells were characterised and histopathology performed. The alveolar type II cells were isolated and cultured in 6 thioguanine (6TG) containing media to select for mutation in the <i>hprt</i> gene.</p> | <p>TiO<sub>2</sub>, anatase, median diameter 180 nm</p>  | <p>NSC: 2<br/>Sonication prior to instillation.</p>   | <p>The inflammatory cells obtained by BAL from the particle-treated animals were found to induce <i>hprt</i>-mutagenesis in a co-cultured rat lung epithelia cell line <i>in vitro</i>.<br/>"Enhanced <i>hprt</i>-mutagenesis was observed with 100 mg/kg, the dose that also elicited persistent lung inflammation, but not with the 10 mg/kg dose."</p> | <p>Reliability: 5</p> <p>This study concerns inflammation mediated effects elicited <i>in vitro</i> by lung cells, with no relevance for the assessment of genotoxicity after oral exposure.</p> | <p>Low</p>              | <p><b>Driscoll et al. (1997)</b></p> |

|  |   |   |   |  |  |  |  |
|--|---|---|---|--|--|--|--|
| <p><i>In vivo</i> DNA deletion assay in the <math>p^{in}</math> locus.</p> <p>C57Bl/6J<math>p^{in}/p^{in}</math> mice; pink-eyed unstable (<math>p^{in}</math>) locus (internal duplication)<br/>Reconstitution of the wild-type <math>p</math> gene can be seen as a single pigmented cell or a clone of pigmented cells on the unpigmented retinal pigment epithelium (RPE) in the transgenic mice and represents a DNA deletion as a permanent genotoxic event</p> <p>Micronucleus assay (Table 2), Comet assay (Table 3), other <i>in vivo</i> assays (Table 4)*</p> | <p>Mice were treated with TiO<sub>2</sub> NPs during embryonic development at a total dose of 500 mg/kg. Offspring were sacrificed at age of 20 days. Water was used as negative control.</p> | <p>TiO<sub>2</sub>NPs (P25), anatase/rutile, 15-24 nm</p>                             | <p>NSC: 2</p> <p>Ultrasonication in water and consideration of agglomeration, reporting is insufficient but indicates presence of both particles and agglomerates</p> | <p><b>Positive</b></p> <p>TiO<sub>2</sub> NPs increased DNA deletion frequency in fetuses.</p> | <p>Reliability: 2</p> <p>“The assessment of genotoxicity in developing embryos was based on method developed in-house, which has not been validated”. (EFSA ANS Panel, 2016)</p> | <p>Limited</p>   | <p><b>Trouiller et al. (2009)*</b></p> |
| <p>Pig-A gene mutation assay in peripheral blood reticulocytes and in total red blood cells of the same animals.<br/>Male B6C3F1 mice</p> <p>Micronucleus assay (Table 2)*</p>   | <p>0.5, 5.0, and 50 mg/kg/day, administered i.p. for 3 days; positive control: 140 mg/kg ENU, Cells analysis over 6 weeks</p>   | <p>TiO<sub>2</sub>NPs, anatase, ellipsoidal shape, minor axes 12.1 ± 3.2 nm (TEM)</p> | <p>NSC: 1</p> <p>Sonication, agglomeration reported for each concentration and confirmation of exposure by measuring Ti</p>   | <p><b>Negative</b></p>   | <p>Reliability: 2</p> <p>Reporting is inconsistent for the route of application (i.p. or i.v.), but upon request the study authors confirmed i.p.</p>                            | <p>Limited</p> <p>The route of administration is not relevant to dietary intake.</p> | <p><b>Sadiq et al. (2012)*</b></p>     |

|  |  |  |   |                 |                |   |                              |
|--|--|--|---|-----------------|----------------|---|------------------------------|
|  |  |  | levels in tissues.  |                 |                |   |                              |
| LacZ gene mutation assay in liver and spleen<br><br>C57Bl/6 transgenic mice (LacZ)<br>Micronucleus assay (Table 2), Comet assay (Table 3)* | i.v. on 2 days. Sacrifice 28 days after last i.v administration<br>0, 10, 15 mg/kg bw,<br><br>Positive control: ENU 120 mg/kg bw, i.p. | TiO <sub>2</sub> NPs (NM-102), anatase, 21-22 nm | NSC:1<br>Nanogenotox protocol and confirmation of exposure by EM (although not all data reported, and EM did not include detection of Ti) | <b>Negative</b> | Reliability: 1 | Limited<br><br>The route of administration is not relevant to dietary intake. | <b>Louro et al. (2014) *</b> |

BAL: bronchoalveolar lavage; ENU: N-ethyl-N-nitrosourea; HPRT: hypoxanthine-guanine phosphoribosyl transferase.

