MUT/2021/03

COMMITTEE ON THE MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT

REVIEW OF GENOTOXICITY OF TITANIUM DIOXIDE

Referral to COM

1. The Food Standards Agency has asked for advice on the genotoxicity of Titanium Dioxide (TiO_2), following a recent re-evaluation from the European Food Safety Authority (EFSA).

Introduction

2. Titanium dioxide, Chemical Abstracts Service (CAS) Registry number 13463-67-7, European Inventory of Existing Commercial Chemical Substances (EINECS) number 236-675-5 and Colour Index (C.I.) number 77891 is an organic substance. The titanium atom is coordinated octahedrally with oxygen, but the position of the octahedral structure differs in the different crystalline forms. Titanium dioxide exists in nature in different crystalline forms - the anatase (tetragonal, CAS Registry number 1317-70-0) and rutile (tetragonal, CAS Registry

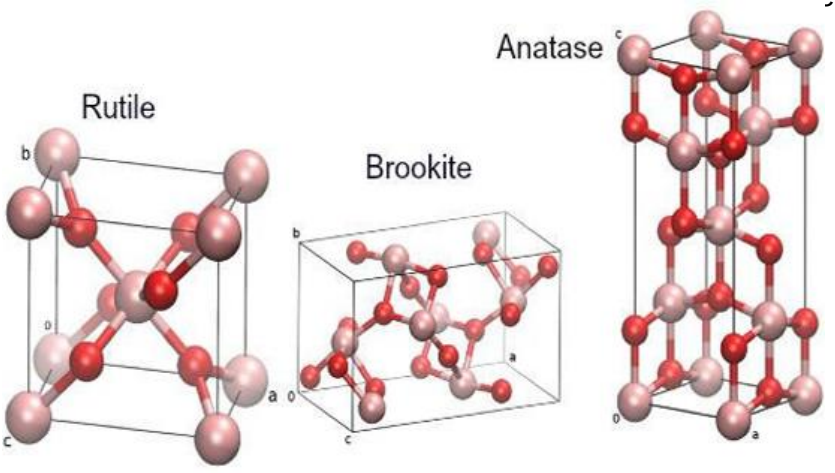

Fig.1: Natural forms of TiO_2[1]

3. Titanium dioxide is an authorised Food Additive in the EU in accordance to Annex with Annex II to Regulation (EC) No 1333/2008 in both anatase and rutile forms (Commission Regulation (EU) No 231/2012) and under GB Food Law

[1] http://www.fangyuan-tio2.com/rutile-anatase-tio2-uses-titanium-dioxide-properties.html

(retained EU law Regulation No 1333/2008 on food additives). Titanium dioxide particles can reflect light over the majority of the visible spectrum and achieve opacity by causing multiple reflections and refractions (EFSA, 2016). As such, it is used in food as a colour to make food more visually appealing, to give colour to food that would otherwise be colourless, or to restore the original appearance of food. It is also widely used in cosmetics and medicines[2].

4. Titanium dioxide has been the subject of multiple safety evaluations: the Scientific Committee on Food (SCF) in 1975 and 1977, by the Joint FAO/WHO Expert Committee of Food Additives (JECFA) in 1969. In 1969, JECFA allocated an acceptable daily intake (ADI) 'not limited except for good manufacturing practice'. In 1975, the SCF did not establish an ADI for titanium dioxide, whereas in 1977, the SCF included titanium dioxide in the category 'colours for which an ADI was not established but which could be used in food'.

5. In 2016, EFSA reviewed the safety of titanium dioxide. One of the largest uncertainties related to the composition of titanium dioxide. EFSA considered that E 171 mainly consisted of micro-sized titanium dioxide particles, with a nano-sized (< 100 nm) fraction less than 3.2% by mass. Uncertainties around the identity and characterisation of E 171 were however highlighted, noting that no limits for the particle size of E 171 were set in the EU specifications (EFSA, 2021). Subsequently, in 2019, and following the evaluation of data submitted by interested operators, the Panel recommended that " the EU specifications for E 171 include the parameter of median minimum external dimension by particle number >100 nm (measured by electron microscopy), which is equivalent to less than 50% of constituent particles by number with a minimum external dimension <100 nm."

6. With regards to genotoxicity, based on the available genotoxicity data and considering other absorption, distribution, metabolism and excretion parameters (ADME) the Panel concluded that orally ingested titanium dioxide particles (micro- and nanosized) were unlikely to represent a genotoxic hazard *in vivo*. This was based on the observations from both *in vitro* and *in vivo* genotoxicity studies. *In vitro*, mixed results were obtained, providing some evidence of genotoxicity for micro- and nano- sized titanium dioxide particles. The Panel noted that most positive results were reported under experimental conditions associated with the induction of oxidative stress and that the genotoxic effects concerned were mainly seen in indicator assays such as the Comet assay. The Panel noted that the reliability of Comet assay for evaluating nanoparticle-induced genotoxicity has been questioned because of the possible secondary induction of DNA damage by nanoparticles during sample processing. They further highlighted that comparing

[2] https://www.efsa.europa.eu/en/news/titanium-dioxide-e171-no-longer-considered-safe-when-used-food-additive

the results obtained in intact cells and isolated nuclei, Ferraro *et al*. (2016) demonstrated that most DNA damage elicited by titanium dioxide nanoparticles in human epithelial cells was produced during the assay performance (ex post damage) rather than during treatment (ex ante damage), through the direct interaction of cytoplasm-internalised nanoparticles with DNA in nucleoids. *In vivo*, overall negative results have been obtained in genotoxicity studies with microsized titanium dioxide pigment. Limited evidence of genotoxicity, if any, is provided by studies with orally administered titanium dioxide nanoparticles. The Panel considered that there was limited or no indication of the genotoxicity of titanium dioxide nanoparticles is provided by studies using an intravenous route of administration, which allows maximum exposure of target tissues (EFSA, 2016). Overall, the Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI). They further recommended that:

- In order to enable the Panel to establish a health-based guidance value (ADI) for the food additive TiO_2 (E 171), additional testing could be performed. An extended 90-day study or a multigeneration or extended-one generation reproduction toxicity study according to the current OECD guidelines could be considered. Such studies should be performed with TiO_2 (E 171) complying with the EU specifications and additionally including a characterisation of the particle size distribution of the test material. However, in deciding on actual testing, considerations of animal welfare need to be balanced against the improvement in the toxicological database within a tiered testing approach.

- The EU specifications for TiO_2 (E 171) should include a characterisation of particle size distribution using appropriate statistical descriptors (e.g. range, median, quartiles) as well as the percentage (in number and by mass) of particles in the nanoscale (with at least one dimension < 100 nm), present in TiO_2 (E 171) used as a food additive. The measuring methodology applied should comply with the EFSA Guidance document (EFSA Scientific Committee, 2011).

- The maximum limits for the impurities of the toxic elements (arsenic, lead, mercury and cadmium) in the EU specification for TiO_2 (E 171) should be revised in order to ensure that TiO_2 (E 171) as a food additive will not be a significant source of exposure to those toxic elements in foods.

7. In 2018 four additional studies were evaluated, including one *in vitro* genotoxicity study in two human colon cancer cell lines. The Panel re-confirmed that E171 did not raise concerns for *in vivo* genotoxicity[3] .

Other evaluations

8. After a report by the French Authorities in 2016, and a proposal for evaluation of titanium dioxide the Committee for Risk Assessment (RAC) of the European Chemicals Agency (ECHA) concluded in June 2017 that titanium dioxide met the criteria to be classified as a substance suspected of causing cancer (category 2) if inhaled. The main mechanism to explain the effects induced by titanium dioxide, in common with effects seen with other substances, was inflammation and an indirect genotoxic effect through production of reactive oxygen species (ROS) arising from the biopersistence and insolubility of all forms of titanium dioxide particles. However, a direct interaction with DNA could not be excluded, since titanium dioxide was found in the cell nucleus in various *in vitro* and *in vivo* studies. This was in line with the International Agency for Research on Cancer (IARC) evaluation which concluded that "titanium dioxide is possible carcinogenic to humans (Group 2B) based on sufficient evidence in experimental animals and inadequate evidence from epidemiological studies.[4]" This was with relation to exposure via inhalation. However, in the same report by the French Authorities the Agency for Food, Environmental and Occupational Health and Safety (ANSES) concluded that there was no carcinogenic concern after oral or dermal administration.

9. In their most recent evaluation, the Scientific Committee on Consumer Safety (SCCS) assessed titanium dioxide used in cosmetic products that lead to exposure by inhalation. With regards to mutagenicity and genotoxicity, the SCCS noted that in the 2010 evaluation, IARC concluded that that most of the in vitro genotoxicity studies with titanium dioxide exposure were negative despite the high rate of false positives and that the EFSA Panel in 2016 considered that the positive genotoxicity results may have been due to experimental conditions associated with the induction of oxidative stress. The SCCS also noted that studies showing a positive association between the so-called group of Poorly Soluble Low Toxicity (PSLT) particles exposures and genotoxicity are generally consistent with the mechanism that sub-toxic concentrations of PSLT particles can cause inflammation and oxidative stress, which may lead to mutations. Oxidative stress is considered the underlying mechanism of the proliferation and genotoxic responses to PSLT particles including titanium dioxide and thus there is a large body of evidence that titanium dioxide has no direct genotoxic potential. The

[3] https://www.efsa.europa.eu/en/efsajournal/pub/5366

[4] https://monographs.iarc.who.int/wp-content/uploads/2018/06/TR42-Full.pdf

SCCS was of the opinion that “The genotoxic effects of titanium dioxide most probably manifest through an indirect mechanism (oxidative stress), or secondary mechanisms (e.g. oxidative stress and inflammation caused by immune cells). The SCCS therefore considers it plausible that there is a practical threshold for this mode of action and therefore a risk assessment could be carried out for its use in cosmetic products.” They concluded that when used in cosmetic products titanium dioxide does not pose a genotoxic risk. (SCCS, 2020)

2021 Evaluation by EFSA

10. Following the review of titanium dioxide specifications in 2019, and based on the fraction of nanoparticles present in E171, the food additive falls under the scope of the EFSA guidance on nanotechnology which was revised in 2018[5] to include ‘a material that is not engineered as nanomaterial but contains a fraction of particles, less than 50% in the number–size distribution, with one or more external dimensions in the size range 1–100 nm’. The proposed amendment to E171 specifications was therefore accompanied by a recommendation for re-assessment of toxicological data in line with the requirements of the 2018 EFSA guidance on nanotechnology.

11. Data evaluated was for the food additive titanium dioxide E171 as well as titanium dioxide other than E171 containing a fraction of nanoparticles <100nm or nano titanium dioxide (TiO_2 NPs). The characterisation of E 171 was previously evaluated by the Panel and it was concluded that, according to data received from interested business operators, less than 50% of constituent particles in E 171 have a minimum external dimension below 100 nm by number. The Panel considered that studies performed with TiO_2 NPs that predominantly consist of particles smaller than 30 nm (e.g. P25) are of limited relevance to the safety assessment of E 171. This is because titanium dioxide particles in pristine E 171 likely form large agglomerates. When dispersion procedures are applied, these agglomerates may de-agglomerate, resulting in increased numbers of ‘free’ nanoparticles. The extent of agglomeration and the number of ‘free’ nanoparticles present maybe further affected by the conditions in food and the gastrointestinal tract (GIT) environment. The data available to EFSA showed that the percentage by number of constituent particles < 30 nm was in the order of 1% or less in samples of pristine E 171 or in E 171 extracted from foods analysed after dispersion. However toxicity studies performed with TiO_2 <30 nm have been considered for completeness of the database and may be relevant with respect to whether a minimum limit for particle size should be included in the EU specifications for E 171.

