

Fifth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive- Studies using the E171 form of TiO₂ (in mice)

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Studies using the E171 form of TiO₂ (in mice)

147. With regards to the effects of TiO₂, ACF were evaluated in the studies by Bettini *et al.*, 2017, Blevins *et al.*, 2019 and the EOGRT study (TDMA, 2020). These are described below. Additional studies have also been evaluated to assess evidence for proliferative changes in the colon.

EOGRT study (LPT, 2020)

148. Details of this study are given in paragraphs 113 - 115 and 136 - 139, but the overall conclusion based on data from this study was that oral exposure to E171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon. In addition, no evidence of morphological changes in the colon were identified during routine microscopic histopathological examination of tissues from this study.

Blevins et al., (2019)

149. The Blevins *et al.* (2019) study used test material E171, anatase, 110 - 115 nm (SEM), 36% of the particles had a particle size 100 nm. Internal exposure was not examined. Six-week-old male Wistar Han IGS rats were exposed to E171 in a standard diet at 4 concentrations between 0 - 5,000 mg/kg diet and a control in two studies each of 7 days (n = 5/group) (equal to 1.8, 4.8, 31.4 and 374 mg/kg bw per day) and one study of 100 days (n = 15/group) and (equal to 1.3, 3.5, 22.4 and 267 mg/kg bw per day). Prior to exposure to E171, animals in groups 1 - 4 were treated with one intraperitoneal injection of a sterile dose of 180 mg/kg bw

dimethylhydrazine dihydrochloride (DMH) (an inducer of GI tract tumours) in 1.5% EDTA-0.9% NaCl, pH 6.5. Animals in groups 5 - 8 were treated with a single sterile dose of 1.5% EDTA-0.9% NaCl, pH 6.5 without DMH.

150. In the 100-day study no treatment related histopathological changes were found in the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E171. Rats that were initiated with DMH only and those which received E171 in the diet after the initiation displayed several histopathological abnormalities. There were two invasive adenocarcinomas in the large intestine in one animal in the 1.3 mg E171/kg bw per day + DMH group, and single adenomas in the large intestines in one animal in the 3.5 mg E171/kg bw per day + DMH group and in one animal in the 22.4 mg E171/kg bw per day + DMH group.

151. There were no other histopathological changes in the large intestines of the other animals treated with DMH. One rat in the 1.3 mg E171/kg bw per day + DMH group and one rat in the 22.4 mg E171/kg bw per day + DMH group had subpleural lymphocytes in the lung, but without any evidence of acute inflammatory changes or hyperplasia.

152. The 100-day study also showed that there was, as expected, a significant increase in ACF/cm² and ABC/cm² in animals pre-treated with 180 mg/kg bw DMH compared to the non-pre-treated groups. E171 doses administered after DMH did not result in statistically significant increases in ACF or ABC. Of note was that ACF and ABC were also seen at low levels in control animals. Overall, no effects of E171 on histopathologic evaluations of ACF were noted although the authors did include a caveat that much of the epithelial surface of the colon samples (proximal, middle and distal) were obscured when observed by light microscopy limiting examination of the entire surface of the colon samples. The Committee noted that Blevins *et al.* (2019) pointed out the limitations of their study, however, authors of other studies did not report on any limitations in their studies.

Bettini et al., (2017)

153. In the Bettini study, in addition to the 7-day studies described in paragraphs 58 and 59, a second group of rats (n = 11 to 12 per group) were treated (or not) with 1,2-dimethylhydrazine (DMH) to induce colon carcinogenesis and were exposed daily to E171 at 200 µg or 10 mg/kg bw/day through drinking water for 100 days. Control animals (n = 12) received water only. Rats were used for flow cytometry and cytokine assays and for gut inflammation and ACF assessments.

154. DMH and TiO₂ treatment at 10 mg/kg bw/day for 100 days significantly increased the total number of aberrant crypts and large ACF per colon when compared with both control animals and the 200 µg/kg bw/day group. However no overall significant difference between the number of ACF per colon between groups of rodents was observed. The authors did not explicitly give their definition of an ACF, however, the authors defined a 'large ACF' as consisting of more than three aberrant crypts per ACF.

155. Spontaneous development of ACF was also observed in 4 rats (from a total of 11) in the E171 group without a colon carcinogenesis initiator. Three or fewer ABCs were observed per ACF in three of these 4 positive samples and one which contained 12 ABCs.

