COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

REVIEW OF EFSA OPINION ON TITANIUM DIOXIDE

Background

1. Titanium dioxide (TiO_2), 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 inorganic 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 number 1317-80-2) being the two most important.

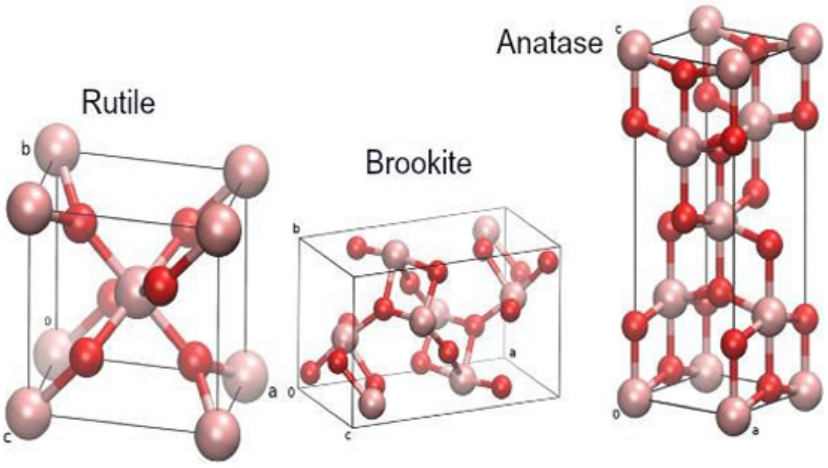

Fig.1: Natural forms of TiO_2[1]

2. 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 (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

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

food that would otherwise be colourless, or to restore the original appearance of food. It is also widely used in cosmetics and medicines[2].

3. Titanium dioxide has been the subject of multiple safety evaluations: the Scientific Committee on Food (SCF) in 1975 and 1977 and 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'.

4. 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."

5. On the basis of the data available, the Panel concluded that the absorption and oral bioavailability of titanium dioxide was low, independent of size. 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*. For the other endpoints, the Panel identified a no-observed adverse effect level (NOAEL) of 2,250 mg/kg bw/d based on a study in rats. Compared to the exposure based on reported use levels and analytical data, the use of E171 was not considered to be of concern.

6. However, the Panel did not establish an ADI due to the lack of an extended 90-day toxicity study or a multi-generation or extended one generation reproduction toxicity study with E171. This is because possible adverse effects were identified in the reproductive system in some studies conducted with test substances that were non-food grade or with inadequately characterised

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

nanomaterial. 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

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

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 in 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 2018, the Dutch Office for Risk Assessment and Research held a workshop on the "potential health effects of the food additive titanium dioxide (E171)", the results of which were published in 2019[5], where overall the need for further studies to further investigate the effects of titanium dioxide exposure-particularly for the endpoints of colon tumours and immunotoxicology based on the data gaps and study limitations of the available database at the time was highlighted. Furthermore the need to better characterise the composition of E171 was noted. In 2020, a review was published that summarised the outcomes of this workshop and additionally aimed to identify and evaluate recent toxicological studies on food-grade titanium dioxide and nano-sized titanium dioxide in *ex-vivo*, *in-vitro*, and *in-vivo* experiments along the gastrointestinal route, and to postulate an Adverse Outcome Pathway (AOP) following ingestion. Adverse effects were identified including the generation of ROS, alterations of the gut microbiota, persistent inflammation, and other effects on the immune system. It was noted that findings were inconsistent between the different species and independent research groups. With regards to the animal studies that reported positive effects on precancerous lesions/tumour formation, it was noted that those were mainly used as research models and a proper investigation of a dose-response relationship was not performed. Based on the available information, it was not

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

[5] https://english.nvwa.nl/documents/consumers/food/safety/documents/opinion-of-buro-on-possible-health-effects-of-the-food-additive-titanium-dioxide-e171

possible to carry out a risk assessment. When considering the mode of action, it was postulated that it was closely related to the ability of titanium dioxide to induce ROS formation and promote inflammation. The potential key events were considered to be persistent inflammation and ROS generation that can result in oxidative stress as well as persistent epithelial cell injury and potentially lead to DNA damage and exert a tumour-promoting effect of E171 seen in some of the studies. Finally, it was noted that it is generally assumed that the round and spherical crystal forms of TiO_2 contribute to the induction of adverse effects to a lesser extent when ingested and similarly, that titanium dioxide nanoparticles are suspected to induce more adverse effects than other particle sizes. However, a study by Proquin *et al.(*2017) was also mentioned, that demonstrated that a mixture of nano- and micro-sized TiO2 particles, as present in E171, induce more adverse effects than the single fractions alone. The authors further expanded on possible interactions of E171 with its direct environment as well as other factors that could potentially affect agglomeration for example and discussed how these could directly affect the properties of titanium dioxide. Therefore, they considered that "it is important to carefully examine and analyse the physicochemical characteristics of TiO_2 particles in its vehicle, as well as in its surrounding matrix as their final milieu, to guarantee a profound assessment of potential adverse health effects of E171 and to adequately compare different studies in the process of risk assessment." (Bischoff *et al.*,2020)

10. 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 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

11. 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[6] 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.

12. The data evaluated was for the food additive titanium dioxide E171 as well as for 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.

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

Kinetics and Metabolism

13. In 2016, the Panel concluded that the absorption of orally administered titanium dioxide was low. Its oral systemic availability (measured either as particles or as titanium (Ti)) was 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.

14. For the re-evaluation, ADME 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[7], the Panel's estimates always included the Ti amount in the liver, which 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)

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

Toxicity

Short term

16. For E171, the Panel considered that no adverse effects were observed in mice (n=4) following administration of E171 at a mean dose of 2 mg/kg bw/d for 21 days. E 171 or water (for controls) was slowly dripped with a pipette into the mouths of mice, 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.

[7] 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.

17. 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). In another short term study (Talbot et al*.,* 2018) the effects of 0,0.1 and 10 mg/kg bw/d exposure to E171 for 60 days on GIT microbial production of short chain fatty acids and mucin O-glycosylation were studied. No effects were observed even at the highest dose tested.

18. 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 of 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. The Panel noted a study reporting histological changes in the myocardium of rats treated with 1200 mg/kg bw/d TiO_2 NPs by gavage for 90 days. Due to the limited reporting the Panel was unable to conclude on the relationship between the observed results and the exposure to TiO_2 NPs (El Din *et al*., 2019)

19. The studies assessing the safety of TiO_2 NPs <30nm were also reported. These are summarised in the table below, using information from the EFSA, 2021 Opinion:

Table 1: TiO2NPs < 30 nm studies in mice and rats

Test System	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**	**Ref**
Male CD-1 mice (n=10/group)	Gavage :0,64,320 mg/kg bw/d for 14 weeks	TiO_2 NPs (26 nm)	Treated mice had increased fasting blood glucose levels from weeks 10. Impaired glucose tolerance was observed (without showing a dose response), but no changes in blood insulin or lipids could be detected.	TiO_2 NPs at both doses led to increases in fasting state plasma glucose, and also to increases in glucose levels in a glucose tolerance test without showing dose response and without differences in plasma insulin levels-indicating inconsistency between the measured outcomes`	Hu *et al.,* 2015