[5] https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2018.5327

Kinetics and Metabolism

12. In 2016, the Panel concluded that the absorption of orally administered titanium dioxide is low. Its oral systemic availability (measured either as particles or as titanium) is estimated to be 0.02–0.1%, and the vast majority was eliminated unchanged in the faeces. The small amount of orally ingested titanium dioxide appeared to be absorbed by Peyer's patches, a group of cells in the gut-associated lymphoid tissue (GALT). It is subsequently distributed to various organs (by order of decreasing concentration: mesenteric lymph nodes, liver, spleen, kidney, lungs, heart and reproductive organs), from which the material disappears with variable half-lives. The ANS Panel noted the potential for tissue accumulation based on the slow elimination of titanium from tissues after intravenous administration with calculated half-lives ranging between 28 and 650 days in different organs (EFSA, 2016). Interpretation of these findings was, however, complicated by the extent of the variability in the background levels of Ti in animals and humans which also prevented the accurate determination of kinetic parameters such as the elimination half-life. In the most recent evaluation the uncertainties around the variability in the environmental, dietary and tissue backgrounds remained as one of the critical aspects when evaluating the toxicokinetics of titanium dioxide. In addition, the challenges in analytical determination of low concentrations of Ti in tissues further complicated obtaining accurate and reliable tissue concentrations and toxicokinetic data.

13. For the re-evaluation was based on observations from both human and animal studies with titanium dioxide that meets the specifications for E171 and titanium dioxide materials other than those that meet the specifications for E171. The estimate of the oral systemic availability of titanium dioxide was updated by multiplying the reported concentration with the respective organ or tissue weights. Subsequently, the sum of the calculated amounts in the different organs was compared to the dose applied to estimate the percentage absorbed. Data were extracted only from those publications in which the analytical method used for the measurement of internal exposure was evaluated as reliable or reliable with some limitations (Appendix D of EFSA opinion). The Panel concluded that "E171 had low systemic availability, probably not greater than 0.5%". This was based on observation from two studies in mice (Comera *et.al,* 2020; Talamini *et al., 2019*). The studies allowed the derivation of estimates of internal dose at 0.01% and 0.1% respectively. The Panel noted that the estimate were based on measurements of Ti concentrations in a limited number of organs and that, despite the uncertainty with regards to what extent titanium dioxide distributes to other organs[6], the Panel's estimates always included the Ti amount in the liver, which

[6] Talamini *et al., 2019:* stomach, large and small intestine, liver, lung, spleen, testes, brain, kidney. Comera *et al.,* 2020Segments of the jejunum, ileum and colon.

accounted for about 12.5% of the Ti amount in the body. They therefore considered that the underestimation in body burden and absorption was therefore unlikely to be more than 5-fold. It was also concluded that “it may pass the placenta. With regards to the studies on TiO_2 NPs, consisting of nanoparticles with primary particle sizes between 7 and 90 nm,the data indicated that these materials have long half-lives (roughly 200–450 days), a potential for accumulation (accumulation factor of 290 to 450) and long time to reach steady state (3–5 years). The oral systemic bioavailability of these materials was higher than for E171 but still low (probably <1%). In tissues from deceased human subjects, titanium dioxide particles were identified in liver and spleen, the low Ti amount of the investigated organs indicating low oral systemic availability of titanium dioxide ingested from a number of sources, including dietary exposure to E 171”.(EFSA, 2021)

14. Further information can be found in pages 14-21 of the EFSA 2021 Opinion (Annex 1).

Toxicity

Short term

15. For E171, the Panel considered that no adverse effects were observed in mice (n=4) following administration of E171 at a mean dose of 2mg/kg bw/d for 21 days. E 171 or water (for controls) was slowly dripped with a pipette into the mice mouths, allowing each drop to be swallowed. The test material was E 171 (35% nano), anatase, 201 nm in suspension (Appendix H; EFSA 2021). The treatment regime was 5 mg E 171/kg bw per day, 3 days per week, for 3 weeks (nine treatments in 21 days, providing an average daily dose of 2 mg E 171/kg bw/d (Talamini *et al.,*2019). No body weight or feed intakes were observed and organ weights were not affected. The Panel noted reports for areas of “necro inflammatory” foci in the livers of exposed mice and considered these deserved attention. However, the Panel could not conclude on the association of this finding with exposure to E 171, due to very limited number of livers examined. The Panel noted the absence of additional endpoints indicative of evidence for liver injury and the fact that these reported changes can variably occur as a background pathology in murine liver.

16. In rats, there were no signs of systemic toxicity following gavage administration of up to 1000 mg/kg bw/d in 90 day studies however they noted that the study had limitations for assessing the toxicological effects of the fraction of nanoparticles. The test material (Appendix H; EFSA, 2021) was E 171, anatase, 150 nm (dynamic light scattering (Han *et al*., 2020).

17. Several studies were identified assessing the safety of materials other than E171 (TiO_2 NPs or TiO_2 containing a fraction of NPs). Study durations varied

between 14- 90 days. From those it was concluded no adverse effects were observed at the highest dose tested (100 mg/kg bw/d, Vasantharaja *et al*. (2015)). Overall, no adverse effects associated with general toxicity were observed in rats orally exposed to E 171, TiO_2 NPs or TiO_2 containing nanoparticles.

18. The studies assessing the safety of TiO_2 NPs <30nm were also reported. These were: mild hyperbilirubinaemia however this was not accompanied by any changes in liver enzymes (Yang *et al*., 2017); the effect size of increased fasting glycaemia and impaired glucose tolerance (Hu *et al*., 2015) which the Panel concluded was not of toxicological relevance and not accompanied by changes in insulin or other changes in lipid metabolism, histopathological changes were reported in the heart (Yu *et al*., 2016); however, these findings were not supported by incidences and severity scores. Histopathological findings indicating inflammation were reported in the liver, but investigations to confirm hepatic injury were not performed (Hong *et al*., 2016). In rats, inconsistent and/or unexplained sex differences in some parameters were reported (e.g. hypobilirubinaemia in females (Chen *et al*. (2015a); heart rate and blood pressure changes in females (Chen *et al*. (2015b); leucocyte changes in females (Heo *et al*., 2020); higher absolute pituitary weights in males (Heo *et al*., 2020); lower blood insulin levels in females, lower C-peptide levels in males and differences in blood concentrations compared to controls in a glucose tolerance test in males (Chen *et al*., 2020b). The Panel considered that the TG changes reported in several studies were likely incidental study findings since the reductions were seen in only one sex and without a clear dose response (Chen *et al*., 2015b), lacked a clear dose response (Vasantharaja *et al*., 2015) or increased in a single dose study (Grissa *et al*., 2015).

19. It was concluded that “effects reported in mouse studies TiO_2 NPs < 30 nm could be associated with accumulation of NPs in various tissues whereas inconsistent findings in rats were considered incidental.” (EFSA, 2021).

20. With regards to reproductive and developmental toxicity, a number of studies available in literature were assessed, in addition to the extended one-generation reproduction toxicity (EOGRT) study. The EOGRT study was commissioned by interested business operators to address data gaps identified in 2016. The protocol was later amended to accommodate the investigation of additional parameters related to the occurrence and titanium dioxide-related induction of aberrant crypt foci (ACF) in the colon; these are preneoplastic lesions that had been reported by Bettini *et al*. (2017) shortly after the completion of the ANS Panel re-evaluation of E 171. Overall, no effects on reproductive and developmental toxicity were observed up to a dose of 1,000 mg E 171/kg bw per day, the highest dose tested in the EOGRT study.

21. No reliable studies were found in the literature addressing reproductive and developmental toxicity of E 171 (EFSA, 2021) and no effect was reported up to a dose of 1,000 mg/kg bw per day for titanium dioxide containing a fraction of

nanoparticles when administered from gestation days (GDs) 6 to 15 (Warheit *et al.,* 2015).

22. Concerning neurotoxicity, no reliable studies performed with E 171 were found in the literature (EFSA, 2021). In studies with TiO_2 NP > 30 nm, neurotoxic effects were observed at the only dose tested of 100 mg/kg bw per day in rats exposed in embryonal life and at the only dose tested of 500 mg/kg bw per day in rats exposed in adult life. In studies using titanium dioxide NPs < 30 nm, effects were seen at doses as low as 2.5 mg/kg bw per day. The findings in studies with E 171 on immunotoxicity and inflammation were considered inconsistent; in studies with TiO_2 NPs > 30 nm effects were seen at a dose of 20 mg/kg bw per day whereas in studies with TiO_2 NPs < 30 nm effects were observed at doses as low as 2.5 mg/kg bw per day.

23. Regarding the newly performed EOGRT study E 171 was administered in the diet. In the F0 generation, E 171 was administered in the diet at doses of 0, 100, 300 or 1,000 mg/kg bw per day from 10 weeks prior to mating until weaning of the F1 generation. The F1 generation received these diets from weaning until postnatal days (PND) 4 or 8 of the F2 generation. The F2 generation was exposed through the milk until the termination of the study on PND 4 or PND 8. Duration of dosing depended on the endpoints under evaluation in the different cohorts, with the longest duration of treatment up to 18 weeks. The Panel concluded that there were no indications of general toxicity, no effect on thyroid or sex hormone levels, no effect on reproductive function and fertility in either male or female rats. Furthermore, no effects were observed on pre- and postnatal development. No effects on neurofunctional endpoints in F1 offspring were observed either (EFSA, 2021).

24. Concerning immunotoxicity, a marginal but statistically significant decrease in antigen-induced IgM levels (-9%) in males of the F1 Cohort 3 only was noted, with no apparent dose-response. However, the Panel noted that there were methodological shortcomings in the design of this part of the EOGRT study. Therefore, the Panel could not conclude on immunotoxicity. In a satellite group of that study, E 171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon. The Panel considered that there was uncertainty regarding the extent of the internal exposure to titanium dioxide nanoparticles (present in E 171) across the range of tested doses (EFSA, 2021). The Panel also noted that there was uncertainty with regards to the extent to which the particle size distribution of the E 171 used in the EOGRT study was reflective of the particle size distributions of E 171 when added to foods as well as the extent to which the particle size distribution of E 171 in transit through the GIT in the EOGRT study was affected by the concentration in the diet (i.e. dose).

Genotoxicity

25. Due to the large volume of studies considered, the Appendices (J, K, L, M,N, O,P) from the EFSA evaluation, summarising the studies including characterisation of the test materials and reliability and NSC scores have been

included at the end of this document (Annex 2). The studies include: new *in vitro* and *in vivo* genotoxicity studies, *in vitro* and *in vivo* genotoxicity studies that have been considered in the 2016 review as well *in vitro* and *in vivo* studies from the OECD dossier (2016).

26. As previously mentioned, the genotoxicity of titanium dioxide was evaluated in 2016 by EFSA. Based on the available data at the time, titanium dioxide was not considered a nanomaterial based on the EU Recommendation on the definition of nanomaterials: "natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm". Therefore, data on titanium dioxide as nanomaterial were not considered as directly applicable to the evaluation of the food additive. As discussed in paragraph 6, mixed results were obtained *in vitro*, evidence of some *in vitro* genotoxicity of micro- and nano-sized titanium dioxide particles. The ANS Panel had considered that most positive results were reported under experimental conditions associated with the induction of oxidative stress (as shown by increased 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), LPO and reactive oxygen species (ROS) generation), and that the genotoxic effects observed mainly concerned indicator assays (comet and H2AX histone phosphorylation), which in some studies were shown not to be associated with permanent chromosome damage such as chromosome breaks visualised as micronuclei (MN) (EFSA, 2016).