156. Growth-promoting effects on colonic preneoplastic lesions were investigated for TiO₂ differentially affecting the viability of normal or preneoplastic cells. This was investigated using the comparative cytotoxicity of food-grade TiO₂ particles on non-mutated and pre neoplastic cells (Apc^{+/+} and Apc Min/+). Results showed that 24-hour exposure to TiO₂ was more cytotoxic to Apc^{+/+} than Apc Min/+ cells.

Additional studies examining evidence of histopathological proliferation in the colon

157. No microscopic evidence of proliferative changes in the intestine have been seen in other chronic toxicity studies with TiO₂ including Warheit, Brown and Donner, Agaki et al and the NCI Unitane ® carcinogenicity 1979 study. The Agaki study was not included in the EFSA review as it was published after the EFSA Opinion, in 2023. The EFSA review also did not include the NCI 1979 study because, although the EFSA ANS Panel did conclude that the study indicated that TiO₂ was not carcinogenic in rats and mice, the Panel scored the study of low reliability as they considered that the analytical information on the test item was insufficient. However, the COT notes that the Titanium Dioxide Manufacturers Association (TDMA) has more recently completed work that shows that the test item used in the NCI 1979 study is comparable to the specifications of E171 today, and so the study should be considered reliable.

Studies using E171 or equivalent form of TiO₂

Han (2020)

158. The 90-day oral repeated dose toxicity study of E171 was carried out in male and female SD rats in a study conducted according to OECD TG 408 and to GLP. Five-week-old male and female Sprague-Dawley rats (50 male and 50 female). Ten rats per sex and per dosage group were randomly assigned to one of four groups (0, 10, 100, or 1,000 mg/kg dosage of E171) administered daily by oral gavage for 13 consecutive weeks (90 days). The control group was provided with the equivalent volume of water. AGS cells (70 - 80 % of confluence) were incubated with E171 (0, 10, 20, 40 µg/mL) for 24 h. Colon sections and AGS cells (human gut epithelial cell line) were fixed, sectioned and images were produced for analysis using a TEM.

159. There were no dose-related changes in the endpoints. Effects observed included E171 deeply penetrating cells lining the stomach tissues at the maximum dosage (1,000 mg/kg), and significantly lower blood IgM (male and female) and GM-CSF (female) levels in the E171-treated animals than in control animals. Colonic superoxide dismutase (SOD)-1 protein levels decreased in males and females and SOD-2 protein levels decreased in females with increasing Titanium accumulation.

160. E171 (40 µg/mL) accumulated in the perinuclear region following exposure of AGS cells for 24 hours. The E171 treatment affected expression of ER stress-related proteins but did not induce cell death up to 40 µg/mL (the maximum dosage in this study).

161. The authors concluded a NOAEL for E171 of less than 1,000 mg/kg for both male and female rats based on the 90-day study and proposed that further chronic toxicity studies be conducted due to the potential for E171 to reduce a host's immune defence function by decreasing antioxidant capacity.

NCI (1979)

162. The Unitane ® 0-220 study (NCI, 1979), a compound which was previously manufactured in the US for use as food-grade TiO₂, was considered by the sub-group due to recent work by the TDMA which demonstrated that the test item tested in this study was comparable to the updated specification of E171. EFSA scored this study low on reliability and discounted the study from their evaluation as they considered that the analytical data on the test item was not sufficient. A 13-week study of male and female B6C3F1 mice and Fischer 344 rats (n = 10 per sex per group) were fed doses of Unitane ® 0-220 (TiO₂, anatase, 98% purity). A further cohort (n = 50 per sex per group) were fed Unitane ® 0-220 via the diet for 103 weeks at concentrations equivalent to 0, 3,250 or 6,500 mg/kg bw/day

and 0, 4,175 or 8,350 mg TiO₂/kg bw/day for male and female mice (respectively) and doses of 0, 1,125 or 2,250 mg/kg bw/day and 0, 1,450 or 2,900 mg/kg bw/day for male and female rats (respectively) and then observed for 1 additional week. Fifty untreated rats of each sex and 50 untreated mice of each sex were used as controls. All remaining rodents were euthanised at 104 weeks. No evidence was observed (in either species) of lesions in the gastrointestinal tract e.g., proliferative non-neoplastic or neoplastic findings. It was also noted following examination of the pathology data that nematodes were recorded in the intestine which introduces an additional potential for GI inflammation which could potentially induce inflammatory and proliferative changes, however none were observed in the study.