**Table 2: *In vivo* chromosome aberrations/ micronucleus 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                          |
|---|--|--|--|---|---|---|------------------------------------|
| <p>Micronucleus test</p> <p>Male B6C3F1 mice (peripheral blood and in bone marrow erythrocytes)</p> | <p>i.p injection on 3 consecutive days, animals euthanised 48-hr after the last treatment. groups of 5 mice;</p> <p>2 experiments in bone marrow:<br/>           1) 250, 500 and 1,000 mg /kg bw (same doses for peripheral blood)<br/>           2) 500, 1,000 and 1,500 mg/kg bw</p> | <p>TiO<sub>2</sub>NPs, anatase (Unitane® 0-220), particle size &gt; 100 nm</p> | <p>NSC: 3<br/>           No information on dispersion.</p>   | <p><b>Equivocal</b><br/>           Not all criteria for a clearly positive result are met (all values were within the range of spontaneous control values observed in this study).</p> <p>Exp.1: MN increase at 1,000 mg/kg bw and statistically significant linear trend; MN increase also in peripheral blood, but not statistically significant</p> <p>Exp 2: MN increase at 1,000 mg/kg bw, but not significant linear trend.</p> | <p>Reliability: 2<br/>           Equivocal results<br/>           No positive control</p> | <p>Limited<br/>           The route of administration is not relevant to dietary intake.<br/>           Not all criteria for a clearly positive result are met.</p> | <p><b>Shelby et al. (1993)</b></p> |

|  |   |  |  |   |  |   |                                  |
|--|---|--|--|---|--|---|----------------------------------|
| Micronucleus test Male B6C3F1 mice (peripheral blood and in bone marrow erythrocytes)                  | Same data of Shelby et al, 1993   | TiO <sub>2</sub> NPs, anatase (Unitane® 0-220), particle size > 100 nm | Same data of Shelby et al., 1993   | See Shelby et al., 1993   | See Shelby et al., 1993  | See Shelby et al., 1993   | <b>Shelby and Witt (1995)</b>    |
| Chromosomal aberration in bone marrow<br><br>Male B6C3F1 mice;   | Single i.p injection, animals euthanised 17 or 36-hr after the injection.<br>625, 1250, 2500 mg/kg bw;<br>groups of 8 animals | TiO <sub>2</sub> NPs, anatase (Unitane® 0-220), particle size > 100 nm | NSC: 3<br>No information on dispersion.  | <b>Negative</b>   | Reliability: 2<br><br>Positive control included, but data not reported.  | Limited<br><br>The route of administration is not relevant to dietary intake. | <b>Shelby and Witt (1995)</b>    |
| Micronucleus assay in peripheral blood erythrocytes<br><br>C57Bl/6J <sup>μn</sup> / <sup>μn</sup> mice | Adults exposed for 5 days via drinking water at 50, 100, 250, and 500 mg/Kg.  | TiO <sub>2</sub> NPs (P25), anatase/rutile, 15-24 nm                   | NSC: 2<br>Ultrasonication in water and consideration of agglomeration, reporting is insufficient but indicates presence of both particles and agglomerates | <b>Positive</b><br><br>Statistically significant increase of MN cell frequency at the highest dose only | Reliability: 3<br><br>Inadequate study protocol<br><br>"the study protocol applied is not appropriate to detect MN in mature (normochromatic) erythrocytes. MN in mature erythrocytes can be used as endpoint only when the treatment period exceeds the lifespan of erythrocytes, e.g. 4 weeks or | Low   | <b>Trouiller et al. (2009) *</b> |

|  |  |   |   |   |  |            |                                      |
|--|--|---|---|---|--|------------|--------------------------------------|
|  |  |   |   |   | more in the mouse (OECD TG 474, 2014). In this work, a far shorter treatment period was applied (5 days), with no positive control to demonstrate the efficacy of treatment.” (EFSA ANS Panel, 2016)   |            |                                      |
| <p>Micronucleus assay in bone marrow</p> <p>Male F1 (CBAxB6) mice</p> <p>Comet assay (Table 3), other <i>in vivo</i> assays (Table 4)*</p> | <p>gavage for 7 days; Animals killed 24-hr after the last dose.</p> <p>TiO<sub>2</sub> 40, 200, 1000 mg/kg</p> | <p>TiO<sub>2</sub>, anatase, 160 nm ± 59.4 nm</p> | <p>NSC: 3<br/>no information on dispersion method</p> | <p><b>Inconclusive</b><br/>no demonstration of bone marrow exposure</p> | <p>Reliability: 3</p> <p>“MN assay performed with a limited protocol, based on the analysis of 1,000 immature erythrocytes per animal instead of the 4,000 recommended (OECD 474, 2014); moreover, the statistical analysis of the experimental results, performed by the chi-square test, is incorrect because it does not consider the animal as a statistical unit, as recommended.</p> | <p>Low</p> | <p><b>Sycheva et al. (2011)*</b></p> |