27. However *in vivo,* overall negative results were obtained in genotoxicity studies with micro-sized titanium dioxide. With regards to TiO_2 NPs, there was limited evidence, if any, of genotoxicity from the orally administered studies. Similarly, limited or no indication of genotoxicity for TiO_2 NPs was observed when the test chemical was administered intravenously. Therefore, the Panel concluded that E171 as a food additive did not raise genotoxicity concerns (EFSA, 2016).

In vitro gene mutation

28. The Panel considered fourteen studies investigating the ability of titanium dioxide to induce gene mutations in mammalian cells. Of those, seven were considered relevant and used for the assessment. These have been summarised in Table 1 below (Information taken from relevant EFSA Appendices):

Table 1:*In vitro* gene mutation studies considered by EFSA.

Test System	Exposure	Characterisation of test substance	Result	EFSA's Reliability Score	EFSA's evaluation of Relevance	Ref

Mammalian cell gene mutation test in V79 (hypoxanthineguanine phosphoribosyl transferase (HPRT or HGPRT) gene)	Particle uptake: 0, 10, 50, 100 µg/ml MTT assay: 1, 10, 25, 50, 100 µg/ml ROS production: 1, 10, 25, 50, 100 µg/ml Exposure: HGPRT gene mutation in V-79 cells: 6 hours Particle uptake: up to 24 hours MTT assay: up to 24 hours ROS production: 6 hours	**TiO_2 NPs, anatase, 12-25nm (TEM),** NCS Score:1	**Positive**:Statistically significant increase in the mutation frequency of HPRT gene at 50 and 100 µg/ml. Particle uptake: Cellular uptake detected by flow cytometry and confirmed by TEM. ROS production (DCFH-DA): statistically significant increase of % of ROS production at all concentrations except at 1 µg/ml	2 Main deviations from OECD TG 476: the cytotoxicity induced by the treatment (relative survival) was not evaluated in the gene mutation experiment. The number of treated cells is not reported	Limited	Jain *et al*.,2017-Appendix J
Mammalian cell gene mutation assay HPRT locus Chinese hamster lung fibroblasts (V79 cells)	0, 5, 20 and 100 µg/mL 2h exposure Positive control: EMS without S9 OECD TG 476 (1997)	**TiO_2 NPs, anatase, 75 nm** NSC: 2 DLS measurement in cell media confirming agglomeration	**Positive**: statistically significant and concentration-related increase in the mutation frequency of HPRT gene.	1	High	Chen *et al*. (2014)-Appendix L
Gene mutation (Spi-) assay gpt delta transgenic mouse primary embryo fibroblasts (MEF) Oxidative stress	0.1,1, 10 and 30µg/mL 3 days exposure without S9 No positive control	**1)TiO_2 NPs, anatase 5 nm** **2) TiO_2 NPs, anatase, 40 nm** **3) TiO_2, anatase, 325 mesh** NSC: 2 sonication and indirect assessment of exposure to particles by flow cytometry	**Positive**: 5 and 40 nm significantly increased mutation yield at 0.1 µg/mL and above; the effect was concentration related with TiO_2 NPs (40nm), as reported by the authors, however, it is not supported by statistical analysis, nor by the visual inspection of the data. The effect was abrogated by the concurrent treatment with the endocytosis inhibitor Nystatin. Treatment of MEF cells with TiO_2 NPs (40 nm), resulted in a concentration	2 No positive control was used. Results of statistical analysis not reported in detail, test system not validated for regulatory purpose	Limited	Xu *et al* (2009) Appendix L

			dependent decrease in cell viability when analysed with MTT assay. **Negative:** TiO_2 -325 mesh			
Mammalian Cell Gene Mutation Test (Hprt) OECD TG 476 Chinese hamster lung (V79-4) fibroblasts	3, 15 and 75 µg/cm2 24h Positive control: MMS	**TiO_2 NPs (NM105), anatase/rutile, 15-24 nm** NSC:1 Two dispersion protocols, good dispersion for protocol 1, and larger agglomeration for protocol 2, giving different size distribution.	**Negative:** No mutagenic effects independently of the dispersion protocol used	1	High	Kazimirova *et al*.,2020- Appendix J
Mammalian cell gene mutation assay HPRT locus CHO-K1 cells	10, 20 or 40 µg/mL for 60 days without S9	**TiO_2 NPs, anatase, < 25 nm (XRD)** NSC: 1 no information on dispersion but exposure confirmed by EM and Ti measurements	**Negative:** no significant increase in gene mutations. No effects on colony forming ability. No cytotoxicity (XTT assay). Cells exposure is demonstrated	2 No positive control; no cytotoxicity observed.	Limited	Wang *et al* (2011)- Appendix L
Mammalian cell gene mutation test (Thymidine kinase (Tk) locus) in mouse lymphoma L5178Y cells (OECD TG 490)	Particle uptake TEM: 0, 1, 100 µg/mL of TiO_2-NPs < 25 nm TiO_2-NPs (50 nm) Mouse lymphoma assay: 0, 1, 10, 100 µg/mL of microparticulated form of titanium dioxide (TiO_2), TiO_2 NPs (24.23 nm) and TiO_2 NPs (50 nm) Exp: Particle uptake TEM: 24 hours	**1) TiO_2 NPs, anatase 24.2 nm (TEM)** **2) TiO_2 NPs, anatase 50.2 nm (TEM)** **3) micro-TiO_2, (no further information available**) NSC: 1 Dispersion measured according to the Nanogenotox protocol and cellular internalisation	Equivocal No statistically significant increase compared to the negative control. The Global Evaluation Factor (GEF) was not exceeded. However, a statistically significant concentration/eff ect trend was observed in 6 separate experiments	2 Methods not reported in details, only reference to 6 publications provided	Limited	Demir *et al*.,2017

		confirmed by TEM. Both NPs and agglomerates observed in the exposed cells.				
Mouse lymphoma gene mutation assay L5178Y TK+/- cells	NM-102: two series of concentrations: 1) 0, 32, 64, 128 and 256 µg/ml 2) 0, 312.5, 625, 1250 and 2500 µg/ml NM-105: two series of concentrations: 1) 0, 32, 64, 128 and 256 µg/ml 2) 0, 625, 1250, 2500 and 5000 µg/ml	**1) TiO_2NPs (NM102), anatase, 21-22 nm 2) TiO_2NPs (NM105), anatase/rutile, 15-24 nm** NSC: 1 Nanogenotox Project dispersion protocol	**Negative** for all forms of TiO_2 NPs tested	1: Only minor deficiency in data reporting.	High	NANOGENOTOX Project, 2013 (Documentation provided to EFSA No. 7 and 8) – Appendix N

29. Other data (seven bacterial reverse mutation studies and one submitted by industry) were considered of low relevance due to limitations in the penetration of particles through the bacterial cell wall and lack of internalisation in bacteria (EFSA Scientific Committee, 2018a).

In vivo gene mutations

30. Six studies considered of high or limited relevance have been reviewed. All studies were performed with TiO_2 NPs <30nm (limited relevance to the safety of E171 but considered for completeness of database and to assess whether a particle size limit should be applied).

Table 2: Summary *of in vivo* studies considered by EFSA

Test System	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's Reliability Score**	**EFSA's evaluation of Relevance**	**Ref**
In vivo DNA deletion assay in the p^{un} locus. C57Bl/6Jp^{un}/p^{un} mice; pink-eyed unstable (p^{un}) locus (internal duplication) Reconstitution of the wild-type *p* gene can	**Oral route:** Mice were treated with TiO2 NPs during embryonic development at a total dose of 500 mg/kg. Offspring were sacrificed at age of 20 days.	**TiO_2NPs (P25), anatase/rutile, 15-24 nm** NSC: 2 Ultrasonication in water and consideration of agglomeration,	**Positive** TiO_2 NPs increased DNA deletion frequency in fetuses.	2: "The assessment of genotoxicity in developing embryos was based on method	Limited	Trouiller *et al*. (2009)- Appendix M

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	Water was used as negative control.	reporting is insufficient but indicates presence of both particles and agglomerates		developed in-house, which has not been validated". (EFSA ANS Panel, 2016)		
Pig-A gene mutation assay in peripheral blood reticulocytes and in total red blood cells of the same animals. Male B6C3F1 mice	0.5, 5.0, and 50 mg/kg/day, **administered by intraperitoneal (i.p.) injection** for 3 days; positive control: 140 mg/kg ENU, Cells analysis over 6 weeks	**TiO_2NPs, anatase, ellipsoidal shape, minor axes 12.1 ± 3.2 nm (TEM)** NSC: 1 Sonication, agglomeration reported for each concentration and confirmation of exposure by measuring Ti levels in tissues	Negative	2 Reporting is inconsistent for the route of application (i.p. or i.v.), but upon request the study authors confirmed i.p.	Limited The route of administration is not relevant to dietary intake.	Sadiq *et al*. (2012)-Appendix M
Pig-a mutation in erythrocytes gpt and Spimutation in liver Male gpt Delta transgenic C57BL/6J 5 mice/group	2, 10 and 50 mg/kg bw, **intravenously (i.v)**, once a week for 4 consecutive weeks positive control: ENU or DEN	**TiO_2NPs (P25), anatase/rutile, 15-24 nm .** NSC:1 Level of dispersion measured for each concentration and cellular internalisation confirmed by EM with Ti detection	**Negative:** No significant increase in the frequency of Pig-a mutant frequency in erythrocytes nor of gpt and Spi- mutants in liver. TiO_2NPs accumulated in liver and localised mainly in Kupffer cells	1	Limited Route of administration not relevant for oral exposure	Suzuki *et al*.,2016-Appendix K
gpt and Spi- mutation in liver Male C57BL/6J gpt delta mice, 6 animals/grou p	0, 2, 10, and 50 mg/kg **i.v.** Exposure: weekly for 4 consecutive weeks. Mice were euthanized on day 90 after the final injection of TiO_2NPs The route of administration is not relevant to dietary intake, but relevant	**TiO_2NPs (P25), anatase/rutile, 15-24 nm** NSC: 1 Dispersion, stability and cellular internalisati on measured and reported.	**Negative:** 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 TiO_2NPs has no mutagenic effect on hepatocytes in mice	2 neither a positive control nor DNA from previous positive control was included, (although it is noted that in a	Limited	Suzuki *et al*, 2020-Appendix K

	for ADME/(geno)tox		90 days after the last administration.	previous study by the same authors (Suzuki *et al*., 2016) the positive controls performed as expected).		
LacZ gene mutation assay in liver and spleen C57Bl/6 transgenic mice (LacZ)	**i.v.** on 2 days. Sacrifice 28 days after last i.v administration 0, 10, 15 mg/kg bw, Positive control: ENU 120 mg/kg bw, **i.p.**	**TiO_2NPs (NM-102), anatase, 21-22 nm** NSC:1 Nanogenotox protocol and confirmation of exposure by EM (although not all data reported, and EM did not include detection of Ti)	**Negative**	1	Limited The route of administration is not relevant to dietary intake	Louro *et al*, 2014-Appendix M
Pig-a Male Sprague-Dawley rats	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 cm2/lung); Six rats were injected with MNU (N-methyl-Nnitrosourea) in **one i.p. injection** 35 days before kill as positive control for the mutation assay administered at a dose of 60 mg/kg	**TiO_2NPs (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)	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-Appendix K

EFSA's concluding remarks

31. "Several in vitro studies demonstrated the ability of TiO_2 NPs to induce gene mutations in cultured mammalian cells. One *in vivo* study indicated the induction of large DNA deletions, however four other studies, that investigated different molecular targets suitable for identification of point mutations and small deletions, gave consistently negative results. Overall, the available experimental

data do not confirm the potential of TiO_2 NPs (< 30 nm) to induce gene mutations *in vivo*." (EFSA, 2021)

In vitro micronuclei/chromosomal aberrations

32. Out of fifty six available studies, fourty three were classified as high or limited relevance and considered for the assessment. Due to the large volume of studies considered, a summary table is not presented here, however the relevant Appendices (J, L, N, P) have been attached in the Annex2.These will also include characterisation of the test material.