Warheit, Brown and Donner (2015)

163. Warheit, Brown and Donner (2015) summarised 3 OECD-type studies of TiO₂ in rats of varying particle sizes and surface coatings. Study 1 was a subchronic 90-day study (OECD TG 408) in which adult male and female rats were fed rutile, surface-coated pigment-grade TiO₂ test particles (d₅₀ $\frac{1}{4}$ 145 nm, 21% nanoparticles) by oral gavage for 90 days with a NOAEL of 1000 mg/kg bw/day. Study 2 was a 28-day repeated-dose oral toxicity study (OECD TG 407) in adult male rats fed two rutile-type, uncoated, pigment-grade TiO₂ test particles (d₅₀ $\frac{1}{4}$ 173nm) by oral gavage at a dose of 24,000 mg/kg bw/day with no test item-related effect. Study 3 was an acute oral toxicity study (OECD TG 425) with female rats were fed surface-treated rutile/anatase nanoscale TiO₂ particles (d₅₀ $\frac{1}{4}$ 73 nm) up to 5,000 mg/kg. The oral LD₅₀ for the test substance was >5,000 mg/kg bw. Collectively, no test item-related adverse effects were found including during microscopic examination of colon.

Studies using the nanoparticle form of TiO₂

Akagi *et al.*, (2023)

164. In the Akagi *et al.*, 2023 study, TiO₂ NPs (6 nm) were examined in male and female rats by repeated oral administration of 10, 100, and 1,000 mg/kg bw/day for 28 days (5 individuals per sex per dosage group group) and of 100, 300, and 1,000 mg/kg bw/day for 90 days (10 individuals per sex per group). The authors reported that no mortality was observed in either study length with no treatment-related effects observed in body weight, urinalysis, hematology, serum

biochemistry or organ weight. Histopathological examination of the 28-day study specimens found TiO₂ particle deposition in the gastro-intestinal lumen, nasal cavity, epithelium and stromal tissue as well as in the 90-day study in Peyer's patches in the ileum, cervical lymph nodes, mediastinal lymph nodes, bronchus-associated lymphoid tissue, and trachea. Despite this, no inflammation or tissue damage were observed. Little evidence was found of TiO₂ absorption or accumulation was found in the liver, kidneys, and spleen. Immunohistochemical analysis showed no extension in colonic crypts of the proliferative cell zone or preneoplastic cytoplasmic/nuclear translocation of β -catenin up to 1000 mg/kg bw/day in males or females. With regard to genotoxic endpoints, no observed increase in micro-nucleated or γ -H2AX positive hepatocytes was found. No effects were observed after repeated oral administration of TiO₂ (6 nm particle size) up to 1,000 mg/kg bw/day.

EFSA review and conclusions

165. The EFSA Panel (2021) noted a considerable variability in the results, which may mask possible effects. The Panel considered that the effect of E171 in producing ACF reported by Bettini et al. (2017) was not replicated in later investigations (EOGRT study and Blevins et al., 2019). One source of uncertainty was that it was noted that there were methodological limitations in Blevins et al. A further source of uncertainty is being unclear to what extent animals were exposed to TiO₂ Nanoparticles in both the EOGRT study and Blevins et al. The Panel concluded that E171 may induce ACF in male rats at a dose of 10 mg/kg bw per day when the test substance is pre-dispersed and stabilised in a liquid medium preventing agglomeration of nanoparticles prior to administration by gavage.

Health Canada review and conclusions

166. As mentioned in paragraph 14, the Health Canada State of the Science report on titanium dioxide had previously been reviewed by the Committee and its conclusions endorsed. The Committee sub-group agreed that the report was comprehensive and included further discussions with pathologists from the Blevins *et al.* study to evaluate the colon findings.

167. Papers included in the Health Canada report which discussed effects on the gut microbiota were reviewed by the sub-group. These include Lamas, Martins Breyner and Houdeau 2020.