Female CD-1 (ICR) mice (n=20/group)	Gavage: 0, 2.5, 5, 10 mg/kg bw/d for 90 days	TiO_2 NPs (5-6 nm)	Body weight gain was statistically significantly decreased in a dose-dependent manner to 30.3 % in all treated groups. Reports of histopathological changes in the heart.	Limited reporting- Panel was not able to conclude on the relationship between TiO_2 NP treatment with decreased body weight gain and histological changes reported	Yu *et al.,*2016
Male CD-1 (ICR) mice (n=40)	Gavage: 0, 2.5, 5, 10 mg/kg bw/d for 90 days	TiO_2 NPs (5-6 nm)	Dose-dependent decrease in body weight gain was observed at all tested doses (approx. 5%, 5% and 7% decrease in BW gain compared to control at 2.5, 5 and 10 mg/kg bw per day, respectively), with statistically significant differences at the two highest doses. Relative liver weights increased by~ 10–15% compared to control; however, absolute liver weights were unchanged. Histological alterations of the liver (lymphocyte infiltration and necrobiosis) were reported. Changes in the liver expression of inflammation-related proteins were also found.	Histopathological data in the liver not accompanied by any other confirmatory investigations. The Panel considered the effects reported as likely an hepatic inflammatory response to TiO_2 NPs	Hong *et al.,* 2016
Male C57BL/6 mice (n=5/group)	Gavage: 0, 250, 500 mg/kg bw/d for 14 days.	TiO_2 NPs (21 nm)	3-fold increase of serum bilirubin (total and indirect) at the highest dose in the absence of inflammation, apoptosis, necrosis and molecular defects in bilirubin metabolism.	The Panel noted structural changes in hepatocytes which were not quantified. However the increases occurred in the absence of any changes in relative liver weight, in other serum markers for liver injury or quantitative histopathological changes in the liver. Changes in the hepatic expression of selected genes were considered either incidental or adaptive, but not evidence of adversity.	Yang *et al.,* 2017
Sprague Dawley rats (n=10/group for 30 days or	Gavage: 0, 2, 10 and 50 mg/kg bw per day with	TiO_2NPs (24 nm)	Increases in white blood cells parameters (white blood cell counts and granulocytes) were observed in female rats	The Panel considered there were limitations in the reporting of histopathological	Chen *et al.,* 2015a

5/sex/group for 90 days)	and without glucose (1.8 g/ kg bw per day) for 30 and 90 days.		after exposure to TiO_2NPs 50 mg/kg bw per day for 90 days and among male rats exposed to TiO_2NPs 50 mg/kg bw per day for 30 (white blood cells counts, lymphocytes, monocytes absolute numbers and in the percentage of lymphocytes and granulocytes) and 90 days (percentage of monocytes); and a decrease in the while blood cells at 90 days in rats exposed to 10 mg/kg bw per day. Authors reported liver oedema, fatty degeneration and necrosis at the highest dose group. No significant pathological changes in kidney, spleen, testis and ovary tissues. Decreased serum TBIL contents and increased GLB levels were observed in the groups treated with higher dose of TiO_2 NPs (at 10 and 50 mg kg− 1 BW) among female rats. Among the male rats, increases in kidney coefficient and BUN level as well as decreases in Crea contents were observed. CK activity in female rats and serum LDH and HBDH in male rats were significantly lower than the control group	changes in the liver and in the absence of changes in serum enzyme activities reflective of liver injury considered effects on the liver as not adverse.	
Sprague Dawley rats (n=10/sex/group)	Gavage 0, 2, 10 and 50 mg/kg bw per day	TiO_2 NPs (24 nm)	Treatment with TiO_2 NPs at all doses had no effect on body weight, feed intake or the relative heart weight. Statistically significant changes in heart rate and blood pressure relative to the control group were recorded in mid- and high-dose females as increased DBP on day 47 and decreased SBP on day57 (no data shown to evaluate a dose response), and mid-dose males as a decreased HR on day 88.The	Although some changes were reported by the authors, The Panel noted that each of these transient changes were limited to one sex and considered them not to be treatment related. The Panel considered that gavage administration of TiO_2NP (24 nm) in doses up to 50 mg/kg bw per day to rats for up to 90 days did	Chen *et al.,* 2015b

			area under curve for SBP was statistically significantly lower for mid-dose females.	not induce any treatment-related effects	
Male Wistar rats (n=6/group)	Gavage: 0, 50, 100 and 200 mg/kg bw/d for 60 days.	TiO_2 NPs (5-12nm)	No data on feed consumption, body weight or mortality were reported. Statistically significant dose-related decreases in RBC (up to 28%),HCT (up to 23%) and haemoglobin (up to 28%) in exposed animals although the decreases in the last parameter were not dose dependent. Mean corpuscular volume (up to 29%), platelets (up to 42%),mean platelet volume (up to 30%) and WBC (up to 235%) were statistically significantly and dose-dependently increased in exposed animals. The authors also reported poikilocytotic hyperchromatic RBCs and abnormally shaped nuclei and hyper-segmented nuclei in lymphocytes and neutrophils in the animals exposed to 100 and 200 mg/kg bw/d.	The Panel considered the reported haematological changes to be of no toxicological significance.	Grissa *et al.*, 2015
Male Sprague Dawley rats	Gavage: 0, 2, 10, 50 mg/kg bw/d for 90 days	TiO_2NPs (29 nm)	Starting from week 8, the 10 and 50 mg/kg bw per day groups showed decreased body weight gains up to about 15%, while food intake was not different between the groups. Serum levels of triglycerides in the 10 and 50 mg/kg bw per day groups were statistically significantly lower than in the control group while serum TC, HDL-C and LDL-C were not affected. In an untargeted metabolomic analysis, 343 of 1,837 lipophilic metabolites were differentially expressed between controls and the 50 mg/kg bw per day group. No	The Panel considered that, while the change in body weight gain may be adverse, other reported changes were of no toxicological significance.	Chen *et al.,* 2020a

			statistically significant differences in organ weights for the heart, spleen, liver, kidney, lung, stomach and testis were observed. No further results were reported		
Sprague Dawley rats (n=5/sex/group)	Gavage: 0, 2, 10 or 50 mg/kg bw per day with or without 1.8 g/kg bw glucose i for 90 days.	TiO_2NPs (24 nm)	Rats treated with glucose and TiO_2NPs (10 and 50 mg/kg bw per day groups) had a significantly reduced levels of HbA1c in female rats. Male rats treated with glucose and TiO_2NPs (2 mg/kg bw group) had a significantly reduced level of GSP. Blood insulin levels were statistically significantly lower than control in females in the 10 or 50 mg/kg bw per day TiO_2NPs groups, but there was no effect in males. In males, C-peptide was significantly lower in the 50 mg/kg bw per day TiO_2NPs group, but no such effect was seen in females. No clear dose–response effect was seen on glucagon levels. In an oral glucose tolerance test, differences were seen in blood glucose concentrations, only in male rats (at 30and 60 min after glucose challenge in the 2 mg/kg bw per day TiO_2NPs group and at 60 min after glucose challenge in the 50 mg/kg bw per day TiO_2NPs + glucose group)	The Panel considered the changes in blood glucose,HbA1c, GSP, insulin, C-peptide, glucagon and glucose tolerance as either not test substance related or irrelevant for the safety evaluation of E 171	Chen *et al.,* 2020b
Wistar albino rats (n=6/group)	Gavage: 100 mg/kg bw/d for 8 weeks	TiO_2NPs (5–10nm)	iO2NP-treated group had a statistically significantly decreased body weight gain and serum cholesterol, glucose and TG concentrations were statistically significantly higher. The authors reported significant changes in plasma oxidative stress markers and an increase in plasma	The Panel noted the changes in glucose levels which are potentially adverse, and considered that the changes in cholesterol and TG are of unclear toxicological relevance.	Grissa *et al., 2017*

			interleukin-6 (IL-6) compared to control.		
Male Sprague Dawley rats (n=10/group)	150 mg/kg bw	TiO_2 NPs (21 nm)	RBC, Hb, WBC, Monocy and EOS were not different compared with control, whereas the Lym and Neutro were statistically significantly increased by treatment with TiO_2 NPs (no units given). ALT increased 2.5-fold,AST 2-fold, LPO 2-fold, TNF-a 5-fold, whereas TBAR decreased 2-fold, GSH 4-fold and testosterone 5.5-fold. In all organs investigated (liver, brain, lung, heart, testis, kidney) 'histopathological' lesions were reported in either 4–6(++)or 7–10 (+++) rats per treated group, with no lesions found in the control group. The reported findings in the liver were congestion, vacuolar degeneration, mononuclear infiltration in the portal area, focal necrosis with mononuclear infiltration; in the brain: haemorrhage, congestion of choroid plexus blvs, chromatolysis, neuronal degeneration, perivascular lymphocytic cuffing; in the lung: congestion, thrombosis, hyalinisation of the blood vessels wall, hyperplasia of peribronchial lymphoid aggregation; in the heart: vacuolar degeneration and myocardial necrosis; in the testis: congestion and coagulative necrosis; in the kidney: congestion and perivascular mononuclear infiltration. It is not mentioned whether the pathologist performing the histopathology was blinded or whether there was a blinded second reading of the slides.	Based on the many flaws in the study reporting (e.g. descriptions of 'histopathological' lesions are unclear, some of the findings are not histopathological lesions, the number of lesions per organ and the number of animals with any lesions are not clearly stated), the Panel was not able to draw any conclusions.	Hassanein and El-Amir, 2017