33. Positive results were reported in three out of seven studied in primary human lymphocytes that were considered of high or limited relevance. In a study, classified of high relevance, a concentration-dependent increase of MN frequency was observed in peripheral blood lymphocytes from healthy subjects and colon cancer patients (Kurzawa-Zegota *et al*., 2017- Appendix J). Positive results in cultures of human peripheral lymphocytes were also reported in two studies with limited relevance (Appendix L:Turkez and Geyikoglu, 2007;Appendix N: Kang *et al*., 2008). Negative or equivocal results were described in four studies classified at high or limited relevance (Appendix N: NANOGENOTOX Project 2013 Documentation provided to EFSA No 7 and 8; Appendix L:Tavares *et al*., 2014; Appendix J: Andreoli *et al*., 2018; Osman *et al*., 2018))

34. Three out of four studies performed with intestinal cells were considered relevant. One study, classified at high relevance, showed negative results with MN assays in Caco-2 cells exposed at different concentrations of TiO_2 NPs (Appendix J: Zijno *et al*., 2015). EFSA considered the outcome of this study to be consistent with the results reported in the same cell line by the NANOGENOTOX Project, 2013 (Documentation provided to EFSA No 7 and 8- Appendix N). A single study showed concentration dependent increase of MN frequency in human colon adenocarcinoma (HCT116) cell line (Appendix J: Proquin *et al*., 2017).

35. Thirteen studies performed with lung cells were classified as relevant. Four out of five studies available in human lung epithelial cells (BEAS-2B) were negative with MN tests after exposure at different concentrations of TiO_2 NPs and for different times ((Appendix N:NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Appendix J:Vales *et al*., 2015; Di Bucchianico *et al*., 2017; Zijno *et al*., 2020). In Falck *et al*. (2009)- Appendix L, negative (rutile, 5,000 nm) and equivocal (anatase, < 25 nm) results were reported. Positive results with the MN test were reported in BEAS-2B cells only using a treatment medium that minimised the nanoparticle agglomeration (Appendix L:Prasad *et al*., 2013). Inconsistent results were reported in studies in human lung carcinoma cell line (A549). Two out of five studies were evaluated as positive (Appendix L: Srivastava *et al*., 2013; Appendix J: Stoccoro *et al*., 2017). Negative results were reported in two studies ((Appendix N: NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Appendix J: Brandao *et al*., 2020) classified at high relevance and in a study with limitations (Appendix L: Jugan *et*

al., 2012). Negative results with the CA test were described in a Chinese hamster lung cell line (CHL/IU cells) (Appendix L: Nakagawa *et al*., 1997).

36. Two studies in human epidermal cell lines (A431, NHEK) to which high relevance was assigned were positive ((Appendix N: NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Appendix L: Shukla *et al*., 2011).

37. Twenty-six studies carried out in various other types of cell lines of different origin, reporting results on MN frequency or on structural CAs were evaluated: eight of them were classified of high relevance and ten of limited relevance. The differences in the results observed in different studies could not be attributed to a certain parameter such as the crystalline form, particle size, degree of aggregation, treatment medium used, concentrations applied and treatment time.

38. The Panel noted that around 60% of the available results were obtained with TiO2 NPs < 30 nm. The majority of in vitro MN or CA tests gave negative results, regardless of the size of the tested particles (55% for TiO2 NPs < 30 nm and 67% for TiO2 NPs > 30). A single study tested E 171 in intestinal cells and reported positive results (Appendix J: Proquin *et al*., 2017).

In vivo micronuclei/chromosomal aberrations

39. Out of twenty-six studied, fifteen were ranked as high or limited relevance and considered for assessment.

Table 3:Summary of micronucleus and chromosomal aberrations *in vivo* studies considered by EFSA

Test System	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's Reliability Score**	**EFSA's evaluation of Relevance**	**Ref**
Micronucleus assay in bone marrow Sprague-Dawley male rats	**Oral route** Intragastric administration once a day for 30 consecutive days 0, 10, 50, 200 mg/kg; 7 rats each group.	**TiO_2 NPs, Anatase 75 ±15 nm** NSC: 2sonication, agglomeration confirmed (reported size 473.6nm)	**Negative** 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	2 No positive control	Limited	Chen *et al*. (2014) Appendix M
Micronucleus test and Mammalian bone marrow chromosomal aberration test	200 and 500 mg/kg per bw 90 daily **oral** administrations by gavage to 5 animals/sex/dose	**TiO_2 NPs, 58.25 8.11 nm** (SEM) (crystalline form unknow)NSC:4 for *in vivo* assay	**In vivo MN test: Positive.** Significant (P<0.01) increase in the mean	2 Data show some inconsistencies in the	Limited	Chakrabarti *et al*. (2019) Appendix K

Swiss-Albino mice		Insufficient information provided on dispersion and only high doses used.	percentages of MNPCEs at the highest dose. The ratio PCE/total erythrocytes not affected by treatments, at any dose. **Chromosome aberration test**: **Positive**. Statistically significant increase (P<0.01) in the incidence of chromosomal aberrations at the highest dose.	comparison of total MNPCEs or aberrant cells and their frequencies.		
Micronucleus test in bone marrow cells male Swiss albino mice: 5 animals/group	**Oral:** gavage 10, 50, 100 mg/kg bw /day for 14 days positive control EMS, single ip 100 mg/kg b.w.	**TiO_2 NPs Anatase, 20–50 nm** NSC:1 Sonication and level of agglomeration reported for each dose, a level of agglomeration observed	**Positive** Dose-related increase on MNPCEs statistically significant only at the highest dose. Data on bone marrow toxicity not reported.	1	High	Shukla *et al*. (2014)Appendix K
Chromosome aberrations Swiss albino male mice: 5 animals/dose	**Oral:** 0.2, 0.4, and 0.8 mg/kg/day by gavage for 28 days Positive control: MMC (by i.p.) Bone marrow sampled 18 h after the last treatment Analysed 150 metaphases per anima	**TiO_2 NPs, Rutile, 21–31 nm**, **(TEM), spherical and rodshaped particles (TEM), 21-31 nm (TEM)** NSC:2 Dispersion measured and high level of agglomeration confirmed.	**Positive** 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	2 Data in Table 1 are not consistent with the scoring of 750 metaphases (150 x 5 animals), as stated in Methods	Limited	Manivannan *et al*. (2020) Appendix K
Micronucleus test in bone marrow cells: 6 animals/dose Male Wistar rats	**Oral:** TiO2 NPs suspended in distilled water at 50, 100 and 200	**TiO_2 NPs, Rutile, 5-12 nm** NSC:2	**Positive** Statistically	2 Treatment determined	Limited	Grissa *et al*. (2015) Appendix K

	mg/kg bw 60 days by gavage	Sonication performed with no additional information	significant and dose related increase on MNPCEs at the two highest doses. Significant decrease of PCE/total erythrocyte ratio at top dose.	distinct hematotoxicity with formation of abnormally shaped red blood cells with Heinz bodies. It is not clear whether this could have bias the scoring of MNPCEs in bone marrow.		
Micronucleus test Male B6C3F1 mice (peripheral blood and in bone marrow erythrocytes)	**I.p. injection:** on 3 consecutive days, animals euthanised 48-hr after the last treatment. groups of 5 mice; 2 experiments in bone marrow: 1) 250, 500 and 1,000 mg /kg bw (same doses for peripheral blood) 2) 500, 1,000 and 1,500 mg/kg bw	**TiO_2 Anatase (Unitane® 0-220)** **> 100 nm** NSC 3 No information on dispersion.	**Equivocal** Not all criteria for a clearly positive result are met (all values were within the range of spontaneous control values observed in this study). 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 Exp 2: MN increase at 1,000 mg/kg bw, but not significant linear trend.	2 Equivocal results No positive control	Limited The route of administration is not relevant to dietary intake. Not all criteria for a clearly positive result are met.	Shelby *et al*. (1993)Appendix M
Chromosomal aberration in bone marrow Male B6C3F1 mice;8 animals/group	**I.p. injection:** animals euthanised 17 or 36-hr after the injection. 625, 1250, 2500 mg/kg bw	**TiO_2 Anatase (Unitane® 0-220)** **> 100 nm** NSC 3 No information on dispersion.	**Negative**	2 Positive control included, but data not reported.	Limited The route of administration is not relevant to dietary intake.	Shelby and Witt (1995)-Appendix M
Micronucleus assay in bone marrow Male Swiss Webster mice- 5 animals/group	**I.p. injection:** administration for 5 consecutive days. Animals sacrificed after 24- hr 0, 500, 1000, 2000 mg/kg bw per day; 5 animals/group. Positive control:	**TiO2NPs, mixture of rutile and anatase (XRD), 44 nm (XDR), polyhedral morphology (TEM**) NSC: 1 exposure	**Positive** and decrease of PCE/NCE	1	Limited The intraperitoneal route of administration applied in this study is not recommended by OECD	El-Ghor *et al*. (2014) Appendix M

	cyclophosphamide	confirmed by Ti measurements in tissues No information on dispersion method			guidelines, as non-physiological; in addition, the route of administration is not relevant to dietary intake	
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) and 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	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) Appendix K
Micronucleus test in bone marrow erythrocytes Male swiss mice, 6-8 weeks old	**i.p. injection** 9.38, 18.75, 37.50, 75, 150 mg/kg bw for five consecutive days	**TiO_2 NPs Anatase, < 30 nm** NSC: 2 Dispersion and stability measured and some level of agglomeration confirmed	**Positive** after 5 days treatment statistically significant and dose dependent increase in micronucleated polychromatic erythrocytes (MNPCEs) and decrease of the PCE:NCE ratio; After a 5 days recovery period since last treatment (sacrifice at day 10), the increase of MNPCEs was still statistically significant at the	1	Limited The relevance of the test system is considered limited as the route of administration is not relevant to dietary intake	Fadoju *et al*. (2019) Appendix K