168. Papers included in the Health Canada report which discussed carcinogenicity and chronic toxicity endpoints were reviewed by the sub-group. These include Bettini et al. 2017, Blevins et al. 2019, EOGRT study (TDMA, 2020), and Han et al. 2020. The Health Canada report concluded that while there were some uncertainties identified which may require further studies, they did not identify consistent evidence of preneoplastic lesions in the colons of rodents exposed to food-grade TiO₂ via the oral route. A single non-guideline study in which rats were exposed to food-grade TiO₂ dispersed in drinking water at doses of ~10 mg/kg bw/d for 100 days (Bettini et al., 2017) resulted in formation of ACF, but these results were not replicated in subsequent dietary studies when higher doses were administered, up to ~236 - 300 mg/kg bw/day for 100 days in a non-guideline study (Blevins et al., 2019) and up to 1000 mg/kg bw/day for ~18 - 19 weeks in a GLP- and OECD guideline-compliant EOGRT study (TDMA, 2020). An OECD guideline-compliant study that administered food-grade TiO₂ dispersed in water to rats via oral gavage at doses up to 1,000 mg/kg bw/day for 90 days showed no histopathological changes in the gastrointestinal tract (GIT) (or any other tissues) (Han et al., 2020). The report concluded that there was no evidence of, and therefore that there was a low level of concern for, carcinogenicity, chronic toxicity, or other non-neoplastic lesions of the GIT. A weight of evidence approach did not identify data gaps of such significance to require a precautionary approach currently.

169. In the final summary the authors concluded that "Health Canada Food Directorate's position is that there is no conclusive scientific evidence that the food additive TiO₂ is a concern for human health".

FSANZ review and conclusions

170. FSANZ noted that there were no chronic toxicity or carcinogenicity studies available in the literature which used oral administration of food-grade TiO₂. The NCI 1979 Unitane study was previously considered suitable for assessing the carcinogenicity of food-grade TiO₂ however FSANZ noted that the EFSA 2021 opinion on TiO₂ raised questions regarding the suitability of use of Unitane 0-220 for this purpose.

171. FSANZ noted EFSA's concerns from the 2021 opinion around the risk of induction for ACF in the colon identified in Bettini et al. (2017). FSANZ' previous assessment of the Bettini et al. (2017) study concluded that this study had limited

relevance in humans due to design limitations including the use of sonicated test item delivered via drinking water and its applicability to the use of TiO₂ as a food additive. This concern was raised by ANSES in 2017 in which it was concluded that the Bettini et al. (2017) study results could not be used for risk assessment without a confirmatory study which included dietary administration of the test item. It was also noted that in their 2018 opinion on TiO₂, EFSA also agreed that these data “were not sufficient to raise a concern on the potential initiation or promotion properties of TiO₂ on colon carcinogenesis”.

172. FSANZ noted that while Bettini et al. (2017) noted evidence suggesting increased incidence of ACF related to oral exposure to TiO₂, two additional studies using sonicated food-grade TiO₂ administered to rats via by gavage for up to 90 days (Blevins et al. 2019 and the unpublished EORGT Study (LPT 2020)) found no evidence of treatment-related histopathological changes in the gastrointestinal tract up to doses of 1000 mg/kg bw/day. In addition, FSANZ also noted that the NCI 2-year bioassay results “found no evidence of carcinogenicity of a test item comparable to food-grade TiO₂ in the colon or other tissues in rats and mice at dietary concentrations up to 50,000 ppm, equivalent to doses of 2250 – 2900 and 6500 – 8350 mg/kg bw/day, respectively”, a finding which was also inconsistent with the Bettini et al. (2017) results.

173. FSANZ concluded that dietary administration of the food-grade test item were considered most relevant to human exposure to TiO₂ in foods and beverages and that the different modes of exposure may explain the difference in results between these studies. Given the increased weighting given to these studies, FSANZ stated that “dietary exposure to food-grade TiO₂ is unlikely to induce pre-neoplastic or neoplastic lesions in the colon or other tissues”.

COT review and conclusions

174. The Committee questioned whether studies in which TiO₂ was administered by methods other than through the diet represented consumption of food. In one study that assessed ACF (Bettini *et al.*, 2017) the test item was administered via water whereas the EOGRT study and Blevins *et al.*, 2019 were dietary. Studies with TiO₂ prepared in solution had bovine serum albumin (BSA) added to provide protein with sonication for up to 1 hour which can affect the material. This may not be representative of the exposure in humans via use in food, due to the sonication affecting the structure of TiO₂.

175. The results from the Bettini study where ACF were identified in rats pre-treated with DMH and in a small subset of animals with TiO₂ alone, exposed to food-grade TiO₂ dispersed in drinking water at doses of ~10 mg/kg bw/d for 100 days, were not replicated in the Blevins or EOGRT dietary studies. In addition, there was no evidence of further proliferative changes in the colon mucosa in these studies or other studies where histopathology of the colon was performed. The committee concluded that there was no conclusive evidence that TiO₂ induced ACF and no evidence to support progression to proliferative lesions in the colon.