	Gavage: 0, 250, 500 and 1,000 mg/kg bw per day for 28 and 90 days	TiO_2NPs (21 nm)	No statistically significant treatment-related differences with respect to body weight gain, food and water intake were observed. No mortality or clinical signs were detected during the exposure period of 28 and 90 days. No effects were detected in a functional observation battery in the last week of the 90-day study. Ophthalmoscopic examination and urinalysis did not show statistically significant differences between the groups. Changes – circulating neutrophils and lymphocytes, blood urea nitrogen and blood Na – occurred without a clear dose response. Nonabnormal gross findings were found at necropsy in treated animals. Changes in some organ weights were considered unrelated to the treatment. On histopathological examination, differences between the control group and the 1,000 mg/kg bw per day group were found.	The Panel considered that the reported changes were within the historical control normal range and therefore of no toxicological significance.	Heo *et al.,* 2020

20. 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).

Reproductive/developmental toxicity

21. With regards to reproductive and developmental toxicity, a number of studies available in the 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 the 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. The results will be discussed later in the paper in detail. 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.

22. 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.,* 2015a).

23. Several studies using TiO_2 NPs <30 nm were reported. These are summarised in the table below. It is worth noting that EFSA considered these of limited relevance to the safety of E171 but included them for completeness of the database and as they may be relevant with respect to whether a minimum limit for particle size should be included in the EU specifications for E 171. Information from the EFSA 2021 opinion was used:

Table 2: Reproductive toxicity studies with TiO_2 NPs<30 nm

Test System	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**	**Ref**
Female mice (presumably NMRI). Numbers varied for different investigations: histology of ovaries, oestrogen and MDA serum levels (7 animals/group), fertility (10 animals/group) and IVF rates(10 animals/group)	Gavage: 100 mg/kg bw/d for 5 weeks	TiO_2NPs(10–25 nm)	Significantly decreased pregnancy rate (70% vs. 100% in the control group), a 20% decrease in litter size and increases in circulating oestrogen (20%) as well as MDA (25%). Degeneration and reduction of follicles, cyst formation and impairment of follicular development in the ovaries of the TiO_2NPs group (no quantitative data). Lower number of oocytes isolated from the exposed group and a higher percentage of developmental arrest before the blastocyst stage after *in vitro* fertilisation. Authors suggested that the observed effects could be the consequence of an indirect effect of TiO_2NPsthrough the generation of increased ROS levels.	The Panel considered that the study shows an impairment of female fertility at a dose of 100 mgTiO_2NPs (10–25 nm)/kg bw per day	Karimipour *et al., 2018*
Male NMRI mice	0, 75, 100, 300 mg/kg	TiO_2 NPs of unknown size	Dose-dependent decreases in testis weight occurred from	The Panel considered that TiO_2NPs (size unknown) from 100 mg/kg bw per day had	Khorsandi et al.,2016

	bw/d for 35 days		a dose of 100mg/kg bw per day.	an effect on testis weight	
Male NMRI mice	Gavage: 300mg/kg bw/d for 35 days	TiO_2NPs (20–30 nm)	Significant decreases in testis weight, circulating and testicular testosterone, testicular catalase (CAT) and superoxide dismutase (SOD) concentrations, sperm counts and s perm motility. Significant increases were found in the percentage of abnormal or degenerative spermatogenic tubules, germ cell apoptosis, testicular MDA concentration and in the percentage of sperm with abnormal morphology.	The Panel considered that testicular toxicity was observed with TiO_2NPs (20–30 nm) at 300 mg/kg bw/d, the only dose tested.	Khorsandi et al., 2017
Male NMRI mice	Gavage: 50 mg/kg bw/d for 35 days	TiO_2NPs (<30nm)	TiO_2NPs significantly reduced testis weight accompanied by reduced serum testosterone, reduced seminiferous tubule diameter and epithelium height and reduced the maturity of the germinal epithelium. Reduced sperm counts, increased sperm abnormalities and reduced sperm motility.	The Panel noted that 50 mg TiO_2NPs/kg bw per day, the only dose tested, resulted in adverse effects on the testis.	Karimi *et al.,*2019
Male ICR mice (n=15)	Gavage: 0, 10, 50, 100 mg/kg bw/d for 30 days	TiO_2NPs (7 nm)	Tight junction damage in the blood–testis barrier (BTB) at 50 and 100 mg/kg bw. Serum testosterone was 50% decreased at the two highest doses tested. Sperm motility was dose-relatedly reduced, accompanied by increased sperm malformation rates.	The Panel noted that the histopathological pictures on BTB were hard to interpret. The Panel considered that TiO_2NPs (7 nm), at 50 or 100 mg/kg bw per day, resulted in a dose-related reduction of sperm motility and increased sperm malformations, accompanied by histological observations in the testis, changes in BTB-related protein levels, changes in MAPK-related levels and reduced circulating testosterone concentrations.	Lu *et al.,* 2020

Mated female Sprague Dawley rats (n=12/group)	Gavage: 0, 0,100,300,100 mg/kg bw/d from GDs 6-19.	TiO_2NPs (21 nm)	No statistically significant differences in general clinical signs, bodyweight, organ weights (absolute and relative to body weight), macroscopic findings. No significant differences for caesarean section parameters and fetal external and visceral examinations.	The Panel considered that no adverse maternal and development al effects were reported with TiO_2NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.	Lee *et al.,* 2019

24. Based on the above, EFSA concluded that "No maternal and developmental effects were observed up to 1,000 mg/kg bw per day, the highest dose tested, in a single rat developmental toxicity study with five different TiO_2 materials, TiO_2 NPs orTiO_2 containing a fraction of nanoparticles (Warheit *et al*., 2015a) (scoring 4 for nanoscale considerations (NSC)[8]). In mice, the effects of TiO_2 NPs < 30 nm on the testis (decreased weight, decreased seminiferous tubule diameter, germ cell apoptosis) and sperm (decreased sperm counts and motility, increased percentage of abnormal spermatozoa) were observed in three studies (Khorsandi et al., 2016, 2017; Karimi *et al*., 2019) at doses ranging from 50 to 300 TiO_2 NPs/kg bw per day. The lowest dose at which the effects were observed was 50 mg TiO_2 NPs/kg bw per day (Karimi *et a*l., 2019). In a mouse study by Lu et al. (2020), no effects were observed at the lowest dose tested, 10 mg/kg bw per day(scoring 4 for NSC). In rats, administration of TiO_2 NPs (21 nm) did not show effects at any dose level in a developmental toxicity study up to 1,000 mg/kg bw per day (Lee et al., 2019, scoring 3 for NSC)." (EFSA, 2021)