			higher dose. All the animals appeared healthy during the exposure duration. No significant changes in the body weights of the animals. Significant changes in the activities of superoxide dismutase, catalase, and levels of reduced glutathione and malondialdehyde were observed in liver and kidney of TiO2 NPs treated mice compared to untreated controls The i.p. administration of TiO2 NPs induced ROS production and altered oxidative stress parameters in liver and kidney			
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) and 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	2 Single sampling was performed exceedingly low baseline incidence of MNPCEs, inconsistency of tabular and graphical data	Limited	Zirak *et al*. (2016)-Appendix K
Micronucleus assay in bone marrow PCE and reticulocytes Male Wistar rat	**i.v. injection:** single dose. 5 mg/kg bw of TiO2 NPs (P25), Groups of 7 animals, sacrificed 24h, 1 week and 4 weeks after injection. For estimation of	**TiO2NPs (P25), anatase/rutile, 15-24 nm** NSC:2 Sonication before administration.	**Positive** MN cells frequency increase in PCE only after 24h, no changes in PCE%. No MN increase in	2 No positive control	Limited The route of administration is not relevant to dietary intake.	Dobrzynska *et al*. (2014)Appendix M

	induction of MN in PCE, cells were stained with solutions of May Grunwald and Giemsa stains. For estimation of induction of MN in reticulocytes, cells were stained with acridine orange		reticulocytes in the same blood smears.			
Micronucleus test in peripheral blood reticulocytes (RETs) Male gpt Delta transgenic C57BL/6J, 5 mice/group	2, 10 and 50 mg/kg bw/week for 4 consecutive weeks **intravenously.** The frequency of MN was determined in the blood specimens collected on day 2 and day 9 after final injection	**TiO_2 NPs (P25) 15–24 nm**, NSC:1 Level of dispersion measured for each concentration and cellular internalisatio n confirmed by EM with Ti detection.	**Negative** No decrease in % reticulocytes: % RETs in the 50 mg/kg TiO2NPtreated group was significantly higher than that in the control on day 2	2 No positive control	Limited The route of administration is not relevant to dietary intake.	Suzuki *et al*. (2016) Appendix K
Micronucleus assay in peripheral blood reticulocytes C57Bl/6 transgenic mice (LacZ)	**i.v. injection** on 2 days. 0, 10, 15 mg/kg bw, Blood collected 42- hr after last i.v Positive control: ENU 120 mg/kg bw, i.p.	**TiO_2 NPs (NM-102), 21–22 nm** NSC:1 Nanogenotox protocol and confirmation of exposure by EM (although not all data reported and EM did not include detection of Ti)	**Negative**	2 Only one sampling time	Limited	Louro *et al*. (2014) Appendix M

40. The studies administered via the oral route (gavage) were given higher weight and four were evaluated as positive for the induction of micronuclei or structural chromosomal aberrations. One (Chen *et al*., 2014) tested negative in the rat bone marrow micronucleus assay, although some evidence of bone marrow exposure was provided by the concurrent analysis of H2AX foci.

41. The studies via intraperitoneal and intravenous injection were considered as supporting evidence. EFSA considered that the *in vivo* studies – one of them of high relevance and the others of limited relevance– "were predominantly positive, independently of the route of exposure. Discrepant results were reported in some studies using comparable dose ranges, species and endpoint, which cannot be traced to size or other specificities of the test material. Rather, it is possible that differences in handling of TiO_2 NPs, and dispersion protocols, which were insufficiently reported for most studies, were important variability factors" (EFSA, 2021).

EFSA's concluding remarks*:*

42. Overall, based on the available lines of evidence, the Panel considered that - on balance - TiO_2 NPs have the potential to induce MN/CA. The Panel noted that a significant portion of the studies was performed using TiO_2 NPs < 30 nm, however some positive results were observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in MN/CA assay was observed.

Comet Assays (*in vivo and in vitro*)

43. *In vitro,* 142 assays were available and out of those 106 were classified as high or limited relevance and considered for risk assessment. The range of titanium dioxide particle size tested was from 2.3 nm to 5 µm. Information on these studies including particle sizes can be found in Appendices J,L,N (Annex2)

44. *In vivo,* eighteen out of fourty-four studies were classified as high or limited relevance (Appendices K, M). Details of the results of the *in vivo- and in vitro* assays can be found in pages 53-57 of the EFSA Opinion (Annex1), however due to the large volume of studies only the main findings have been summarised in this paper.

45. *In vitro* the majority of the studies performed on colon cancer cells tested positive in the Comet assay, showing an increase in DNA damage, i.e. strand breaks or strand breaks and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites (Fpg detects oxidised purines). Test particle sizes varied from 15-150 nm, with two performed with E171 specification titanium dioxide[7]. Some of the studies have been found to be negative (Dorier *et al*., 2019- Appendix J) test materials 1)E171 (118 ± 53 nm,2) TiO_2 NPs, anatase, 12±3 nm[A12] and 3) TiO_2 NPs (NM105), anatase/rutile, 15-24 nm or equivocal- test material TiO_2 (NM-100), anatase, 50-150 nm (Vila *et al*., 2018- Appendix J).

46. All five studies performed on human peripheral blood mononuclear cells (PBMC) were positive, most of them for strand breaks (Demir *et al*., 2013; Cowie *et al*., 2015; Kurzawa-Zegota *et al*., 2017; Andreoli *et al*., 2018; Kazimirova *et al*., 2019) and also for Fpg- and Endo III-sensitive sites (Demir *et al*., 2013). One of these studies showed a negative response in some donors (Kazimirova *et al*., 2019).Test particle sizes varied from 2.3 to 60 nm (Appendix J).

[7] E171 (39% nano)- Proquin *et al*., 2017, Appendix J & E 171, anatase (0.2% rutile), 390 nm (DLS)- Brown *et al.,* 2019, Appendix J.

47. Two studies performed with human lymphoblastoid TK6 cells, showed DNA damage after exposure to titanium dioxide particles- test material size from 15-150 nm (Appendix J: Cowie *et al*., 2015; El Yamani *et al*., 2017) and two studies were negative. Test materials were TiO_2NPs (NM105), anatase/rutile, 15-24 nm (Magdolenova *et al*., 2012) and TiO_2NPs, anatase, ellipsoidal shape (TEM), 10x 30 nm, minor axes 12.1 ± 3.2 nm(Woodruff *et al*., 2012).Information on these studies available in appendix L.

48. Fourteen studies (9 considered high relevance) used a lung model and the majority showed positive results for strand breaks for test materials size ranging from 12 to <5000nm (Appendix L: Falck *et al*., 2009; Karlsson *et al*., 2009; Jugan *et al*., 2012; Prasad *et al*., 2013; Appendix N: NANOGENOTOX project, 2013 Documentation provided to EFSA No 7, 8 and 10; Appendix J: Cowie *et al*., 2015; Wang *et al*.,2015; Biola-Clier *et al*., 2017; El Yamani *et al*., 2017; Stoccoro *et al*., 2017; Murugadoss *et al*., 2020; Zijno *et al*., 2020) as well as oxidised DNA lesions (Di Bucchianico *et al*., 2017; El Yamani *et al*., 2017; Stoccoro *et al*., 2017; Zijno *et al*., 2020). There were two negative studies for strand breaks- test materials size ranging from 21-150 nm (Appendix J: Vales *et al*., 2015; Di Bucchianico *et al*., 2017).

49. The majority of Comet assays in other cell types such as HepG2, THP-1, BeWo b30 placenta, HEK293, cerebral endothelial cells, HeLa, HUVECs, TH-1,GM07492, MCF-7, L-02 human fetus hepatocytes, NHEK normal keratinocytes, HEp-2 derived from HeLa, A431 keratinocytes, EUE human embryonic epithelial cells showed positive results (Appendix L: Osman *et al*., 2010; Shukla *et al*., 2011, 2013; Demir *et al*., 2013Appendix N: NANOGENOTOX project, 2013 Documentation provided to EFSA No 7, 8 and 10); Appendix J: Cowie *et al*., 2015, Shi *et al*., 2015; Ferraro *et al*., 2016; Brown *et al*., 2019; Liao *et al*., 2019; Murugadoss *et al*., 2020; Kumar *et al*., 2020). Test material sizes varied from10 to 390 nm, with one material (Brown et al., 2019) E171 specification titanium dioxide (see footnote no.7, page 23) with four (test material sizes ranging from 5 to 49 nm) testing negative (Appendix L: Woodruff *et al*., 2012;Appendix J: Franchi *et al*., 2015; Sramkova *et al*., 2019; Elje *et al*., 2020) and two (test material sizes ranging from 15-150 nm) equivocal. (Appendix L: Magdolenova *et al*., 2012; Appendix J: Brzicova *et al*., 2019).

50. The majority of assays in cells from monkey, rat or hamster origin were also positive. Three from four different types of titanium dioxide tested in mouse lymphoma L5178Y cells by Nakagawa *et al*. (1997) were negative (anatase 21 nm, rutile 255 nm and rutile 420 nm) and one was positive (anatase 255 nm). In a study of Brown *et al*. (2019), E 171 ((anatase (0.2% rutile), 390 nm (DLS)) was positive for strand breaks in all studied cell lines, and positive for oxidised DNA lesions only in one of them (HepG2) (Brown *et al*., 2019).

51. The Panel noted that around 5% of available studies were obtained with titanium dioxide <30nm, however no clear dependence of the positive effect on particle size was observed. The majority of in vitro comet assay gave positive results, regardless of the size of the tested particles (87% positive findings for titanium dioxide particles > 30 nm and 78% positive findings for titanium dioxide NPs < 30 nm). Five studies of high or limited relevance investigated, by the in vitro Comet assay, the effect of E 171 treatment; 4 studies were positive for strand breaks and 1 negative.

52. *In vivo*, out of 9 studies administered by oral gavage 6 tested positive (Appendix K: Sycheva *et al*.,2011[8];Appendix M: Shukla *et al*., 2014; Grissa *et al*., 2015; Shi *et al*., 2015; Manivannan *et al*., 2020; Murugadoss *et al*., 2020). Test material sizes varied from -160 nm[9], doses from -2000mg/kg bw/d and treatment times from 7-60 days. Three were negative (Appendix K: Bettini et al., 2017; Martins et al., 2017; Jensen et al., 2019.) Two of those included E171[10] (Bettini et al., 2017 Jensen et al., 2019) and other TiO_2 NPs. The study doses and duration were 50 and mg/kg once a week, for 10 weeks (Jensen *et al.,* 2019), 10mg/kg bw/d for 7 days (Bettini *et al.*,2017) and 0.5mg/kg bw/d for 45 days (Martins *et al.,*2017) To identify possible factors responsible for the different outcomes of the assays, the Panel took into consideration physico-chemical characteristics of TiO_2 NPs (crystalline form, size of constituent particles, shape and agglomeration state), time of exposure, doses and target tissues. No obvious correlation could be identified between specific physicochemical properties of the titanium dioxide particles and the outcome of the assays, the time of exposure nor the administered titanium dioxide particle doses. The Panel calculated a cumulative dose by integrating dose and time of treatment, however this factor alone appeared not to be the main determinant of assay results. The Panel noted that the majority of the positive results were obtained from organs of the reticulo-endothelial system.

53. Studies via intravenous and intratracheal instillation administration were also considered. The Panel considered that the induction of DNA damage in liver following intra-tracheal instillation demonstrates a systemic effect which is possibly triggered by an inflammatory response observed in the lung.

EFSA concluding remarks

54. Based on the results of the *in vitro* and *in vivo* comet assays, the Panel concluded that "TiO_2 particles have the potential to induce DNA damage. The

[8] Positive for bone marrow, negative for liver and brain

[9] Murugadoss *et al*.,(2020) dosed at 10, 50, 250 µg/ animal

[10] **Bettini *et al.,*** 2017*:* 1) E 171, anatase, 20- 340 nm (118 nm) (TEM); 44.7% (< 100 nm 2) TiO2NPs (NM-105), anatase/rutile, 15-24 nm and **Jensen *et al.,*** 2019 E171, anatase (0.2% rutile), three size group s of particles : 135 ± 46 nm, 305 ± 61, 900 ± 247 nm (TEM image)

Panel noted that a significant portion of the studies were performed using TiO_2 NPs < 30 nm, however some positive results were also observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in Comet assay was observed" (EFSA, 2021).