|   |   |  |   |                 |  |   |                              |
|---|---|--|---|-----------------|--|---|------------------------------|
|   |   |  |   |                 | Finally, the biological significance of the small and not dose-related relative increase in MN cells in treated animals compared with controls should be evaluated based on the distribution of historical control values, which were not available in this study." (EFSA ANS Panel, 2016) |   |                              |
| Micronucleus assay in peripheral blood reticulocytes.<br><br>Male B6C3F1 mice | 0.5, 5.0, and 50 mg/kg/day, administered <b>i.p.</b> for 3 days;<br><br>Positive control: 140 mg/kg ENU, <b>i.p.</b> %MN-RET frequencies were monitored one day following the last treatment. | TiO <sub>2</sub> NPs, anatase, ellipsoidal shape, minor axes 12.1 ± 3.2 nm (TEM) | NSC: 1<br>Sonication, agglomeration reported for each concentration and confirmation of exposure by measuring Ti levels in tissues. | <b>Negative</b> | Reliability: 2<br><br>Reporting is inconsistent for the route of application (i.p. or i.v.), but upon request the study authors confirmed i.p.<br><br>TiO <sub>2</sub> in bone marrow was also measured: exposure of target tissue is demonstrated   | Limited<br><br>The route of administration is not relevant to dietary intake. | <b>Sadiq et al. (2012) *</b> |
| Micronucleus assay in bone marrow of ICR mice.                                | Single <b>i.v.</b> injection, MN analysis after 14 days   | TiO <sub>2</sub> NPs, anatase, 42.3 nm (SEM).                                    | NSC: 2<br>Sonication.   | <b>Negative</b> | Reliability: 3<br><br>"the sampling time applied in this study (14 days after treatment) is  | Low   | <b>Xu et al. (2013)</b>      |



|  |   |  |   |  |  |         |                                |
|--|---|--|---|--|--|---------|--------------------------------|
|  | 140, 300, 645, and 1387 mg/kg bw of TiO <sub>2</sub> NPs. 8 mice per group (4 males and 4 females).<br><br>Positive control: Cyclophosphamide (20 mg/kg, two i.p. injections, at 24 and 48 h before mice were sacrificed, respectively) |  |   |  | not appropriate for the test method applied, and considered this study not relevant for risk assessment." (EFSA ANS Panel, 2016) |         |                                |
| Micronucleus assay in bone marrow<br><br>Sprague-Dawley male rats<br><br>Other <i>in vivo</i> studies (Table 4)* | <b>Intragastric</b> administration once a day for 30 consecutive days<br><br>0, 10, 50, 200 mg/kg; 7 rats each group.   | TiO <sub>2</sub> NPs, anatase, 75 ± 15 nm        | NSC: 2 sonication, agglomeration confirmed (reported size 473.6nm)  | <b>Negative</b><br><br>No changes in PCE/NCE, however, a significant and dose-related increase in γH2AX foci in bone marrow cells, observed at the end of treatment (at the two highest doses), which is an evidence of bone marrow exposure | Reliability: 2<br><br>No positive control  | Limited | <b>Chen et al. (2014)*, **</b> |
| Micronucleus assay in peripheral blood   | <b>i.v.</b> on 2 days. 0, 10, 15 mg/kg bw, Blood collected 42-hr after last i.v.  | TiO <sub>2</sub> NPs (NM-102), anatase, 21-22 nm | NSC: 1 Nanogenotox protocol and confirmation of exposure by EM (although not all data reported and EM did | <b>Negative</b>  | Reliability: 2<br><br>Only one sampling time   | Limited | <b>Louro et al. (2014) *</b>   |

|  |  |   |   |   |   |   |                                   |
|--|--|---|---|---|---|---|-----------------------------------|
| C57Bl/6 transgenic mice ( <i>LacZ</i> )  | Positive control: ENU 120 mg/kg bw, i.p.   |   | not include detection of Ti)  |   |   |   |                                   |
| Micronucleus assay in bone marrow PCE and reticulocytes<br>Male Wistar rats<br><br>Comet assay (Table 3)*                            | <b>i.v.</b> single dose. 5 mg/kg bw of TiO <sub>2</sub> NPs (P25), Groups of 7 animals, sacrificed 24h, 1 week and 4 weeks after injection.<br>For estimation of induction of MN in PCE, cells were stained with solutions of May-Grunwald and Giemsa stains<br><br>For estimation of induction of MN in reticulocytes, cells were stained with acridine orange. | TiO <sub>2</sub> NPs (P25), anatase/rutile, 15-24 nm  | NSC: 2<br>Sonication before administration.   | <b>Positive</b><br><br>MN cells frequency increase in PCE only after 24h, no changes in PCE%. No MN increase in reticulocytes in the same blood smears. | Reliability: 2<br><br>No positive control | Limited<br><br>The route of administration is not relevant to dietary intake.   | <b>Dobrzynska et al. (2014) *</b> |
| Micronucleus assay in bone marrow<br><br>Male Swiss Webster mice<br><br>Comet assay (Table 3), other <i>in vivo</i> assay (Table 4)* | <b>i.p.</b> administration for 5 consecutive days. Animals sacrificed after 24-hr<br><br>0, 500, 1000, 2000 mg/kg bw per day; 5 animals/group.<br><br>Positive control: cyclophosphamide   | TiO <sub>2</sub> NPs, mixture of rutile and anatase (XRD), 44 nm (XDR), polyhedral morphology (TEM) | NSC: 1<br>exposure confirmed by Ti measurements in tissues<br><br>No information on dispersion method | <b>Positive</b><br>and decrease of PCE/NCE  | Reliability: 1                            | Limited<br><br>The intraperitoneal route of administration applied in this study is not recommended by OECD guidelines, as non- | <b>El-Ghor et al. (2014) *</b>    |