Neurotoxicity

25. Concerning neurotoxicity, no reliable studies performed with E 171 were found in the literature (EFSA, 2021). In studies with TiO_2 NP > 30 nm, increased hippocampal apoptosis and reduced hippocampal neurogenesis after both gestational and lactational exposure observed in offspring (PND 1 in gestation group, PND 22 in lactation group) of female Wistar rats (n=6/group) exposed from GD 2 to 21 at 100 mg/kg bw/d TiO_2 (<100nm) (Ebrahimzadeh *et al*., 2017; scoring 3 for NSC). At the only dose tested of TiO_2 NPs (90 nm- range 40-140nm) 500

[8] This refers to EFSA's scoring system of nanoscale considerations on the description of the test materials. The lower the score, the higher the confidence for assessing toxicological effects of the fraction of small particles, so when a study is scored as a 1, it is suitable to assess the safety of nanoparticles but if scored as a 4 then the results are not really relevant.

mg/kg bw/d for 14 days, male albino rats n=20/group) adverse effects in CNS which were possibly related to oxidative stress were reported (Kandeil *et al.,2019;* NSC score 3). The Panel concluded that "these data show that oral TiO_2NPs administered to rats during embryofetal and early postnatal development reduced hippocampal neurogenesis at 100 mg/kg bw per day, and that oral administration to adult rats produced adverse effects in the brain consistent with oxidative stress at 500 mg/kg bw per day" (EFSA, 2021).

26. In studies using titanium dioxide NPs < 30 nm, effects were seen at doses as low as 2.5 mg/kg bw per day. This was in a study in mice (n = 20/group) dosed by gavage at 0,2.5,5 and 10 mg/kg bw/d for 35 days with TiO_2 NPs, where reduced volume of the hippocampus and the polymorph layer of the dentate gyrus as well as reduced density and total number of dentate gyrus granular cells were observed even at the lowest dose (Rahnama *et al*., 2020; NSC score 4). Zhang *et al.,* 2020 *(*NCS score 3) reported no effect on body weight or histopathology of gut or brain, but significant decrease of the richness and evenness of gut microbiota, elevated gut HuC/D and TuJ1 and marked reduction of serotonergic markers Sstr1 and Sstr2 in the gut but not in the cerebral cortex. The results suggested an effect on the enteric nervous system. However, gut–brain peptides secreted by endocrine cells and enteric neurons, and inflammatory cytokines were not affected by treatment. In the open field test, centre field activity was statistically significantly reduced by the treatment, which was consistent with anxiety-like behaviour, but MWM learning and spatial memory were unaffected in male mice (n=15/group) dosed at 0 and 150 mg/kg bw/d TiO_2 NPs (21nm) for 30 days. The Panel considered that TiO_2NPs (21 nm) at 150 mg/kg bw per day, the only dose tested, altered gut microbiota, without pathological changes in small intestine and brain.

27. The rat studies are summarised below. EFSA considered that, in rats, "the most sensitive endpoint was reduced brain cholinesterase activity (about 35–50%)and increased brain Na, K-ATPase activity (about 2-fold), observed with TiO_2NPs (21 nm) at all doses tested, in fem ale albino rats dosed for 14 days, as reported by Canli *et al*. (2020) (scoring 4 for NSC). In this study, rats (n = 6/group) were dosed by gavage with TiO_2NPs (21 nm) at 0, 0.5, 5 or 50 mg/kg bw per day" (EFSA, 2021).

Table 3: Reproductive studies with TiO_2 NPs <30nm

Test System	Exposure	Characterisation of test substance	Result	EFSA's evaluation	Ref
Male Wistar rats	Gavage: 0,50,100,200 mg/kg bw/d for 60 days	TiO_2 NPs(5-10 nm)	Reduced brain cholinesterase at 100 and 200 mg/kg bw per day (no dose-response). Reduced plasma cholinesterase activity at all doses tested (35%, 50% and 50% at 50, 100 and 200	Panel noted the methodology of the authors did not indicate whether plasma cholinesterase activity represented acetylcholinesterase or	Grissa *et al*. (2016)

			mg/kg bw per day, respectively). Cerebral cortex GFAP-positive cell counts were dose-dependently increased at 100 and 200 mg/kg bw/d	butyrylcholinesterase or both (but considered it was probably both) and noted that TiO_2 NPs reduced brain cholinesterase activity	
Albino rats (n=6/group)	Gavage: 0,0.5, 5, 50 mg/kg bw/d for 14 days	TiO_2 (21nm)	One death at 0.5 mg/kg bw per day group (no further details reported), but no other notable clinical signs. No effect on liver total, reduced or oxidised glutathione (tGSH, rGSH or GSSG)or the ratio between reduced and oxidised glutathione (GSH/GSSG ratio), or on kidney and intestine ATPase activity. Brain Na/K-ATPase activity was significantly increased (approximately 2-fold) at 0.5 and 5 mg/kg bw per day, Mg-ATPase and total ATPase activity at 5 mg/kg bw per day. Brain cholinesterase activity was significantly reduced at all doses (by about 50%, 35% and 50% at 0.5, 5and 50 mg/kg bw per day, respectively, i.e. no dose response) The authors reported that TEM demonstrated the presence of TiO_2 particles in the liver, kidney and brain which 'seemed dose dependent '	The Panel noted that verification of the elemental composition of the particles of interest was not performed in the liver, kidney and brain. This apparent 200 -fold difference in potency adds to uncertainty; possible contributory factors include differences in test substance dispersion and internal exposure between Grissa *et al*, (2016) and the current study	Canli *et al.,* 2020

28. With regards to the studies reporting effects on cholinesterase activity the Panel noted that: "the most sensitive endpoint in adult rats was reduced (dose related) brain cholinesterase activity and increased brain Na/K-ATPase activity, observed at 0.5 mg/kg bw per day (in females dosed for 14 days), the lowest of three doses tested, reported by Canli *et al*. (2020) with TiO_2NPs (21 nm). However, Grissa *et al*. (2016) reported reduced brain cholinesterase activity at 100 but not 50 mg/kg bw per day (in males dosed for 60 days with TiO_2NPs (5–10 nm)). This apparent 200-fold difference in potency adds to uncertainty."

29. Developmental effects were seen in both mice and rat studies. In pre- and perinatal CD-1 mice (n = 6/group), dosed with TIO_2 NPs (6-7 nm) by gavage at

0,1,2 and 3 mg/kg bw/d from GD 7 to PND 21, inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function were seen in *ex vivo* hippocampal CA1 neurons at all does tested (Zhou *et al.,*2017, NSC score 2). In rats, (pre- and perinatal) passive avoidance behaviour was altered in offspring of female rats dosed at 100 mg/kg bw/d TiO_2 NPs (10 nm) during lactation (Mohammadipour *et al.*, 2016; NSC score 3). Ebrahimzadeh *et al* (2017, NSC score 3) reported increased hippocampal apoptosis and reduced offspring hippocampal neurogenesis following maternal dosing at 100 mg/kg bw/d TiO_2 NPs during gestation and lactation. The Panel concluded that developmental effects were observed at this dose level of TiO_2 NPs (10 nm) and that the effects seen on the brain structure and function in these studies were mutually plausible, given that passive avoidance behaviour is related to hippocampal functioning.