Other Studies:

55. Numerous other studies investigating titanium dioxide exposure and DNA damage were reported. Due to the large volume of studies only the main findings are summarised. These include studies on DNA binding, γH2AX foci and other markers of DNA Damage, Oxidised DNA bases, Reactive oxygen species, Epigenetic DNA methylation and Cell transformation. (pages 57-60 of the EFSA Opinion). The results of the above studies were used to attempt to establish a mode of action (page 60-62 of EFSA Opinion).

56. Overall, combining the available lines of evidence, the Panel concluded that "TiO_2 particles had the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations. No clear correlation was observed between the physico-chemical properties of TiO_2 NPs, such a crystalline form, size of constituent particles, shape and agglomeration state, and the outcome of either *in vitro* or *in vivo* genotoxicity assays. The Panel considered that there is some evidence for internalisation of TiO_2 nanoparticles in the nucleus and mitochondria" (EFSA, 2021).

57. Furthermore they concluded that there is evidence for several modes of action for genotoxicity that may operate in parallel (excerpt from EFSA, 2021):

- Direct interaction of TiO_2 nanoparticles with DNA (there is no proof for covalent binding).
- Direct formation of reactive (oxygen) species due to intrinsic properties of TiO_2 nanoparticles.
- Reactive (oxygen) species formation via TiO_2 particles-induced inflammation.
- Reactive (oxygen) species formation via interference of TiO_2 nanoparticles with mitochondrial function.

58. Additionally, there are indications that TiO_2 particles may:

- induce epigenetic modifications affecting the expression of genes involved in the maintenance of genome function (e.g. downregulation of some genes involved in DNA repair pathways).

- interact with proteins involved in the control of chromosome segregation and the spindle apparatus.

59. The Panel concluded that “the relative contribution of the modes of action mentioned above to the genotoxicity elicited by TiO_2 particles is unknown and there is uncertainty on whether a threshold mode of action could be assumed. Even if it was assumed that all modes of action would be indirect, the available data would not allow identification of a threshold dose. Therefore, the Panel concluded that a concern for genotoxicity of TiO_2 particles that may be present in E 171 cannot be ruled out. A cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified” (EFSA, 2021).

Overall EFSA conclusions:

60. Concerning the genotoxicity studies, combining the available lines of evidence, the Panel concluded that “TiO_2 particles have the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations. No clear correlation was observed between the physico-chemical properties of TiO_2 particles – such as crystalline form, size of constituent particles, shape and agglomeration state – and the outcome of *in vitro* or *in vivo* genotoxicity assays” (*i.e* a cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified). The Panel also concluded that “several modes of action (MOA) may operate in parallel and the relative contributions of the different molecular mechanisms resulting in the genotoxicity of TiO_2 particles are unknown. Based on the available data, no conclusion could be drawn as to whether the genotoxicity of TiO_2 particles is mediated by a mode (s) of action with a threshold(s)”. Therefore, the Panel concluded that a concern for genotoxicity of TiO_2 particles cannot be ruled out.

61. With regards to other endpoints the Panel concluded (excerpt from EFSA, 2021): “that the absorption of TiO_2 particles is low, however they can accumulate in the body due to their long half-life; studies on general and organ toxicity, including the newly performed EOGRT study with E 171, did not indicate adverse effects up to a dose of 1,000 mg/kg bw per day. Also, no effects were seen in studies retrieved from the literature with TiO_2 NP > 30 nm up to the highest dose tested of 100 mg/kg bw per day. No effects on reproductive and developmental toxicity up to a dose of 1,000 mg/kg bw per day, the highest dose tested, were observed in the EOGRT study with E 171. No other reliable studies were found in the literature addressing these effects with E 171;some findings regarding immunotoxicity and inflammation with E 171 as well as neurotoxicity with TiO_2 NPs may be indicative of adverse effects. They also considered that there are indications of the induction of aberrant crypt foci with E 171 and that no studies appropriately designed and conducted to investigate the potential carcinogenicity of TiO_2 nanoparticles were available.”

62. Overall, on the basis of all currently available evidence along with all the uncertainties, in particular the fact that genotoxicity concern could not be ruled out, the Panel concluded that E 171 can no longer be considered as safe when used as a food additive.

63. The Panel, after evaluating the scientific evidence available, has identified uncertainties related to the following points:

- The size distribution of the particles in marketed E 171 that consumers are exposed to, related to the different types of E 171, as presented in the EFSA FAF Panel (2019) opinion.
- The processes used by industry when using E 171 in food and to what extent these processes may affect the degree of agglomeration and thus internal exposure.
- State of agglomeration i.e. presence of 'free' (non-agglomerated) particles of tested material in GIT of the animals and its effect on absorption.
- Representativity of different tested materials used in toxicity and genotoxicity studies for the food additive E 171 when used in food.
- Differences in the physico-chemical properties of the different tested materials and the extent of their impact on the observed results.
- Interference in the measurements of Ti/TiO_2 in blood, tissues or organs with the most widely used analytical technique, i.e. ICP-MS, and its impact on the reliability of tissue concentration data.
- Confidence in the limited kinetic data as the basis for estimating half-lives and accumulation and for assessment of internal exposure and, related to that, the extent of systemic availability.
- None of the rodent studies were sufficiently long to cover the time needed for reaching the steady state for accumulation and this impacted the interpretation of the study results.
- Relative contribution of different molecular mechanisms leading to the production of ROS resulting in the genotoxicity of TiO_2 (inflammation, interaction with mitochondria, intrinsic potential of TiO_2 to generate ROS).
- Several modes of action for the genotoxicity may operate in parallel. The relative contributions of different molecular mechanisms elicited by

TiO_2 particles are unknown; it is unclear if a threshold mode of action could be assumed.

- Nature of the interactions between DNA and TiO_2 particles leading to conformational changes in DNA (EFSA, 2021).

Questions for the COM

64. Members are asked to consider the EFSA opinion:

i) Do Members consider that the weight of evidence supports EFSA's evaluation of the genotoxicity studies with respect to a) nano TiO_2 and b) micro TiO_2? If not, what are the COM's conclusions based on the available information?

ii) Do members consider that the available information demonstrates a direct genotoxic mechanism or do they consider on the balance of probabilities a thresholded mode of action is a) probable and b) likely. Members are asked to indicate the size threshold for genotoxic effects based on the data available based on their response. Members are asked to indicate what evidence would be required to increase or decrease their confidence in these conclusions.

iii) Do Members consider a cut-off value for TiO_2 particle size above which genotoxicity is unlikely and if so can they comment on the magnitude of genotoxic risks associated with 3.2% (the current E171), 1 % and 0.1% of smaller particles in such material?

iv) Could member comment on whether the conclusion "that a concern for genotoxicity of TiO_2 particles cannot be ruled out" is meaningful from a risk communication perspective?

v) There are a number of risk management options that could minimise the risks if a concern for genotoxicity of TiO_2 particles cannot be ruled out, Members are asked to outline the risks and the uncertainties associated with the following options a) do nothing, b) restrict the presence of small particles to less than x% and c) a ban.

Food Standards Agency

May 2021

Abbreviations:

ACF – Aberrant crypt foci

ADI – Acceptable Daily Intake

ADME – Absorption, Distribution, Metabolism, Excretion

ANSES – Agency for Food, Environmental and Occupational Health and Safety

C.I. – Colour Index

CAS – Chemical Abstract Service

ECHA – European Chemicals Agency

EFSA – European Food Safety Authority

EINECS – European Inventory of Existing Commercial Chemical Substances

EOGRT – Extended one-generation reproduction toxicity

GALT – Gut- associated lymphoid tissue

GIT – Gastrointestinal Tract

IARC – International Agency for Research on Cancer

JECFA – Joint FAO/WHO Expert Committee of Food Additives

MN – Micronuclei

PND – Post natal days

PSLT – Poorly Soluble Low Toxicity

RAC – Committee for Risk Assessment

ROS – Reactive Oxygen species

SCCS - Scientific Committee on Consumer Safety

SCF – Scientific Committee on Food

TiO_2 – Titanium Dioxide

TiO_2 NPs – Titanium Dioxide Nanoparticles

References

Andreoli C, Leter G, De Berardis B, Degan P, De Angelis I, Pacchierotti F, Crebelli R, Barone F and Zijno A (2018): Critical issues in genotoxicity assessment of TiO2 nanoparticles by human peripheral blood mononuclear cells, *Journal of Applied Toxicology*: JAT, 38, 1471–1482.

Bettini S, Boutet-Robinet E, Cartier C, Comera C, Gaultier E, Dupuy J, Naud N, Tache S, Grysan P, Reguer S,Thieriet N, Refregiers M, Thiaudiere D, Cravedi JP, Carriere M, Audinot JN, Pierre FH, Guzylack-Piriou L andHoudeau E (2017): Food-grade TiO2impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon, *Scientific Reports*, 7, 40373.

Biola-Clier M, Beal D, Caillat S, Libert S, Armand L, Herlin-Boime N, Sauvaigo S, Douki T and Carriere M (2017): Comparison of the DNA damage response in BEAS-2B and A549 cells exposed to titanium dioxide nanoparticles, *Mutagenesis,* 32, 161–172.

Brandao F, Fernandez-Bertolez N, Rosario F, Bessa MJ, Fraga S, Pasaro E, Teixeira JP, Laffon B, Valdiglesias V and Costa C (2020): Genotoxicity of TiO2nanoparticles in four different human cell lines (A549, HEPG2, A172 andSH-SY5Y), *Nanomaterials*, 10(3), 412. doi: 10.3390/nano10030412.

Brown DM, Danielsen PH, Derr R, Moelijker N, Fowler P, Stone V, Hendriks G, Moller P and Kermanizadeh A (2019): The mechanism-based toxicity screening of particles with use in the food and nutrition sector via the ToxTracker reporter system, *Toxicology in vitro: an international journal published in association with BIBRA*, 61.

Brzicova T, Javorkova E, Vrbova K, Zajicova A, Holan V, Pinkas D, Philimonenko V, Sikorova J, Klema J, Topinka Jand Rossner Jr P (2019): Molecular responses in THP-1 macrophage-like cells exposed to diverse nanoparticles, *Nanomaterials* (Basel, Switzerland) 9(5), 687. https://doi.org/10.3390/nano9050687

Chakrabarti S, Goyary D, Karmakar S and Chattopadhyay P (2019): Exploration of cytotoxic and genotoxic endpoints following sub-chronic oral exposure to titanium dioxide nanoparticles, *Toxicology and Industrial Health*, 35, 577–592.

Chen Z, Han S, Zheng P, Zhou D, Zhou S and Jia G (2020b): Effect of oral exposure to titanium dioxide nanoparticles on lipid metabolism in Sprague-Dawley rats, *Nanoscale*, 12, 5973–5986.

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J and Jia G (2014): Genotoxic evaluation oftitanium dioxide nanoparticles in vivo and in vitro *Toxicology Letters*, 226, 314–319.

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J and Jia G (2014): Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro, *Toxicology Letters*, 226, 314–319.