|   |   |  |   |   |   |   |                                    |
|---|---|--|---|---|---|---|------------------------------------|
|   |   |  |   |   |   | physiological; in addition, the route of administration is not relevant to dietary intake |                                    |
| <p>Micronucleus assay in Peripheral blood reticulocytes (RET)</p> <p>Sprague–Dawley CrI:CD (SD) rats M, F</p> | <p>Single oral dose administered by <b>gavage</b></p> <p>500, 1,000 or 2,000 mg/kg bw<br/>5 animals/sex per dose</p> <p>Positive control: Cyclophosphamide</p> <p>Peripheral blood collected 48 and 72 h after dosing</p> <p>20,000 RET/animal analysed by flow cytometry</p> | <p>5 TiO<sub>2</sub> tested:</p> <p>1) TiO<sub>2</sub>NPs, mixture (89% anatase/11% rutile), hydrodynamic diameter 43 nm (XSDC), shape irregular (TEM)</p> | <p>NSC: 4</p> <p>High doses and high level of agglomeration reported for the TiO<sub>2</sub>NPs, in particular for TiO<sub>2</sub>NPs, mixture (89% anatase/11% rutile) and TiO<sub>2</sub>NPs (rutile)</p> | <p><b>Inconclusive</b></p> <p>no statistically significant increase of micronucleated reticulocytes (MN-RET) at any dose or sampling time.</p> <p>However, no statistically significant decreases in %RET among total erythrocytes and no signs of toxicity reported at any dose.</p> <p>No detectable dose-dependent increases in TiO<sub>2</sub> NPs content over controls were measured in blood (48 or 72 h) or liver (72 h) following TiO<sub>2</sub> NPs administration</p> | <p>Reliability: 3</p> <p>Lack of demonstration of target tissue exposure was indicated by % RET determination. No relevant systemic exposure to TiO<sub>2</sub> was indicated by toxicokinetic data either.</p> | <p>Low</p>  | <p><b>Donner et al. (2016)</b></p> |

|  |  |   |  |  |   |     |  |
|--|--|---|--|--|---|-----|--|
|  |  | 2) TiO <sub>2</sub> NPs, anatase, hydrodynamic diameter 42 nm (XSDC), shape irregular (TEM) | NSC: 4<br>High doses and high level of agglomeration reported for the TiO <sub>2</sub> NPs, mixture (89% anatase/11% rutile) and TiO <sub>2</sub> NPs (rutile) | <b>Inconclusive</b><br>Due to lack of demonstration of target tissue exposure. Statistically significant slight increase of MN in males at 1000 mg/kg 48h after treatment and at the highest dose, after 72 h, and in females at 2000 mg/kg after 72 h. The statistical significance increases reported were similar to other negative control groups from the same study. Therefore, this increase was considered as biologically not relevant. | Reliability: 3<br><br>Lack of demonstration of target tissue exposure was indicated by % RET determination. No relevant systemic exposure to TiO <sub>2</sub> was indicated by toxicokinetic data either. | Low |  |
|--|--|---|--|--|---|-----|--|

|  |  |   |   |  |   |            |  |
|--|--|---|---|--|---|------------|--|
|  |  | <p>3) TiO<sub>2</sub>NPs, rutile, hydrodynamic diameter, 47 nm (XSDC), shape rod-like (TEM)</p>                 | <p>NSC: 4<br/>High doses and high level of agglomeration reported for the TiO<sub>2</sub>NPs, mixture (89% anatase/11% rutile) and TiO<sub>2</sub>NPs (rutile)</p>    | <p><b>Inconclusive</b><br/>due to lack of demonstration of target tissue exposure. no statistically significant increase of MN-RET at any dose or sampling time<br/>No statistically significant decreases in %RET among total erythrocytes<br/>No signs of toxicity were reported at any dose level</p> | <p>Reliability: 3<br/><br/>Lack of demonstration of target tissue exposure was indicated by % RET determination. No relevant systemic exposure to TiO<sub>2</sub> was indicated by toxicokinetic data either.</p> | <p>Low</p> |  |
|  |  | <p>4) TiO<sub>2</sub> (27% nano) (TEM), anatase, hydrodynamic diameter 153 nm (XSDC), shape irregular (TEM)</p> | <p>NSC: 4<br/>High doses. Ultrafine materials "as dosed" were determined to be predominantly agglomerated, whereas the pigmentary materials were better dispersed</p> | <p><b>Inconclusive</b><br/>due to lack of demonstration of target tissue exposure. no statistically significant increase of MN-RET at any dose or sampling time. No statistically significant decreases in %RET among total erythrocytes</p>   | <p>Reliability 3<br/><br/>Lack of demonstration of target tissue exposure was indicated by % RET determination. No relevant systemic exposure to TiO<sub>2</sub> was indicated by toxicokinetic data either.</p>  | <p>Low</p> |  |

