EOGRT Study - Toxicity of Titanium Dioxide as a Food Additive

In this guide

In this guide

This is a paper for discussion.

This does not represent the views of the Committee and should not be cited.

161. The Extended One Generation Reproductive Toxicity (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 (preneoplastic lesions that had been reported by Bettini et al. (2017). Methodology: Test Material, Doses, Administration of Treatment.

162. The test material: Titanium dioxide E171-E, Particle size (ECD); (number measurement, primary particle size) x10 = 0.070 µm x50 = 0.110 µm x90 = 0.180 µm via the diet.

163. The doses used were: Group 1: 0 mg/kg b.w./day, Group 2: 100 mg/kg b.w./day, Group 3: 300 mg/kg b.w./day, 4: 1000 mg/kg b.w./day 20 male and 20 female rats evaluated. The concentration of the test item in the diet was adjusted based on the mean group food consumption per sex. The concentration was adjusted weekly using the food consumption values from the previous week.

164. The test item was administered in graduated doses to several groups of males and females prior to, during and after mating until weaning of the F1 and F2 Generation. The F1 Generation was dosed in the same way as the F0 Generation after weaning. Until weaning, the exposure of the F1 Pups to the test item was indirectly through the breast milk, however the pups additionally received the test item directly when commencing feeding by themselves during the last week of the lactation period. The duration of dosing depended on the requested endpoints for the different cohorts of the F1 Generation. Cohort 1B animals were maintained on treatment beyond PND 90 and bred to obtain an F2 Generation. Detailed examination of key developmental endpoints, such as offspring viability, neonatal health, developmental status at birth, and physical and functional development until adulthood, was performed to identify specific target organs in the offspring. Possible endocrine disruptor effects of the test item were also examined.

Evaluation of Sexual Function and Fertility

Male fertility:

165. An overview of the results for male fertility parameters is reported in Annex A. No statistically significant or dose-related effects on sperm motility, total spermatids/gram testis, percentage of abnormal spermatozoa and male mating index were observed in the F0 generation. The slight decrease in the number of successful matings at doses of 300 and 1,000 mg/kg bw per day appears unrelated to the male partners, as all males that failed to impregnate their females showed normal sperm motility and sperm counts. Only one of the high-dose males was found to have a lower testicular spermatid content (50% of the group mean), a finding that was also associated with a slightly lower testis weight (85% of the group mean). The number of abnormal sperm was low in all dose groups and remained below 2% in the few males in which abnormal sperm were

found.

166. The Panel noted that the epidydimal sperm parameters were not evaluated but that this deviation has no effect on the final conclusion of the study. There were no effects on any of the sperm endpoints in the cohort 1A.

Female fertility:

167. An overview of results for female fertility parameters is reported in Annex A. No effects on mean oestrus cycle duration were noted in F0 and F1 (cohort 1B) parental generations and all F0 females in the control, 100, 300 and 1,000 mg/kg bw per day groups mated. In the F1 generation 2 and 3 animals from the mid- and the high-dose groups, respectively, were erroneously removed from the study, before mating had been unequivocally confirmed. All other females mated, except one F1 female in the 100 mg/kg bw per day group. With few exceptions, mating occurred at the first oestrus after the females were housed with males. No effects of treatment were observed. The pregnancy rate was slightly lower in the F0 generation at 300 and 1,000 mg/kg bw per day (100, 96, 92 and 92%). As this finding was not confirmed in the F1 generation (100, 95, 94 and100%) the Panel considered it as incidental and not treatment-related.

168. No effects were noted on pregnancy duration, number of implantation sites and post-implantation loss. Although they occurred in the mid- and high-dose groups, three single total litter losses, either from total resorption of all embryos or from death of the litter during or shortly before birth, were not considered to be due to treatment. This is because the two F0 dams had unusually small litters of two pups each, which were stillborn, and the F1 dam showed total resorptions of eight implants at necropsy after failing to litter. Live litter sizes and litter weights were comparable to control values in all dose groups in the F0 and the F1 generation.

169. The EFSA Panel concluded that there were no indications of effects on general toxicity, thyroid or sex hormone levels, reproductive function and fertility in either male or female rats, no effects on pre- and postnatal development or on neurofunctional endpoints in F1 offspring.

170. The 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 TiO2NP 30 nm up to the highest dose tested of 100 mg/kg bw per day.