Chen Z, Wang Y, Zhuo L, Chen S, Zhao L, Chen T, Li Y, Zhang W, Gao X, Li P, Wang H and Jia G (2015a): Interaction of titanium dioxide nanoparticles with glucose on young rats after oral administration, *Nanomedicine*: Nanotechnology, Biology, and Medicine, 11, 1633–1642.

Chen Z, Wang Y, Zhuo L, Chen S, Zhao L, Luan X, Wang H and Jia G (2015b): Effect of titanium dioxide nanoparticles on the cardiovascular system after oral administration, *Toxicology Letters*, 239, 123–130.

Comera C, Cartier C, Gaultier E, Catrice O, Panouille Q, El Hamdi S, Tirez K, Nelissen I, Theodorou V and Houdeau E (2020): Jejunal villus absorption and paracellular tight junction permeability are major routes for early intestinal uptake of food-grade TiO2particles: an *in vivo* and *ex vivo* study in mice, *Part Fibre Toxicol*, 17, 26.

Cowie H, Magdolenova Z, Saunders M, Drlickova M, Correia CS, Halamoda KB, Gombau L, Guadagnini R, LorenzoY, Walker L, Fjellsbo LM, Huk A, Rinna A, Tran L, Volkovova K, Boland S, Juillerat-Jeanneret L, Marano F, CollinsAR and Dusinska M (2015): Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles, *Nanotoxicology*, 9, 57–65.

Demir E, Burgucu D, Turna F, Aksakal S and Kaya B (2013): Determination of TiO2, ZrO2, and Al2O3nanoparticleson genotoxic responses in human peripheral blood lymphocytes and cultured embryonic kidney cells, *Journal of Toxicology and Environmental Health*, Part A: Current Issues, 76, 990–1002.

Demir E, Creus A and Marcos R (2017): Titanium dioxide and zinc oxide nanoparticles are not mutagenic in the mouse, *Fresenius Environmental Bulletin*, 26, 1001–1016.

Di Bucchianico S, Cappellini F, Le Bihanic F, Zhang Y, Dreij K and Karlsson HL (2017): Genotoxicity of TiO2nanoparticles assessed by mini-gel comet assay and micronucleus scoring with flow cytometry, *Mutagenesis*,32, 127–137.

Dobrzynska MM, Gajowik A, Radzikowska J, Lankoff A, Dusinska M and Kruszewski M (2014): Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats *in vivo*, *Toxicology*, 315, 86–91.

Dorier M, Tisseyre C, Dussert F, Beal D, Arnal ME, Douki T, Valdiglesias V, Laffon B, Fraga S, Brandao F, Herlin-Boime N, Barreau Rabilloud T and Carriere M (2019): Toxicological impact of acute exposure to E171 foodadditive and TiO2nanoparticles on a co-culture of Caco-2 and HT29-MTX intestinal cells*, Mutation Research*,845.

ECHA (European Chemicals Agency) (2017): Committee for risk assessment RAC opinion proposing harmonised classification and labelling at EU level of titanium dioxide. EC Number: 236-675-5.

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food) (2016): Re-evaluation of titanium dioxide (E 171) as a food additive, *EFSA Journal 2016*;14(9):4545, 83, doi: https://doi.org/10.2903/j.efsa.2016.4545

EFSA Scientific Committee (2018a): Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: part 1, human and animal health, *EFSA Journal* 2018;16(7):5327,95, https://doi.org/10.2903/j.efsa.2018.5327

El Yamani N, Collins AR, Runden-Pran E, Fjellsbo LM, Shaposhnikov S, Zienolddiny S and Dusinska M (2017): In vitro genotoxicity testing of four reference metal nanomaterials, titanium dioxide, zinc oxide, cerium oxide and silver: towards reliable hazard assessment, *Mutagenesis*, 32, 117–126.

El-Ghor AA, Noshy MM, Gala A and Mohamed HRH (2014): Normalization of nano-sized TiO2-induced clastogenicity, genotoxicity and mutagenicity by chlorophyllin administration in mice brain, liver, and bone marrow cells, *Toxicological Sciences*, 142, 21–32.

Elje E, Mariussen E, Moriones OH, Bastus NG, Puntes V, Kohl Y, Dusinska M and Runden-Pran E (2020): Hepato(geno)toxicity assessment of nanoparticles in a HEPG2 liver spheroid model, *Nanomaterials*, 10.

Fadoju O, Ogunsuyi O, Akanni O, Alabi O, Alimba C, Adaramoye O, Cambier S, Eswara S, Gutleb AC and Bakare A (2019): Evaluation of cytogenotoxicity and oxidative stress parameters in male Swiss mice co-exposed to titanium dioxide and zinc oxide nanoparticles*, Environmental Toxicology and Pharmacology,* 70.

Falck GCM, Lindberg HK, Suhonen S, Vippola M, Vanhala E, Catalan J, Savolainen K and Norppa H (2009): Genotoxic effects of nanosized and fine TiO2, *Human and Experimental Toxicology*, 28, 339–352

Ferraro D, Anselmi-Tamburini U, Tredici IG, Ricci V and Sommi P (2016): Overestimation of nanoparticles-induced DNA damage determined by the comet assay, *Nanotoxicology*, 10, 861–870.

Franchi LP, Manshian BB, de Souza TA, Soenen SJ, Matsubara EY, Rosolen JM and Takahashi CS (2015): Cyto- and genotoxic effects of metallic nanoparticles in untransformed human fibroblast, *Toxicology in Vitro: An International Journal Published in ASSOCIATION with BIBRA*, 29, 1319–1331.

Grissa I, Elghoul J, Ezzi L, Chakroun S, Kerkeni E, Hassine M, El Mir L, Mehdi M, Ben Cheikh H and Haouas Z (2015): Anemia and genotoxicity induced by sub-chronic intragastric treatment of rats with titanium dioxide nanoparticles, *Mutation Research Genetic Toxicology and Environmental Mutagenesis*, 794, 25–31.

Grissa I, Elghoul J, Ezzi L, Chakroun S, Kerkeni E, Hassine M, El Mir L, Mehdi M, Ben Cheikh H and Haouas Z (2015): Anemia and genotoxicity induced by sub-chronic intragastric treatment of rats with titanium dioxide nanoparticles, *Mutation Research Genetic Toxicology and Environmental Mutagenesis*, 794, 25–31.

Han HY, Yang MJ, Yoon C, Lee GH, Kim DW, Kim TW, Kwak M, Heo MB, Lee TG, Kim S, Oh JH, Lim HJ, Oh I, YoonS and Park EJ (2020): Toxicity of orally administered food-grade titanium dioxide nanoparticles, *Journal of Applied Toxicology,* doi: 10.1002/jat.4099.

Heo MB, Kwak M, An KS, Kim HJ, Ryu HY, Lee SM, Song KS, Kim IY, Kwon JH and Lee TG (2020): Oral toxicity of titanium dioxide P25 at repeated dose 28-day and 90-day in rats, *Part Fibre Toxicol*, 17, 34.

Hong J, Hong FS, Ze YG and Zhang YQ (2016): The nano-TiO2exposure can induce hepatic inflammation involving in a JAK-STAT signalling pathway, *Journal of Nanoparticle Research*, 18, 9.

Hu H, Guo Q, Wang C, Ma X, He H, Oh Y, Feng Y, Wu Q and Gu N (2015): Titanium dioxide nanoparticles increase plasma glucose via reactive oxygen species-induced insulin resistance in mice, *Journal of Applied Toxicology*, 35, 1122–1132.

Jain AK, Senapati VA, Singh D, Dubey K, Maurya R and Pandey AK (2017): Impact of anatase titanium dioxide nanoparticles on mutagenic and genotoxic

response in Chinese hamster lung fibroblast cells (V-79): the role of cellular uptake, *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, 105, 127–139.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (1970): Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives. Specifications for the identity and purity of food additives and their toxicological evaluation, *WHO Technical Report Series*, No 445. World Health Organization, Geneva, Switzerland.

Jensen DM, Lohr M, Sheykhzade M, Lykkesfeldt J, Wils RS, Loft S and Moller P (2019): Telomere length and genotoxicity in the lung of rats following intragastric exposure to food-grade titanium dioxide and vegetable carbon particles, *Mutagenesis,* 34, 203–214

Jugan M-L, Barillet S, Simon-Deckers A, Herlin-Boime N, Sauvaigo S, Douki T and Carriere M (2012): Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells, *Nanotoxicology*, 6, 501–513.

Kang SJ, Kim BM, Lee YJ and Chung HW (2008): Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes*,* *Environmental and Molecular Mutagenesis*, 49, 399–405.

Karlsson HL, Gustafsson J, Cronholm P and Moller L (2009): Size-dependent toxicity of metal oxide particles – a comparison between nano- and micrometer size, *Toxicology Letters*, 188, 112– 118.

Kazimirova A, Baranokova M, Staruchova M, Drlickova M, Volkovova K and Dusinska M (2019): Titanium dioxide nanoparticles tested for genotoxicity with the comet and micronucleus assays in vitro, ex vivo and in vivo, *Mutation Research*, 843, 57–65.

Kazimirova A, El Yamani N, Rubio L, Garcıa-Rodrıguez A, Barancokova M, Marcos R and Dusinska M (2020): Effects of titanium dioxide nanoparticles on the hprt gene mutations in V79 hamster cells, *Nanomaterials*, 10.

Kumar S, Hussain A, Bhushan B and Kaul G (2020): Comparative toxicity assessment of nano- and bulk-phase titanium dioxide particles on the human mammary gland in vitro, *Human and Experimental Toxicology*, 39,1475–1486

Kurzawa-Zegota M, Sharma V, Najafzadeh M, Reynolds PD, Davies JP, Shukla RK, Dhawan A and Anderson D (2017): Titanium dioxide nanoparticles induce DNA damage in peripheral blood lymphocytes from polyposis coli, colon cancer

patients and healthy individuals: an ex vivo/in vitro study, *Journal of Nanoscience and Nanotechnology*, 17, 9274–9285.

Liao F, Chen L, Liu Y, Zhao D, Peng W, Wang W and Feng S (2019): The size-dependent genotoxic potentials of titanium dioxide nanoparticles to endothelial cells, *Environmental Toxicology*, 34, 1199–1207.

Lotfi A, Zirak RG, Moghadam MS and Pazooki N (2016): Effects of the interaction of nanorutile TiO2with vincristine sulfate on chromosomal abnormalities in vivo, *International Journal of Advanced Biotechnology and Research*,7, 1083–1093.

Louro H, Tavares A, Vital N, Costa PM, Alverca E, Zwart E, de Jong W, Fessard V, Lavinha J and Silva MJ (2014): Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using LacZ plasmid based transgenic mice, *Environmental and Molecular Mutagenesis*, 55, 500–509.

Magdolenova Z, Bilani D, Pojana G, Fjellsbø LM, Hudecova A, Hasplova K, Marcomini A and Dusinska M (2012): Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity, *Journal of Environmental Monitoring*, 14, 455.

Manivannan J, Banerjee R and Mukherjee A (2020): Genotoxicity analysis of rutile titanium dioxide nanoparticles in mice after 28 days of repeated oral administration, *Nucleus-India*, 63, 17–24.

Martins Jr ADC, Azevedo LF, de Souza Rocha CC, Carneiro MFH, Venancio VP, de Almeida MR, Antunes LMG, de Carvalho Hott R, Rodrigues JL, Ogunjimi AT, Adeyemi JA and Barbosa Jr F (2017): Evaluation of distribution, redox parameters, and genotoxicity in Wistar rats co-exposed to silver and titanium dioxide nanoparticles, *Journal of Toxicology and Environmental Health*. Part A, 80, 1156–1165.