|  |  |  |  |   |   |            |  |
|--|--|--|--|---|---|------------|--|
|  |  |  |  | <p>No signs of toxicity were reported at any dose level</p> <p>No detectable dose-dependent increases in TiO<sub>2</sub> content over controls were measured in blood (48 or 72 h) or liver (72 h) following TiO<sub>2</sub> administration</p>   |   |            |  |
|  |  | <p>5) TiO<sub>2</sub> (11% nano) (TEM), rutile, hydrodynamic diameter 195 nm (XSDC), shape irregular (TEM)</p> | <p>NSC: 4</p> <p>High doses. Ultrafine materials "as dosed" were determined to be predominantly agglomerated, whereas the pigmentary materials were better dispersed</p> | <p><b>Inconclusive</b></p> <p>due to lack of demonstration of target tissue exposure. no statistically significant increase of MN-RET at any dose or sampling time</p> <p>No statistically significant decreases in %RET among total erythrocytes</p> <p>No signs of toxicity were reported at any dose level</p> | <p>Reliability: 3</p> <p>Lack of demonstration of target tissue exposure was indicated by % RET determination. No relevant systemic exposure to TiO<sub>2</sub> was indicated by toxicokinetic data either.</p> | <p>Low</p> |  |

NCE: Normochromatic erythrocytes; PCE: polychromatic erythrocytes; RET: reticulocytes; TEM: Transmission electron microscopy

**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                        |
|--|--|--|--|--|--|-------------------------|----------------------------------|
| Comet assay in peripheral blood (white blood cells)<br><br>C57Bl/6J $p^{un}/p^{un}$ mice | Adults exposed for 5 days via drinking water at 500 mg/Kg. | TiO <sub>2</sub> NPs (P25), anatase/rutile, 15-24 nm     | NSC: 2<br>Ultrasonication in water and consideration of agglomeration, reporting is insufficient but indicates presence of both particles and agglomerates | <b>Inconclusive</b><br>TiO <sub>2</sub> NPs increased DNA strand breaks<br><br>mRNA levels (measured in peripheral blood cells) of 3 pro-inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-8) and 3 anti-inflammatory cytokines (TGF- $\beta$ , IL-10, IL-4): TNF- $\alpha$ , IFN- $\gamma$ , and IL-8 were significantly upregulated. No changes in the gene expression for TGF- $\beta$ , IL-10, IL-4 | Reliability: 3<br><br>"The Comet assay performed in peripheral blood did not include the evaluation of cytotoxicity, which is mandatory in this assay (OECD TG489, 2014). Results are reported as Olive tail moment. Moreover, due to the exiguity of the difference between treated and control groups, | Low                     | <b>Trouiller et al. (2009) *</b> |

|  |   |  |  |   |  |         |                                |
|--|---|--|--|---|--|---------|--------------------------------|
|  |   |  |  |   | the biological significance of the effect reported should be evaluated based on the distribution of historical control values, which were not available in this study.” (EFSA ANS Panel, 2016) |         |                                |
| C57Bl/6J $p^{in}/p^{in}$ mice<br><br>8-OHdG measured in liver cells (oxidative DNA damage) | Adults exposed for 5 days via drinking water at 500 mg/Kg.  | TiO <sub>2</sub> NPs (P25), anatase/rutile, 15-24 nm | NSC: 2<br>Ultrasonication in water and consideration of agglomeration, reporting is insufficient but indicates presence of both particles and agglomerates | <b>Positive</b><br>Statistically significant increase of 8-OHdG at the highest dose | Reliability 2<br><br>Only one dose;<br>No positive control;<br>Method and results not reported in full detail  | Limited |                                |
| Alkaline comet assay in bone marrow, liver, brain<br><br>Male F1 (CBAxB6) mice             | <b>gavage</b> for 7 days. Animals killed 24-hr after the last dose. TiO <sub>2</sub> 40 and 200 mg/kg<br><br>5 animals/group<br><br>100 randomly selected comets/organ/animal were analysed | TiO <sub>2</sub> , anatase, 160 nm ± 59.4 nm         | NSC: 3<br>no information on dispersion method reported.  | Bone marrow:<br><b>Positive</b><br><br>Liver and brain:<br><b>Negative</b>          | Reliability: 2<br><br>No positive control. Only 100 comets/organ were analysed<br>No information on target organ toxicity except the unchanged PCE/NCE as                                      | Limited | <b>Sycheva et al. (2011) *</b> |