30. Overall, EFSA concluded that: “autophagy and oxidative stress and reduced mitochondrial function) in ex vivo hippocampal neurons of weanling mice after dosing TiO_2NPs (6–7 nm) during gestation and early lactation at a dose of 1 mg/bw per day, the lowest dose tested. In adult female rats (Canli *et al*., 2020; scoring 3 for NSC), adverse effects (reduced brain cholinesterase, and increased brain Na/K-ATPase activity) were observed with TiO_2NPs (21 nm) at 0.5 mg/kg bw per day, the lowest of three doses tested, in a 14-day study.” (EFSA, 2021)

Immunotoxicity

31. The findings in studies with E 171 on immunotoxicity and inflammation were considered inconsistent. In mice, study reported no adverse effects at 100 mg/kg bw/d (Riedle *et al.,* 2020; NSC score 1), whilst reduction of colonic crypt length, an increase in colon macrophages and CD8 cells inIL-10, TNF-α and IL-6 mRNA at doses of 10 and 50 mg/kg bw/d (Pinget *et al*, 2019; NSC score 2). 2 and 5mg/kg bw/d of E171 increased inflammatory parameters were observed (Talamini *et al*, 2019; Urrutia-Ortega *et al.*, 2016). Furthermore, E171 had no effect on tumour formation but could potentiate intestinal tumour formation in mice exposed to azoxymethane/dextran sulfate sodium (Urrutia-Ortega *et al*, 2016). In rats, a statistically significantly decrease in granulocyte-macrophage colony-stimulating factor (GM-CSF) plasma level (~40%)was observed at the highest dose (10, 100 and 100 mg/kg bw/d for 90 days) (Han *et al.*2020). It was considered that, “GM-CSF is involved in haemopoiesis which may explain the modest but statistically significant decrease in immunoglobulin (Ig) M level (~ 10%)” (EFSA, 2021). Bettini *et al* (2017)- NSC score of 1- increased inflammatory parameters were observed at the only dose tested (10mg/kg bw/d); which were not confirmed in a study by Blevins *et al* (2019)., which did not report any effects at up to 267 mg/kg bw/d. The Panel noted that this study scored 3 for NSC. Pages 115-120 of the EFSA opinion provide further details on the methodology and findings.

32. In gavage studies with TiO_2 NPs > 30 nm effects were seen at a dose of 20 mg/kg bw per day in rats (90 days, dosed at 20 and 40 mg/kg bw/d) in a study by Hashem *et al.*, 2020. This was the only study reported in rats whereas in the two mice studies reported, following dosing with TiO_2 NPs for either 5 or 7 days at 5,

50 and 500 mg/kg bw/d, inflammatory responses were seen in the stomach even at the lowest dose tested (Mohamed, 2015; *Li et al.,*2019). No effects were reported in a study with 1mg/kg bw/d TiO_2 NPs (25,50 or 80 nm) investigating effects on the histology of the spleen). In studies with TiO_2 NPs < 30 nm effects were observed at doses as low as 2.5 mg/kg bw per day in mice. These are summarised in the table below:

Table 4: Immunotoxicity studies with TiO_2 NPs <30nm

Test System	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**	**Ref**
Female CD-1 (ICR) mice (n=20/group)	Gavage: 2.5, 5 and 10 mg/kg bw/d for 90 days	TiO_2 NPs (5-6 nm)	Inflammatory lesions and tissue damage histopathologically- more pronounced at mid and high doses. Expression of NF-κB, and of pro-inflammatory cytokines TNF-α ,IL-1β, IL-6 and IFN-γ expression were increased in a dose-dependent fashion (statistically significant increase up to 1.8-fold compared with the control); expression of the NF-κB inhibitor I-κB was decreased in a dose-dependent fashion (statistically significant decrease up to1.55-fold compared with the control), as evidenced by western blotting	The Panel considered that these data indicate an effect of TiO_2NPs (5–6 nm) exposure at all dose levels tested, as evidenced by histopathological lesions, corroborated by intermediate endpoints indicating disturbance of intracellular ion homeostasis that were adrenergic receptors in the heart. These lesions are accompanied by increases in the expression of intermediate inflammatory endpoints. The Panel noted effects on inflammatory mediators with TiO_2NPs (5–6 nm) at all doses tested and corroborated by histopathological lesions.	Yu *et al.,* 2016
Male C57BL/6 mice (n=10/group)	Gavage: 100 mg/kg bw/d for 28 days	TiO_2 NPs (20 nm) anatase; TiO_2 NPs (15 nm) rutile	No effects on body weight. Particles observed in spleen however no histopathological changes. No histopathological changes in lung, jejunum, kidney, liver, brain. Increased length of villi in colon, irregularly arranged epithelial cells.	The Panel considered that these data support an effect of TiO_2NPs on the microbiota, but as no immunological parameters other than the histopathology of the spleen were included in this study, any consequence(s) associated with these changes in terms of inflammation and the	Li *et al.,* 2018

			Rutile NPs had a more pronounced influence on the gut microbiota than anatase NPs. The most influenced phylum was Proteobacteria, which was significantly increased by rutile NPs but not by anatase NPs. At the genus level, Rhodococcus was enriched by rutile NPs, Prevotella was significantly decreased by both the TiO_2NPs.	immune system remain uncertain.	
C57BL/6J mice (n=30/group)	Gavage: 150 mg/kg bw/d for 30 days	TiO_2 NPs (21nm)	Significantly changed richness and composition of the gut microbiota. No changes in parameters indicating inflammation (IL-6 and IL-1b) in either intestines or brain were observed.	The Panel considered that exposure to TiO_2NPs (21 nm) leads to changes in the microbiota composition, but the study does not indicate a local or systemic inflammatory action.	Zhang *et al*., 2020
Sprague Dawley rats (n=10/group)	Gavage: 0, 2, 10, 50 mg/kg bw/d, glucose (1.8 g/kg bw/d) TiO_2NPs (0, 2, 10 and 50 mg/mg bw /d) + glucose (1.8 g/kg bw per day) for 30 or 90 days.	TiO_2 24nm	No significant histopathological changes were observed in the spleen in all groups. Increases in white blood cell counts and granulocyte in female rats after exposure to TiO_2NPs 50 mg/kg bw/d for 90 days and In male rats exposed to TiO_2NPs 50 mg/kg bw per day for 30 days: increase in white blood cells counts, lymphocytes, monocytes absolute numbers and in the percentage of lymphocytes and granulocytes. At 90 days increase in percentage of monocytes. Decrease in the white blood cells at 90 days in rats exposed to 10 mg/kg bw/d.	The Panel considered that the increase in leucocytes may suggest an inflammatory response induced by TiO_2NPs (24 nm) at the highest dose tested (50 mg/kg bw per day).	Chen *et al*., 2015a
Sprague Dawley rats (n=6/group)	Gavage: 0,2,10,50 mg/kg bw/d for 30 days	TiO_2 NPs (29 nm)	Histopathologically, reduced numbers of goblet cells were found as a result of exposure, as well as inflammatory infiltration, while in serum	N/A	Chen *et al.,* 2019

			increased IL-6 expression was observed		
Male Wistar rats (n=8/group)	Gavage: 0, 50,100, 200 mg/kg bw/d (5 times/week for 8 weeks)	TiO_2 NPs (5-12nm)	Statistically significant dose-related increase in the level of NO in 100 and 200 mg/kg bw/d TiO_2NPs groups was observed together with a statistically significant increase in brain TNF-α in 200 mg/kg bw/d TiO_2 NPs group. The increase was dose-related for both parameters	The Panel noted changes for the above-mentioned inflammatory markers at doses starting from100 mg TiO_2NPs (5–12 nm)/kg bw per day	Grissa *et al.,* 2020

33. The Panel concluded "that these studies indicate immune dysregulatory activity of E 171, evidenced by several immune-related and inflammatory markers. These effects were not observed up 50 mg E 171/kg bw per day. In three single dose level studies with E 171, effects were noted at lower doses, i.e. 2, 5 and 10 mg/kg bw per day. Effects of E 171 may, at least in part, stem from the activity of the fraction of the smaller TiO_2particles, as studies with these particles also indicate inflammatory effects of exposure to TiO_2NPs (5–6 nm) at 2.5 mg/k g per day" (EFSA 2021).