Developmental Toxicity

171. Pre-and postnatal lethality and structural abnormalities: No treatment-related pre- or postnatal loss was observed in the F0 and F1 generations. The average litter size at birth in all dose groups was comparable or higher than in the control group and the sex ratio was unaffected. No external or internal abnormalities were detected in F1 and F2 pups at termination.

172. Growth and sexual development: An overview of the results related to growth and sexual development for the F1 and F2 generations is included in Annex A. No treatment-related effects were observed in birth weights and growth of the pups. There were no indications for any androgenic and/or oestrogenic effects on the male and female anogenital distance (AGD) and the retention of nipples in males.

173. The mean age at vaginal opening was comparable between control and treated groups. The statistically significant lower body weight on the day of vaginal opening in cohort 1A at 300 mg/kg bw per day was not considered to be biologically relevant due to the slightly higher litter sizes in all treated groups. A divergence from the required method was examination of balanopreputial gland cleavage instead of examining balanopreputial separation which does not comply with the OECD TG 443 and therefore cannot be considered a measure of puberty in males.

174. Neurofunctional screening: Male and female F1 cohort 2A offspring were tested for auditory startle response between PND 23 and 25, and for a functional observation battery including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64. No differences in the response to an auditory startle stimulus were observed between the control and all the tested doses. Compared to controls, an increase in hindlimb splay was observed in females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

175. To check whether the significant differences in grip strength and hindlimb splay could be due to systematic bias in group testing order, the testing order was checked The Panel considered that there was no systematic bias in group testing order and that this was therefore not a plausible explanation for the observed group differences. Grip strength and hindlimb splay belong to the same domain of neurological function, i.e. motor function and/or sensory–motor coordination. However, the effects observed (i.e. increase in hindlimbs play and

increase in mean forelimb grip strength) seem to point in opposite directions when it comes to muscle strength. In particular, an increase in hindlimb splay can be interpreted as muscular weakness whereas an increase in mean forelimb grip strength could be indicative of myotonia.

176. The Panel noted that the effects observed were not correlated to any other changes (e.g. alterations in muscle tone, righting reflex, gait, wire manoeuvre, posture). No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in the functional observation battery measurements or locomotor activity were noted.

177. Furthermore, there were no notable histopathological findings in brain or in peripheral nerve (sciatic). Based on all the above considerations, the Panel considered that the effects on grip strength and hindlimb splay were not treatment-related. However, the Panel noted that quantitative information on peripheral nerves was not available. Overall, the Panel considered that E 171 had no adverse effects on neurofunctional endpoints in F1 cohort 2A offspring at the doses used.

178. EFSA conclusions on developmental toxicity results of the EOGRT study: No effects of E 171 on pre- and postnatal development were observed. Data on the attainment of puberty in males (i.e. an appropriate assessment of the timing of the balanopreputial separation) were missing. The Panel did not consider this to be critical in this case.

Developmental Immunotoxicity

179. Effects on developmental immunotoxicity were determined in the F1 cohort 3 animals through an examination of their ability to raise an antibody response to a foreign antigen. Animals are sensitised and the primary IgM antibody response to the sensitising antigen, in this case to keyhole limpethaemocyanin (KLH) antigen, is measured. The ability of the test compound to modulate serum anti-KLH antibody titre is taken as indicative of a developmental immunotoxic effect. A KLH-immunised control group also exposed to a known immunosuppressant (i.e. cyclophosphamide (CY), resulting in at least 50% inhibition in serum IgM anti-KLH titre, is considered crucial for the verification of assay performance.

180. These data can be considered in combination with additional data related to potential immunotoxic effects. In the F1 cohort 1A animals, the

following may contribute to the general assessment for immunotoxicity: weight and histopathology of the spleen, thymus and lymph nodes, as well as bone marrow histopathology, total and differential peripheral WBC count and splenic lymphocyte subpopulation distribution. T-cell-dependent anti-KLH response (KLH assay). Determination of serum anti KLH-IgM antibodies was performed in F1 cohort 3 (10/sex per group, PND 53–61) using an enzyme-linked immunosorbent assay (ELISA).