|  |  |  |   |  |  |  |                            |
|--|--|--|---|--|--|--|----------------------------|
|  |  |  |   |  | reported for the MN assay in bone marrow.                              |  |                            |
| comet assay in lung cells<br>Male Crl: CD (SD) rats                          | <p>1) single <b>intratracheal</b> (INT) instillation: 1.0, 5.0 mg/kg bw<br/>- rats sacrificed 3h or 24h after the treatment</p> <p>2) repeated <b>intratracheal</b> instillation: 0.2 or 1.0 mg/kg bw once a week for 5 weeks, rats sacrificed 3h after last treatment</p> <p>Positive control: EMS (administered orally at 3h before sacrifice)</p> | TiO <sub>2</sub> NPs, anatase, 5 nm  | NSC: 1<br>Information on dispersion and stability is provided                               | <p>1) single INT instillation: <b>Negative.</b></p> <p>2) repeated INT instillation: <b>Negative</b></p>     | Reliability: 1   | Limited<br><br>The route of administration is not relevant to dietary intake                                       | <b>Naya et al. (2012)</b>  |
| Comet assay on bronchoalveolar lavage (BAL) cells<br><br>Female C57BL/6 mice | <p><b>intratracheal</b> instillation of a single dose of 54 µg per animal (approximately 2.8 mg/kg bw); animals sacrificed 24h after dose administration.</p> <p>Positive control: H<sub>2</sub>O<sub>2</sub> exposed A549 cells</p>   | TiO <sub>2</sub> NPs, mixture of anatase (92.2%) with 7.8% rutile (XRD); rutile 19 nm and anatase 12 nm, | NSC: 2<br>Suspensions were sonicated and information provided confirms a high agglomeration | <p><b>Negative</b></p> <p>Inflammation was observed (cellular composition and gene expression analysis).</p> | Reliability: 2<br><br>Sampling time not fully in line with OECD TG 489 | Low<br><br>Due to the study limitations and to the route of administration which is not relevant to dietary intake | <b>Saber et al. (2012)</b> |

|  |  |   |   |                                      |   |  |                                   |
|--|--|---|---|--------------------------------------|---|--|-----------------------------------|
|  |  |   |   |                                      |   |  |                                   |
| Comet assay in liver and spleen<br><br>C57Bl/6 transgenic mice ( <i>LacZ</i> ) | <b>i.v.</b> on 2 days<br>Sacrifice 28 days after last i.v.<br>0, 10, 15 mg/kg bw;<br>Positive control: ENU 120 mg/kg bw, i.p.  | TiO <sub>2</sub> NPs (NM-102), anatase, 21-22 nm  | NSC: 1<br>Nanogenotox protocol and confirmation of exposure by EM (although not all data reported and EM did not include detection of Ti) | <b>Inconclusive</b>                  | Reliability: 3<br><br>Inappropriate sampling time for Comet assay   | Low  | <b>Louro et al. (2014) *</b>      |
| Comet assay in bone marrow leukocytes<br><br>Male Wistar rats                  | <b>i.v.</b> single dose.<br>5 mg/kg bw of TiO <sub>2</sub> NPs (P25),<br>Groups of 7 animals, sacrificed 24h, 1 week and 4 weeks after injection                                   | TiO <sub>2</sub> NPs (NM-105), anatase/rutile, 15-24 nm   | NSC: 2<br>Sonication before administration.   | <b>Negative</b>                      | Reliability: 2<br><br>No positive control; only one dose level; sampling time not fully in line with OECD TG 489.                 | Limited<br><br>The route of administration is not relevant to dietary intake.  | <b>Dobrzynska et al. (2014) *</b> |
| Comet assay in bone marrow, liver, brain<br><br>Male Swiss Webster mice        | <b>i.p.</b> administration for 5 consecutive days. Animals sacrificed after 24h<br><br>0, 500, 1000, 2000 mg/kg bw per day; 5 animals/group.<br>Positive control: cyclophosphamide | TiO <sub>2</sub> NPs, mixture of rutile and anatase (XRD), 44 nm (XDR), polyhedral morphology (TEM) | NSC: 1<br>exposure confirmed by Ti measurements in tissues<br><br>No information on dispersion method                                     | <b>Positive</b> in all three tissues | Reliability: 2<br><br>"highly significant and dose-dependent increases in tail length, % DNA and tail moment were obtained in the | Limited<br><br>The intraperitoneal route of administration applied in this study is not recommended by OECD guidelines, as | <b>Ei-Ghor et al. (2014) *</b>    |