34. With regards to the gut microbiota, a number of studies were considered, however the Panel was unable to conclude on the effects of E171 on the gastrointestinal tract. This was because, although changes were seen in response to exposure to E171 and other forms of TiO_2 NPs, there is currently no consensus as to when these changes should be considered adverse.

EOGRT study

35. Regarding the newly performed EOGRT study, a summary of the findings is presented here, however full details can be found in pages 32-45 of the EFSA Opinion. 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 day (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.

36. 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).

37. From blood and urine samples it was determined that at least a small fraction of the Ti in the mother's diet was absorbed- whilst data from the F2 generation were consistent with exposure via the placenta and possibly milk. 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).

38. 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. These were that: "treatment with CY was not performed at the same time as the rest of F1 cohort 3, without a separate control for the CY response, conducted at the same time (Documentation provided to EFSA No 11). Since the results from the CY positive control were not valid, the sensitivity of the test was not demonstrated. It was noted that the assay conditions may have not been optimal resulting in an apparent low antibody response to keyhole limpet haemocyanin (KLH) when compared to literature (Gore et al., 2004), as also pointed out by the study authors (Documentation provided to EFSA No 11)" (EFSA, 2021).Therefore, the Panel could not conclude on immunotoxicity.

39. In a satellite group of the study, E 171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon. The satellite F0 animals were treated with 0, 100, 300 or 1000 mg/kg bw/d E171 and terminated after weaning. Although a mild increase in the morphological variability (increased size and intensity of the staining of a small portion) of the crypts in the two caudal parts of colon was observed in seven animals (including controls); when assessed by the study pathologist they were deemed as inconsistent with the appearance and definition of ACF. Based on the description in the report, the Panel agreed with this conclusion. Furthermore, the incidence of these single crypts observed in the mid and high doses was not significantly different from the control. The findings of this study were consistent with Blevins *et al.,* (2019) where male Wistar rats were dosed at the equivalent of 1.8, 4.8, 31.4, 374 mg/kg bw/d for 7 days and 1.3, 3.5, 22.4 or 267 mg/kg bw/d for 100 days. However the Panel noted that there was considerable variability in the results, which may mask possible effects. Furthermore, the Panel noted that the examination for presence of ACF and ABC was not performed on the whole colon but was limited to three 2 cm long samples (one from the proximal, mid-portion and the distal parts) (EFSA, 2021). In contrast, as discussed previously, a study by Bettini *et al.*(2017) where Wistar rats were exposed by gavage to 10 mg/kg bw/d E171 for a week or 100 days, aberrant

crypts were examined in the colon. Although the authors did not explicitly define an ACF the Panel presumed it was one or more aberrant crypts/ACF based on the authors definition of a 'large' ACF as consisting of more than three aberrant crypts per ACF. Some of the animals were also treated with a genotoxic carcinogen (DMN) and it was also concluded that E171 increased the numbers of ACF initiated by the genotoxic carcinogen. The Panel considered that E 171 may induce ACF in male rats at a dose of 10 mg/kg bw per day in this study.

40. Overall, the Panel noted that the effect of titanium dioxide inducing ACF without prior initiation that was reported in Bettini *et al.* has not been replicated in any other studies. They further noted the limitations of the Blevins *et al*. study and the fact that there was uncertainty over the extent to which the animals were exposed to TiO_2 NPs in both the EOGRT and Blevins *et al.* study. The Panel concluded that E 171 may induce ACF in male rats at a dose of 10 mg/kg bw per day when it is dispersed in test vehicle preventing agglomeration of NPs prior to administration. The Panel noted that there is literature indicating that ACFs may be a risk factor for human colorectal cancer (Anderson et al., 2012; Drew et al., 2018; Quintanilla et al., 2019; Hong et al., 2019; Clapperet al., 2020; Kowalczyk et al., 2020; Siskova et al., 2020).

Genotoxicity

41. 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 3). 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). The passage below contains some of the text presented to the COM in June of 2021 (paper presented in Annex 2). The COM considered the genotoxicity data presented and their preliminary conclusions are presented at the end of this section.

42. 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.

43. Mixed results were obtained *in vitro*, with 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). Furthermore, the Panel noted 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. Finally, based on the available data, they considered 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 (EFSA, 2016).

44. 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 mutations

45. 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.

46. Other data (seven bacterial reverse mutation studies from the literature 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

47. Six studies considered of high or limited relevance were 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).

EFSA's concluding remarks

48. "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

49. Out of fifty six available studies, forty three were classified as high or limited relevance and considered for the assessment. Due to the large volume of studies considered, relevant Appendices (J, L, N, P) have been attached in Annex 3 of the opinion. These will also include characterisation of the test material.

50. 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).

51. 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).

52. 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).

53. 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).

54. 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.

55. 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

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

57. 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.

58. 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:

59. “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". (EFSA, 2021)

Comet Assays *(in vivo and in vitro)*

60. *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 (Annex 3)

61. *In vivo,* eighteen out of forty-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.

62. *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[9]. 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).

63. 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).

64. 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.

65. Fourteen studies (9 considered high relevance) used a lung model and the majority showed positive results for strand breaks for test materials size ranging

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

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).

66. 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).

67. 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).

68. The Panel noted that around 5% of available studies were obtained with titanium dioxide < 30 nm, 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.

69. *In vivo*, out of 9 studies administered by oral gavage 6 tested positive (Appendix K: Sycheva *et al*.,2011[10];Appendix M: Shukla *et al*., 2014; Grissa *et al*.,

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

2015; Shi *et al*., 2015; Manivannan *et al*., 2020; Murugadoss *et al*., 2020). Test material sizes varied from -160 nm[11], 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[12] (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.

70. 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

71. 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 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:

72. 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

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

[12] **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 groups of particles : 135 ± 46 nm, 305 ± 61, 900 ± 247 nm (TEM image)

Opinion). The results of the above studies were used to attempt to establish a mode of action (page 60-62 of the EFSA Opinion).

73. 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).

74. Furthermore the Panel 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.

75. 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.

76. The EFSA 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).

COM discussion

77. A paper was presented to the Committee on Mutagenicity in June, summarising the EFSA Opinion. The Members considered the available data. It should be noted that the minutes of the meeting are not yet agreed or available

and therefore this is a preliminary report of the discussions and conclusions of the COM. The minutes will be presented to the COT as soon as they become available.

78. The COM questioned the quality of the dataset. Overall the Committee noted that the data used was heterogenous with regards to the test substance thus making the evaluation of the studies, interpretation of the results and drawing conclusions difficult. It was also noted that studies using non-standard methodologies were considered and questions arose with regards to the use of data from laboratories not proficient in genotoxicity testing. Furthermore, the limited reliability score (as scored by EFSA) for the vast majority of the studies also raised concerns over the robustness of the data and its analysis in a weight of evidence evaluation. Conversely, the overall balance (i.e. publication bias) of studies reporting positive effects as opposed to negative effects in literature was also considered as one of the limitations of the dataset. There is lack of a good dataset with defined test compounds to allow for confidence in the assessment. The, up to recently, poorly defined E171 specifications further add to the uncertainty.

79. With regards to the mode of action, the COM considered that the evidence indicates a secondary, thresholded mechanism as opposed to direct DNA damage. Whilst a lot of *in vitro* studies reported positive effects, they appeared to be attributed to the nano-fraction. The *in vivo* studies were mostly negative/equivocal. Since the nano-fraction is less than 3.2% in E171, it was also noted that exposure as well as the low oral bioavailability and the possibility of agglomeration in the gastrointestinal tract should be considered when evaluating the risk.

80. Based on the available evidence, the COM did not agree with EFSA as the current dataset does not allow conclusions to be drawn. A more reliable, robust dataset and further review of the studies considered by EFSA would be needed in order for a conclusion to be made based on reliable data. Furthermore it was noted that, although there were indications that the nano-fraction could potentially be responsible for the positive responses, there was no discrimination between nano- and micro-fraction in the EFSA opinion and a lot of emphasis was put on the nanoparticle dataset, which only accounts for a small fraction of E171. The COM noted that micro-sized TiO_2 potentially has a different toxicological profile than nano-TiO_2 and therefore excluding the smaller TiO_2 particles from E171, if possible, would likely reduce the risk.