Murugadoss S, Brassinne F, Sebaihi N, Petry J, Cokic SM, Van Landuyt KL, Godderis L, Mast J, Lison D, Hoet PHand van den Brule S (2020): Agglomeration of titanium dioxide nanoparticles increases toxicological responses in vitro and in vivo, *Part Fibre Toxicol*, 17, 10.

Nakagawa Y, Wakuri S, Sakamoto K and Tanaka N (1997): The photogenotoxicity of titanium dioxide particles, *Mutation Research*, 394, 125–132.

OECD (2016): Environment directorate joint meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology. Titanium

dioxide: summary of the dossier. Vol No. 73. (Organisation for Economic Co-operation and Development), ENV/JM/MONO(2016) 25.

Osman IF, Baumgartner A, Cemeli E, Fletcher JN and Anderson D (2010): Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells. *Nanomedicine*, 5, 1193–1203

Osman IF, Najafzadeh M, Sharma V, Shukla RK, Jacob BK, Dhawan A and Anderson D (2018): TiO2NPs induce DNAdamage in lymphocytes from healthy individuals and patients with respiratory diseases-an ex vivo/in vitro study, *Journal of Nanoscience and Nanotechnology*, 18, 544–555.

Prasad RY, Wallace K, Daniel KM, Tennant AH, Zucker RM, Strickland J, Dreher K, Kligerman AD, Blackman CF and DeMarini DM (2013): Effect of treatment media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle, *ACS Nano*, 3, 1929–1942

Proquin H, Rodriguez-Ibarra C, Moonen CG, Urrutia Ortega IM, Briede JJ, de Kok TM, van Loveren H and ChirinoYI (2017): Titanium dioxide food additive (E171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions, *Mutagenesis*, 32, 139–149.

Relier C, Dubreuil M, Lozano Garcıa O, Cordelli E, Mejia J, Eleuteri P, Robidel F, Loret T, Pacchierotti F, Lucas S,Lacroix G and Trouiller B (2017) Study of TiO2 P25 nanoparticles genotoxicity on lung, blood, and liver cells in lung overload and non-overload conditions after repeated respiratory exposure in rats, *Toxicological Sciences*,156, 2.

Sadiq R, Bhalli JA, Yan J, Woodruff RS, Pearce MG, Li Y, Mustafa T, Watanabe F, Pack LM, Biris A, Khan QM and Chen T (2012): Genotoxicity of TiO2 anatase nanoparticles 383 in B6C3F1 male mice evaluated using Pig-a and flow cytometric micronucleus assays. *Mutation Research*, 745, 65–72.
SCF (Scientific Committee for Food) (1975): Reports of the Scientific Committee for Food: first series, p. 17.Available online:
http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_01.pdf

SCF (Scientific Committee for Food) (1977): Reports of the Scientific Committee for Food: Fourth Series, p. 27.Available online:
http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_04.pdf

Scientific Committee on Consumer Safety (SCCS) (2020): Opinion on Titanium Dioxide used in cosmetic products that lead to exposure by inhalation, SCCS/1717/20. Available online:

https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_238.pdf

Shelby MD and Witt KL (1995) Comparison of results from mouse bone marrow aberration and micronucleus test, *Environmental and Molecular Mutagenesis*, 25, 302–313.

Shelby MD, Erexson GL, Hook GJ and Tice RR (1993): Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals, *Environmental and Molecular Mutagenesis*, 21, 160–179.

Shi Z, Niu Y, Wang Q, Shi L, Guo H, Liu Y, Zhu Y, Liu S, Liu C, Chen X and Zhang R (2015): Reduction of DNA damage induced by titanium dioxide nanoparticles through Nrf2 in vitro and in vivo, *Journal of Hazardous Materials*, 298, 310–319.

Shukla RK, Kumar A, Vallabani NV, Pandey AK and Dhawan A (2014): Titanium dioxide nanoparticle-induced oxidative stress triggers DNA damage and hepatic injury in mice. *Nanomedicine,* 9, 9.

Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S and Dhawan A (2011): ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells, *Toxicology In Vitro*, 25, 231–241

Sramkova M, Kozics K, Masanova V, Uhnakova I, Razga F, Nemethova V, Mazancova P, Kapka-Skrzypczak L,Kruszewski M, Novotova M, Puntes VF and Gabelova A (2019): Kidney nanotoxicity studied in human renal proximal tubule epithelial cell line TH1, *Mutation Research-Genetic Toxicology and Environmental Mutagenesis*,845, 9.

Srivastava RK, Rahman Q, Kashyap MP, Singh AK, Jain G, Jahan S, Lohani M, Llantow M and Pant AB (2013):Nanotitanium dioxide induces genotoxicity and apoptosis in human lung cancer cell line, A549, *Human and Experimental Toxicology*, 32, 153–166

Stoccoro A, Di Bucchianico S, Coppede F, Ponti J, Uboldi C, Blosi M, Delpivo C, Ortelli S, Costa AL and Migliore L (2017): Multiple endpoints to evaluate pristine and remediated titanium dioxide nanoparticles genotoxicity in lung epithelial A549 cells. *Toxicology Letters*, 276, 48–61.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS (2016): Genotoxicity assessment of intravenously injected titanium

dioxide nanoparticles in gpt delta transgenic mice, *Mutation Research Genetic Toxicology and Environmental Mutagenesis*, 802, 30–37.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS (2020): Genotoxicity assessment of titanium dioxide nanoparticle accumulation of 90 days in the liver of gpt delta transgenic mice. *Genes and Environment,* 42 (7), https://doi.org/10.1186/s41021-020-0146-3

Sycheva LP, Zhurkova VS, Iurchenkoa VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK and Durnev A (2011): Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo, *Mutation Research*, 726, 8–14.

Talamini L, Gimondi S, Violatto MB, Fiordaliso F, Pedica F, Tran NL, Sitia G, Aureli F, Raggi A, Nelissen I, Cubadda F,Bigini P and Diomede L (2019): Repeated administration of the food additive E171 to mice results in accumulation in intestine and liver and promotes an inflammatory status. *Nanotoxicology*, 13, 1087–1101.

Tavares AM, Louro H, Antunes S, Quarre S, Simar S, De Temmerman P-J, Verleysen E, Mast J, Jensen KA, NorppaH, Nesslany F and Silva MJ (2014): Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes.,*Toxicology In Vitro,* 28, 60–69.

Trouiller B, Reliene R, Westbrook A, Solaimani P and Schiestl RH (2009): Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Research*, 69, 8784–8789.

Turkez H and Geyikoglu F,(2007): An in vitro blood culture for evaluating the genotoxicity of titanium dioxide: the responses of antioxidant enzymes, *Toxicology and Industrial Health*, 23, 19–23.

Vales G, Rubio L and Marcos R (2015): Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells, *Nanotoxicology*, 9, 568–578.

Vasantharaja D, Ramalingam V and Reddy GA (2015): Oral toxic exposure of titanium dioxide nanoparticles on serum biochemical changes in adult male Wistar rats*. Nanomedicine Journal*, 2, 46–53.

Vila L, Garcia-Rodriguez A, Marcos R and Hernandez A (2018): Titanium dioxide nanoparticles translocate through differentiated Caco-2 cell monolayers, without

disrupting the barrier functionality or inducing genotoxic damage, *Journal of Applied Toxicology*: JAT, 38, 1195–1205.

Wang S, Hunter LA, Arslan Z, Wilkerson MG and Wickliffe JK (2011): Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells, *Environmental and Molecular Mutagenesis*, 52, 614–622.

Wang Y, Cui H, Zhou J, Li F, Wang J, Chen M and Liu Q (2015): Cytotoxicity, DNA damage, and apoptosis induced by titanium dioxide nanoparticles in human non-small cell lung cancer A549 cells, *Environmental Science and Pollution Research International*, 22, 5519–5530.

Warheit DB, Boatman R and Brown SC (2015): Developmental toxicity studies with 6 forms of titanium dioxide test materials (3 pigment-different grade & 3 nanoscale) demonstrate an absence of effects in orally-exposed rats, *Regulatory Toxicology and Pharmacology*, 73, 887–896

Woodruff RS, Li Y, Yan J, Bishop M, Jones MY, Watanabe F, Biris AS, Rice P, Zhou T and Chen T (2012): Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay, *Journal of Applied Toxicology*, 32, 934–943.
Xu A, Chai Y, Nohmi T and Hei TK (2009): Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells, *Particle and Fibre Technology*, 6, 3.

Yang J, Luo M, Tan Z, Dai M, Xie M, Lin J, Hua H, Ma Q, Zhao J and Liu A (2017): Oral administration of nano-titanium dioxide particle disrupts hepatic metabolic functions in a mouse model, *Environmental Toxicology and Pharmacology*, 49, 112–118.

Yu X, Hong F and Zhang YQ (2016): Cardiac inflammation involving in PKCe or ERK1/2-activated NF-jB signalling pathway in mice following exposure to titanium dioxide nanoparticles, *Journal of Hazardous Materials*, 313, 68–77.

Zijno A, Cavallo D, Di Felice G, Ponti J, Barletta B, Butteroni C, Corinti S, De Berardis B, Palamides J, Ursini CL,Fresegna AM, Ciervo A, Maiello R and Barone F (2020): Use of a common European approach for nanomaterials ’testing to support regulation: a case study on titanium and silicon dioxide representative nanomaterials, *Journal of Applied Toxicology,* 40 (11*),* 1511-1525

Zijno A, Cavallo D, Di Felice G, Ponti J, Barletta B, Butteroni C, Corinti S, De Berardis B, Palamides J, Ursini CL,Fresegna AM, Ciervo A, Maiello R and Barone

F (2020): Use of a common European approach for nanomaterials ’testing to support regulation: a case study on titanium and silicon dioxide representative nanomaterials, *Journal of Applied Toxicology*.

Zijno A, De Angelis I, De Berardis B, Andreoli C, Russo MT, Pietraforte D, Scorza G, Degan P, Ponti J, Rossi F andBarone F (2015): Different mechanisms are involved in oxidative DNA damage and genotoxicity induction by ZnO and TiO2nanoparticles in human colon carcinoma cells, *Toxicology in Vitro*: An International Journal Published In Association With BIBRA, 29, 1503–1512.

Zirak RG, Lotfi A and Moghadam MS (2016): Effects of the interaction of nano anatase TiO2with bleomycin sulfate on chromosomal abnormalities *in vivo, International Journal of Advanced Biotechnology and Research*, 7,1094–1108.

Annex 1 - MUT/2021/03

COMMITTEE ON THE MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT

EFSA 2021: Safety assessment of titanium dioxide (E171) as a food additive

Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6585

Annex 2 - MUT/2021/03

COMMITTEE ON THE MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT

EFSA 2021: Safety assessment of titanium dioxide (E171) as a food additive- Appendices relevant to genotoxicity studies:

- *in vitro* and *in vivo* studies retrieved from the literature search (Appendices J, K),
- *in vitro* and *in vivo* studies considered in the re-evaluation of E 171 (EFSA ANS Panel, 2016) (Appendices L, M),
- *in vitro* and *in vivo* studies reported in the OECD (2016) ((published papers and results from NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7, 8, 9 and 10)) (Appendices N, O) and
- *in vitro* studies submitted by IBOs (Documentation provided to EFSA No 14 and 15) (Appendix P)