|  |  |   |  |   |  |   |  |
|--|--|---|--|---|--|---|--|
|  |  |   |  |   | absence of adequate measurements of cytotoxicity" (EFSA ANS Panel, 2016)   | non-physiological; in addition, the route of administration is not relevant to dietary intake |  |
| <p>Comet assay on the gastric mucosa</p> <p>Male Swiss Webster, 10-12 weeks old, 15/ group (5/sampling time)</p> <p>Titanium content estimation: inductively coupled plasma mass spectrometry</p> <p>Quantitative DNA fragmentation assay (colorimetric diphenylamine assay)</p> <p>Laddered DNA fragmentation</p> | <p><b>Gavage;</b></p> <p>5, 50 or 500 mg/kg bw per day, for 5 days</p> <p>animals sacrificed after 24 h, 7 days, and 14 days</p> | <p>TiO<sub>2</sub>NPs, mixture of rutile (77%) and anatase (22%) (XRD), 43 nm (XDR), 46.2 nm (TEM), polyhedral morphology (TEM)</p> | <p>NSC: 1</p> <p>Dispersion and stability measured after sonication. Presence of agglomeration confirmed but dose-dependent TiO<sub>2</sub> accumulation in gastric cells maintained after end of exposure confirms cellular internalisation</p> | <p>Comet: <b>Inconclusive;</b> dose and time dependent increase in tail intensity with unclear impact of tissue toxicity</p> <p>Orally administrated TiO<sub>2</sub>NPs (measured as Titanium) in mice persists up to 2 weeks in stomach</p> <p>Quantitative DNA fragmentation: statistically significant increase in percentage DNA fragmentation in TiO<sub>2</sub> NPs-treated groups, which was highly correlated with the sampling</p> | <p>Reliability 3</p> <p>No positive control; Severe damage (necrosis, apoptosis, inflammation) to the target tissue.</p> | <p>Low</p>  | <p><b>Mohamed et al., (2015) *</b></p> |

|  |  |  |  |  |  |  |  |
|--|--|--|--|--|--|--|--|
| <p>assay (apoptosis)</p> <p>Histopathological examination</p> <p>Oxidative stress assays: malondialdehyde (MDA) and nitric oxide (NO).</p> <p>Other <i>in vivo</i> assays (Table 4)*</p> |  |  |  | <p>time and TiO<sub>2</sub> NPs dose</p> <p>Apoptosis: statistically significant dose-dependent increase in the percentage of fragmented DNA at all 3 doses administered and all 3 sampling times (dose- and time-dependent manner).</p> <p>Histopathology: The stomachs of treated mice showed histopathological injuries with dose-related and duration-related increasing severity showing necrosis and gastritis after two weeks</p> <p>Oxidative stress: Dose- and time-dependent statistically significant increase in both MDA and NO</p> |  |  |  |
|--|--|--|--|--|--|--|--|

|  |  |  |  |  |  |  |  |
|--|--|--|--|--|--|--|--|
|  |  |  |  | levels; reductions in GSH and CAT levels |  |  |  |
|--|--|--|--|--|--|--|--|

**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                        |
|---|--|--|--|--|---|---|----------------------------------|
| <b>γH2AX</b>  |  |  |  |  |   |   |                                  |
| C57Bl/6J $p^{in}/p^{in}$ mice<br>γH2AX assay in bone marrow cells | Adults exposed for 5 days via drinking water at 50, 100, 250, and 500 mg/Kg. | TiO <sub>2</sub> NPs (P25), anatase/rutile, 15-24 nm     | NSC: 2 Ultrasonication in water and consideration of agglomeration, reporting is insufficient but indicates presence of both particles and agglomerates. | <b>Positive</b><br>Dose-dependent increase of γH2AX positive cells | Reliability: 2<br><br>lack of positive control method poorly reported | Limited<br><br>γH2AX is not a standardised test | <b>Trouiller et al. (2009) *</b> |
| γH2AX assay (to evaluate DNA strand breaks) in bone marrow cells  | Intragastric administration once a day for 30 consecutive days               | TiO <sub>2</sub> NPs, anatase, 75 ± 15 nm                | NSC: 2 sonication, agglomeration confirmed (reported size 473.6nm)   | <b>Positive</b><br>a significant and dose-related increase in      | Reliability: 2<br><br>No positive control                             | Limited<br><br>γH2AX                            | <b>Chen et al. (2014) *</b>      |