176. The Committee considered that although small numbers of ACF were observed (Bettini *et al.*, 2017) in some animals exposed to TiO₂ alone, these could not necessarily be attributed to TiO₂, as ACF were also present in control groups without exposure to TiO₂ in other studies (Blevins *et al.*, EOGRT). Additionally, none of the studies distinguished between hyperplastic or dysplastic ACF in any groups of control or treated animals. The Committee also concluded that there was very little evidence that the effects of TiO₂ were systemic at the doses tested but also that TiO₂ measurements were not accurate in the analysis of TiO₂ in the tissues. One explanation for why the NOAEL is 1,000 mg/kg bw/day, the highest dose tested, is because the absorption is low.

Genotoxicity

177. An overview of the genotoxicity studies evaluated is included in the COM statement and supporting documents published on their website. A link to COM narratives which detail the studies that they have assessed will be included once published.

178. Add in brief overview of COM methodology – RAG ratings to understand the COM conclusions. To be included once COM documents have been finalised.

EFSA review and conclusions

179. EFSA considered that several in vitro studies demonstrated that TiO₂ NPs can induce gene mutations in cultured mammalian cells. Five in vivo studies were considered, one indicated the induction of large DNA deletions, however the remaining four studies, that could identify point mutations and small deletions, gave consistently negative results. They concluded that the available experimental data do not confirm the potential of TiO₂ NPs (30 nm) to induce gene mutations in vivo.

180. Taking into account the available evidence, EFSA considered that - on balance - TiO₂ NPs have the potential to induce micronuclei (MN)/chromosomal aberrations (CA). They noted “that a significant portion of the studies was performed using TiO₂ NPs 30 nm, however some positive results were observed with TiO₂ particles > 30 nm and no clear dependence of the particle size on positive effects in MN/CA assay was observed” (EFSA, 2021).

181. EFSA concluded that based on the results of the in vitro and in vivo comet assays, TiO₂ particles have the potential to induce DNA damage. As noted for MN and CA effects, “a significant portion of the studies were performed using TiO₂ NPs 30 nm, however some positive results were also observed with TiO₂ particles > 30 nm and no clear dependence of the particle size on positive effects in Comet assay was observed” (EFSA, 2021).

182. EFSA also concluded that there is evidence, from both in vitro and in vivo studies, for interaction(s) of TiO₂ NPs with DNA. However, due to the techniques employed, it was not possible to determine whether these interactions involved covalent or non-covalent binding.

Health Canada review and conclusions

183. Health Canada’s Food Directorate notes that, based on the currently available studies including the EOGRT study (LPT 2020), food grade TiO₂ is not genotoxic in-vivo. However, they recognise that the studies available on this endpoint are limited and additional OECD guideline-compliant studies are recommended to confirm this. They conclude that any adverse effects found related to oral exposure were based on the findings from non-standard studies in which the form of TiO₂ administered homogenized suspensions of particles with ultrasonic dispersion and that these do not fully represent exposure to food grade TiO₂.

FSANZ review and conclusions

184. No in-vivo genotoxicity studies were found that used food-grade TiO₂ with dietary administration were found. FSANZ identified four in-vivo genotoxicity studies, two studies using orally-administered food-grade TiO₂ (Bettini et al., 2017 and Jensen et al., 2019) and two studies using intraperitoneal administration of Unitane (0-220) (Shelby et al, 1993 and Shelby and Witt, 1995). These latter two studies assessing genotoxicity by the NCI/NTP were identified by the US National Toxicology Program and were assumed to use Unitane 0-220 as the test

item was not described (Shelby et al, 1993 and Shelby and Witt, 1995)

185. FSANZ noted that DNA damage was not observed either in the two comet assays (food-grade TiO₂, oral gavage) or the micronucleus and chromosomal aberration studies (Unitane 0-220, intraperitoneal injection). In-vitro studies (GLP- and OECD test guideline-compliant, food-grade TiO₂) found no evidence of mutagenicity in mammalian cells nor clastogenicity or aneugenecity or cellular uptake (micronucleus assay using human peripheral blood lymphocytes). There was some observation of uptake and internalisation by A549 cells however no particles were detected in the nucleus. FSANZ noted that “the absence of confirmed cellular uptake in the in vitro genotoxicity studies may limit confidence in the negative results, although alternatively the absence of direct exposure of the nucleus to food-grade TiO₂ in these studies may indicate a low intrinsic hazard from a direct genotoxicity perspective (OECD 2014)”.