81. COM also considered that the wording of EFSA's conclusion was not helpful from a risk communication perspective as, based on the overall quality and equivocality of the dataset further refinement is needed in order for a conclusion on the safety of TiO_2 with regards to genotoxicity can be confidently made. As it stands the conclusion is highly risk adverse based on the weak evidence available, and it might create unnecessary concern to the public.

Carcinogenicity

82. The Panel noted that no suitable studies were located to assess carcinogenicity. With regards to the study used in the 2016 evaluation, the Panel considered that the study was not appropriate to evaluate the carcinogenic potential of TiO_2 NPs (EFSA, 2021).

Exposure

83. Details on the exposure can be found in pages 62-71 of the EFSA Opinion.

84. The Panel estimated the chronic dietary exposure to E 171 for the following population groups: infants, toddlers, children, adolescents, adults and the elderly. The methodology to estimate dietary exposure to E 171 in the current assessment and the different scenarios – maximum level exposure assessment scenario, refined exposure assessment scenarios (brand-loyal and non-brand-loyal) and food supplements consumers only exposure assessment scenario (EFSA, 2021).

85. In the maximum level exposure assessment scenario, mean exposure to E 171 from its use as a food additive ranged from 0.06 mg/kg bw/d in infants to 12.8 mg/kg bw/d in toddlers. The 95th percentile ranged from 0.2 mg/kg bw per day in infants to 31.4 mg/kg bw/d in toddlers. In the brand-loyal refined exposure assessment scenario, mean exposure to E 171 from its use as a food additive ranged from 0.05 mg/kg bw/d in infants to 10.0 mg/kg bw/d in toddlers. At the 95th percentile, exposure ranged from 0.1 mg/kg bw per day in infants to 28.0 mg/kg bw/d in toddlers. In the non-brand-loyal scenario, mean exposure ranged from 0.03 mg/kg bw/d in infants to 6.9 mg/kg bw per day in children. At the 95th percentile, exposure ranged from 0.1 mg/kg bw per day in infants to 27.5 mg/kg bw per day in toddlers.

86. For the food supplements consumers only (results reported in Appendix T), mean exposure to E 171 from its use as a food additive ranged from 0.8 mg/kg bw per day for adults to 11.7 mg/kg bw/d for children. The 95th percentile ranged from 3.1 mg/kg bw per day for the elderly to 41 mg/kg bw per day for children.

87. Based on a number of uncertainties identified by EFSA (Table 13, p.69) it was determined that they resulted in an overestimation of the exposure to E171 from its use as a food additive.

88. Oral exposure via other routes was also discussed, however exposures via medicines or cosmetics were not considered. Similarly, due to the large variation of E171 specifications, exposure to TiO_2 NPs could not be estimated accurately.

Overall EFSA conclusions:

89. 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.

90. 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."

91. 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.

92. 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[13].

- 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.

[13] https://www.efsa.europa.eu/en/efsajournal/pub/5760

- 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).

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

93. It is also worth noting that following the EFSA's Panel on Additives Panel on Food Additive and Flavourings (FAF) evaluation of the safety of titanium dioxide, the FEEDAP endorsed the conclusion and considered that it also applied to titanium dioxide when used as a feed additive for all animal species. They concluded that the genotoxicity of titanium dioxide particles could not be ruled out and this raised potential concerns on safety for the target species (especially long living and reproductive animals). On this point the conclusion was made on the basis that no studies were submitted by the applicant to support the safety of titanium dioxide for the target species and considering that titanium dioxide is intended for use in all animal species, the fact that there were no specific studies available designed to assess the safety for the target species, that genotoxicity could not be ruled out.

94. Furthermore no conclusion could be reached for the safety of titanium dioxide for consumers or the environment. For consumers, this was on the basis of the findings of the FAF Panel and also that there was no available information on potential exposure of consumers to titanium dioxide particles in food products from animals that were fed the additive. For the environment there was absence of adequate data to allow for evaluation of the safety of titanium dioxide particles.

95. For users, no data was available to allow for evaluation of the effects of the additive on the skin and the eyes. It was concluded that inhalation of the dust represents a risk to the users, as titanium dioxide is potentially carcinogenic (based on the IARC and RAC classifications) if inhaled and that the dusting potential of the anatase form was very high (150g/kg). The concern for genotoxicity of titanium dioxide particles could not be ruled out, which the Panel noted that it should be considered as an additional potential concern to users handling the additive.(EFSA, 2021a)

Questions for the Committee

96. Members are asked to consider the EFSA opinion:

i) Do Members consider that the weight of evidence supports EFSA's evaluation and conclusions?

ii) In light of the COM discussions, do the Members agree with the COM's comments on risk communication? Do Members have any comments and recommendations with regards to the wording that should be used for the risk communication perspective?

iii) Do the Members agree with EFSA's conclusion that no differentiation could be made with regards to size/form of Titanium dioxide and different aspects of toxicity?

iv) Do Members have any other comments?

Secretariat

June 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

AOP – Adverse Outcome Pathway

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

FAF – Panel on Food Additive and Flavourings

FEEDAP - Panel on Additives and Products or Substances used in Animal Feed

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

NOAEL – No Observed Adverse Effect Level

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

Anderson JC, Swede H, Rustagi T, Protiva P, Pleau D, Brenner BM, Rajan TV, Heinen CD, Levine JB Rosenberg DW (2012):Aberrant crypt foci as predictors of colorectal neoplasia on repeat colonoscopy, *Cancer Causes &Control*, 23, 355–361.

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.

Bischoff, N. S., de Kok, T. M., Sijm, D., van Breda, S. G., Briedé, J. J., Castenmiller, J., Opperhuizen, A., Chirino, Y. I., Dirven, H., Gott, D., Houdeau, E., Oomen, A. G., Poulsen, M., Rogler, G., & van Loveren, H. (2020): Possible Adverse Effects of Food Additive E171 (Titanium Dioxide) Related to Particle Specific Human Toxicity, Including the Immune System, *International journal of molecular sciences*, 22(1), 207. https://doi.org/10.3390/ijms22010207

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

Canli EG, Gumus C, Canli M and Ila HB (2020) :The effects of titanium nanoparticles on enzymatic and non-enzymatic biomarkers in female Wistar rats, *Drug and Chemical Toxicology*, 1–9.

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, 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.

Chen Z, Zheng P, Han S, Zhang J, Li Z, Zhou S and Jia G (2020a): Tissue-specific oxidative stress and element distribution after oral exposure to titanium dioxide nanoparticles in rats, *Nanoscale*, 12, 20033–20046.

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.

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.

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.

Drew DA, Mo A, Grady JJ, Stevens RG, Levine JB, Brenner BM, Anderson JC, Forouhar F, O'Brien MJ, Devers TJ, Rosenberg DW (2018): Proximal aberrant crypt foci associate with synchronous neoplasia and are primed for neoplastic progression, *Molecular Cancer Research*, 16, 486 –495.

Ebrahimzadeh BA, Mohammadipour A, Fazel A, Haghir H, Rafatpanah H, Hosseini M and Rajabzadeh A (2017): Maternal exposure to titanium dioxide nanoparticles during pregnancy and lactation alters offspring hippocampal mRNA BAX and Bcl-2 levels, induces apoptosis and decreases neurogenesis, Experimental and toxicologic pathology*: official journal of the Gesellschaft fur Toxikologische Pathologie*, 69, 329–337

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 FEEDAP (2021a): Safety and efficacy of a feed additive consisting of titanium dioxide for all animal species (Titanium Dioxide Manufacturers

Association) , *EFSA Journal 2021*;19(6):6630, doi: https://doi.org/10.2903/j.efsa.2021.6630

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-Din EAA, Mostafa HE, Samak MA, Mohamed EM and El-Shafei DA (2019): Could curcumin ameliorate titanium dioxide nanoparticles effect on the heart? A histopathological, immunohistochemical, and genotoxic study, *Environmental Science and Pollution Research International*, 26, 21556–21564.