|   |  |  |   |   |   |   |                                 |
|---|--|--|---|---|---|---|---------------------------------|
| Sprague-Dawley male rats  | 0, 10, 50, 200 mg/kg; 7 rats each group.   |  |   | γH2AX foci in bone marrow cells, observed at the end of treatment (at the two highest doses)            |   | is not a standardised test  |                                 |
| <b>Genomic instability</b>  |  |  |   |   |   |   |                                 |
| single strand conformation polymorphism (SSCP) analysis, p53 mutation in liver and brain cells (PCR+electrophoresis)<br><br>Male Swiss Webster mice | <b>i.p.</b> administration for 5 consecutive days. Animals sacrificed after 24-hr<br><br>0, 500, 1000, 2000 mg/kg bw per day; 5 animals/group. | TiO <sub>2</sub> NPs, mixture of rutile and anatase (XRD), 44 nm (XDR), polyhedral morphology (TEM)                            | NSC: 1 exposure confirmed by Ti measurements in tissues<br><br>No information on dispersion method  | Increased frequency of mutations in brain and liver   | Reliability: 3<br>Negative control data not reported in table format.<br><br>"the screening of mutations in exons 5–8 of the p53 gene is not considered an actual genotoxicity test and has not received adequate validation". (EFSA ANS Panel, 2016) | Low<br><br>The intraperitoneal route of administration applied in this study is not recommended by OECD guidelines, as non-physiological; in addition the route of administration is not relevant to dietary intake | <b>El-Ghor et al. (2014)*</b>   |
| Single strand conformation polymorphism (SSCP) analysis of p53 exons 5 to 8<br><br>Male Swiss Webster, 10-12 weeks old, 15/ group (5/sampling time) | <b>Gavage;</b><br>5, 50 or 500 mg/kg bw per day, for 5 days;<br><br>animals sacrificed after   | TiO <sub>2</sub> NPs, mixture of rutile (77%) and anatase (22%) (XRD), 43 nm (XDR), 46.2 nm (TEM), polyhedral morphology (TEM) | NSC: 1<br><br>Dispersion and stability measured after sonication. Presence of agglomeration confirmed but dose-dependent TiO <sub>2</sub> NPs | SSCP analysis: TiO <sub>2</sub> NPs induced mutation frequencies in p53 exons (5-8) in a dose- and time | Reliability: 3<br><br>"the Panel noted that the modest increase in single-strand conformation polymorphism of the p53 exons 3 and 8 cannot be   | Low   | <b>Mohamed et al., (2015) *</b> |

|  |   |   |   |  |  |  |                                  |
|--|---|---|---|--|--|--|----------------------------------|
| <p>titanium content estimation: inductively coupled plasma mass spectrometry</p>   | <p>24 h, 7 days, and 14 days</p>  |   | <p>accumulation in gastric cells maintained after end of exposure confirms cellular internalisation</p> | <p>dependent manner.<br/><br/>Orally administrated TiO<sub>2</sub>NPs (measured as Titanium) in mice persists up to 2 weeks in stomach</p> | <p>taken as an evidence of mutagenicity without confirmatory sequencing data". (EFSA ANS Panel, 2016).<br/><br/>The test method used to investigate P53 mutation was not adequate and not standardised for regulatory purposes.</p>                              |  |                                  |
| <p>Female Wistar rats</p> <p>Cells from bronchoalveolar lavage fluid (BALF)</p> <p>Analysis on the cell free supernatant of BALF:<br/>- fibronectin in BALF<br/>-TNF-<math>\alpha</math> activity with a cell lytic assay<br/>-phospholipids</p> <p>Analysis in lung tissue:<br/>-proliferation marker Ki-67</p> | <p>0.15, 0.3, 0.6, and 1.2 mg of TiO<sub>2</sub>NPs (P25) <b>intratracheal</b> instillation</p> | <p>TiO<sub>2</sub>NPs (P25), anatase/rutile, 15-24 nm</p> | <p>NSC: 2<br/>Suspensions were sonicated and information provided confirms a high agglomeration</p>     | <p>No increase in 8-oxoguanine.<br/><br/>Increase of some inflammation biomarkers.</p>   | <p>Reliability: 5<br/>"The Panel noted that such studies, especially when assessing genotoxicity at site of direct contact with nanoparticles, have limited relevance for the safety assessment of oral exposure to TiO<sub>2</sub>." (EFSA ANS Panel, 2016)</p> | <p>Low<br/><br/>Due to the study limitations and the route of administration is not relevant to dietary intake</p> | <p><b>Rehn et al. (2003)</b></p> |

|   |  |   |   |  |  |            |                                       |
|---|--|---|---|--|--|------------|---------------------------------------|
| <p>-detection and quantification of 8-oxoguanine by immunocytological assay and image analysis</p>  |  |   |   |  |  |            |                                       |
| <p>Micronucleus assay in Epithelial cells from forestomach and colon<br/>Cells were analysed for: the presence of MN, nuclear protrusions, atypical nuclei, mitosis, bi-nucleated cells, condensed chromatin and pyknosis in the forestomach. Apoptotic index.</p> <p>Micronucleus assay in Testis: spermatids analysed for the presence of micronucleated cells, bi- and multinucleated cells, and apoptotic figures, recorded as cells with apoptotic bodies, i.e. a nucleus in the form of fragments of disintegrated chromatin. Male F1 (CBAxB6) mice</p> | <p>TiO<sub>2</sub> 40, 200, 1000 mg/kg for 7 days via <b>gavage</b>.<br/>Animals killed 24-hr after the last dose.</p> | <p>TiO<sub>2</sub>, anatase, 160 nm ± 59.4 nm</p> | <p>NSC: 3<br/>no information on dispersion method reported.</p> | <p>Negative in all tissues analysed, Cytotoxicity was observed</p> | <p>Reliability: 5<br/>"The 'poly-organ karyological assay' is not a validated assay for risk assessment. Moreover, the parameters evaluated, i.e. mitotic index, apoptosis and nuclear abnormalities of spermatids, are not adequate to evaluate genotoxicity". (EFSA ANS Panel, 2016)</p> | <p>Low</p> | <p><b>Sycheva et al. (2011) *</b></p> |