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.

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.

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.

Hashem MM, Abo-El-Sooud K, Abd-Elhakim YM, Badr YA, El-Metwally AE,Bahy-El-Dien A (2020): The long-termoral exposure to titanium dioxide impaired immune functions and triggered cytotoxic and genotoxic impacts in rats, *Journal of Trace Elements in Medicine and Biology*, 60.

Hassanein KM and El-Amir YO (2017): Protective effects of thymoquinone and avenanthramides on titanium dioxide nanoparticles induced toxicity in Sprague-Dawley rats. *Pathology, Research and Practice*, 213, 13–22

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 BY, Ideta T, Lemos BS, Igarashi Y, Tan Y, DiSiena M, Mo A, Birk JW, Forouhar F, Devers TJ, Weinstock GM, Rosenberg DW (2019): Characterization of mucosal dysbiosis of early colonic neoplasia. NPJ Precis Oncol, 3,29.

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.

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.

Kandeil MA, Mohammed ET, Hashem KS, Aleya L and Abdel-Daim MM (2019) : Moringa seed extract alleviates titanium oxide nanoparticles (TiO2-NPs)-induced cerebral oxidative damage, and increases cerebralmitochondrial viability, *Environmental Science and Pollution Research International*.

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.

Karimi S, Khorsandi L and Nejaddehbashi F (2019): Protective effects of Curcumin on testicular toxicity induced by titanium dioxide nanoparticles in mice, *JBRA Assisted Reproduction*, 23, 344–351.

Karimipour M, Zirak JM, Ahmadi A and Jafari A (2018): Oral administration of titanium dioxide nanoparticle through ovarian tissue alterations impairs mice embryonic development*, International Journal of Reproductive Biomedicine* (Yazd, Iran), 16, 397–404.

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.

Khorsandi L, Orazizadeh M, Mansouri E, Hemadi M and Moradi-Gharibvand N (2016): Morphometric and stereological assessment of the effects of titanium dioxide nanoparticles on the mouse testicular tissue, *Bratislavske lekarske listy*,117, 659–664.

Khorsandi L, Orazizadeh M, Moradi-Gharibvand N, Hemadi M and Mansouri E, (2017): Beneficial effects of quercetin on titanium dioxide nanoparticles induced spermatogenesis defects in mice, *Environmental Science and Pollution Research International*; 24, 5595–5606.

Kowalczyk M, Orłowski M, Klepacki Ł, Zinkiewicz K, Kurpiewski W, Kaczerska D, Pesta W, Zielinski E , Siermontowski P (2020):Rectal aberrant crypt foci (ACF) as a predictor of benign and malignant neoplastic lesions in the large intestine*, BMC Cancer*, 20, 133.

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.

Lee J, Jeong JS, Kim SY, Park MK, Choi SD, Kim UJ, Park K, Jeong EJ, Nam SY and Yu WJ (2019): Titanium dioxide nanoparticles oral exposure to pregnant rats and its distribution, Particle and Fibre Toxicology, 16, 31.

Li XB, Zhang YS, Li B, Cui J, Gao N, Sun H, Meng QT, Wu SS, Bo JZ, Yan LC, Wu J , Chen R (2019): Prebiotic protects against anatase titanium dioxide nanoparticles-induced microbiota-mediated colonic barrier defects, *Nanoimpact,* 14, 9.

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.

Lu T, Ling C, Hu M, Meng X, Deng Y, An H, Li L, Hu Y, Wang H, Song G and Guo S (2020): Effect of nano-titanium dioxide on blood-testis barrier and MAPK signaling pathway in male mice, Biological *Trace Element Research*.

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.

Mohamed HR (2015): Estimation of TiO2nanoparticle-induced genotoxicity persistence and possible chronic gastritis-induction in mice, *Food and Chemical Toxicology: AN International Journal Published for the British Industrial Biological Research Association*, 83, 76–83.

Mohammadipour A, Hosseini M, Fazel A, Haghir H, Rafatpanah H, Pourganji M and Bideskan AE (2016) :The effects of exposure to titanium dioxide nanoparticles during lactation period on learning and memory of rat offspring,*Toxicology and Industrial Health*, 32, 221–228.

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.

Quintanilla I, Loopez-Ceroon M, Jimeno M, Cuatrecasas M, Zabalza M, Moreira L, Alonso V, Rodrıguez de Miguel C,Munoz J, Castellvi-Bel S, Llach J, Castells A, Balaguer F, Camps J, Pellise M, (2019): Rectal aberrant crypt foci in humans are not surrogate markers for colorectal cancer risk, *Clin Transl Gastroenterol*, 10, e00047

Rahnama S, Hassanpour A, Yadegari M, Anvari M and Hosseini-sharifabad M (2020) : Effect of titanium dioxide nanoparticles on the stereological parameters of the dentate gyrus and the morphology of granular hippocampal neurons in mice, *International Journal of Morphology*, 38, 1623–1630.

Riedle S, Wills JW, Miniter M, Otter DE, Singh H, Brown AP, Micklethwaite S, Rees P, Jugdaohsingh R, Roy NC,Hewitt RE and Powell JJ (2020) :A murine oral-exposure model for nano- and micro-particulates: demonstrating human relevance with food-grade titanium dioxide, *Nano-Micro Small*, 16, 2000486.

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

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

Siskova A, Cervena K, Kral J, Hucl T, Vodicka P, Vymetalkova V (2020):Colorectal adenomas-genetics and searching for new molecular screening biomarkers*, International Journal of Molecular Sciences*, 21, 3260

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.

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.

Talbot P, Radziwill-Bienkowska JM, Kamphuis JBJ, Steenkeste K, Bettini S, Robert V, Noordine ML, Mayeur C,Gaultier E, Langella P, Robbe-Masselot C, Houdeau E, Thomas, Mercier-Bonin M (2018): Food-grade TiO2is trapped by intestinal mucus in vitro but does not impair mucin O-glycosylation short-chain fatty acid synthesis in vivo: implications for gut barrier protection. *Journal of Nanobiotechnology*, 16, 53

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.

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.

Urrutia-Ortega IM, Garduno-Balderas LG, Delgado-Buenrostro NL, Freyre-Fonseca V, Flores-Flores JO, Gonzalez-Robles A, Pedraza-Chaverri J, Hernandez-Pando R, Rodriguez-Sosa M, Leon-Cabrera S, Terrazas LI, vanLoveren H, Chirino YI (2016) :Food-grade titanium dioxide exposure exacerbates tumor formation in colitis associated cancer model, *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, 93, 20–31.

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 (2015b): 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
Warheit DB, Brown SC and Donner EM (2015a): Acute and subchronic oral toxicity studies in rats with nanoscale and pigment grade titanium dioxide particles*, Food and Chemical Toxicology*, 84, 208–224.

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.
Zhou Y, Hong F, Tian Y, Zhao X, Hong J, Ze Y and Wang L (2017) : Nanoparticulate titanium dioxide-inhibited dendritic development is involved in apoptosis and autophagy of hippocampal neurons in offspring mice, *Toxicology Research*, 6, 889–901.

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, 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 - TOX/2021/36

COMMITTEE ON TOXICITY 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 - TOX/2021/36

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

MUT/2021/03: Review of genotoxicity of Titanium Dioxide.

MUT_2021_03 TiO2.pdf

Annex 3 - TOX/2021/36

COMMITTEE ON TOXICITY 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)