181. The animals were sacrificed 5 days after intravenous bolus injection (tail vein) of KLH, blood was withdrawn and the level of anti-KLH IgM was measured in serum. In addition, satellite animals of F1 (10/sex, PND 55) were immunised with KLH and treated with CY (single administration of 40 mg/kg bw by gavage on the same day of KLH treatment) to provide a positive control (for an inhibition of immune response).

182. A slight, but statistically significant decrease in the antigen specific IgM level was measured at the highest dose tested (1,000 mg/kg bw per day) in males only (–9%) and without an apparent dose response. In addition, the Panel noted 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.

183. The sensitivity of the test was not demonstrated due to invalid CY positive control results. It was noted that the assay conditions may have not been optimal resulting in an apparent low antibody response to KLH when compared to literature (Gore et al., 2004).

184. It was considered that all tested animals in the study had a weak immunogenic response to KLH that was insufficient to identify a T-cell-dependent immunotoxic effect of E 171 therefore no conclusion can be drawn on the effect of E 171 on the developing immune system. The Panel agreed with the conclusion of the study authors.

185. Assessment of pathology, haematology and splenic lymphocyte subpopulations At necropsy, pathology of lymphoid organs, haematology and lymphocyte subpopulations in the spleen were investigated. The following lymphocyte subpopulations were determined via flow cytometry analysis (FACS): T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells. The Panel noted that haematology, spleen weight and histopathology of lymphoid organs in animals from F1 cohort 1A did not indicate any dose-related effects.

186. For the splenic lymphocyte subpopulation analysis, no statistically significant differences were observed in the percentage of T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells of any of the treated groups compared to control in both sexes. The study authors concluded that no test substance-related effect was observed on the proportion of the examined lymphocyte subtypes. The Panel agreed with the study author conclusion that the splenic lymphocyte subpopulations in this cohort were not affected. However, the Panel considered that an isolated observation in F1 cohort 1A is not sufficient to conclude on immunotoxicity.

187. Compared to the animals of F1 cohort 1A, F1 cohort, 3 animals showed a shift in the lymphocyte subpopulation that indicated activation of the immune system by injection of KLH and concluded that increased B-cell proliferation may have led to the production of antigen-specific antibodies. In the F1 cohort 3 animals, no differences in the relative size of the lymphocyte subpopulations were observed between the control group and the E 171-treated groups, after immunisation of the animals with KLH.

188. The proposed reason was that the B-cell shift in F1 cohort 3 was caused by KLH immunisation, supported by the fact that there was no such shift found for the positive control animals that were sensitised to KLH and treated with CY. It was also considered that KLH induced an immune reaction, and that this response was influenced by CY as expected; KLH would increase the percentage of splenic B cells and decrease the percentage of T cells.

189. The conclusion was that the immune response was affected by CY but was not adversely affected by the TiO2 test substance. The Panel did not agree with the conclusion of the study authors that a shift to B cells by KLH was substantiated. The Panel considered that it is incorrect to compare the groups of F1 cohort 1A and of F1 cohort 3 because the groups of animals of F1 cohort 3 had a different age than that of the animals in F1 cohort 1A at the time of sacrifice (PND 87–96 vs. PND 53–61, respectively). In addition, the FACS analyses on the splenic cell suspensions were not all performed in the same round of analysis but were performed separately, while it is known that this may have influenced staining and subsequent quantification. The authors suggested that even if the positive CY control did not perform as expected, the data still indicate there is no effect of E 171 on sensitisation to KLH.

190. It is worth noting that the EFSA Panel did not agree with this conclusion and overall considered that the data did not allow to conclude on developmental immunotoxicity with respect to E 171.

Immunotoxicity Summary

191. 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.

192. The Panel noted that there were methodological shortcomings in the design of this part of the EOGRT study. Therefore, the Panel could not conclude on immunotoxicity.

193. Some findings regarding immunotoxicity and inflammation with E 171 as well as neurotoxicity with TiO2 Nanoparticles may be indicative of adverse effects including indications of an induction of ACF with E 171.

Neurotoxicity

194. EOGRT Study - Male and female offspring were tested for auditory startle response between PND 23and 25, including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64. No differences in the response to an auditory startle stimulus were observed and an increase in hindlimb splay was observed in females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

195. Grip strength and hindlimb splay belong to the same domain of neurological function, however, the increase in hindlimb splay and increase in mean forelimb grip strength are opposed in this case - increases in hindlimb splay indicate muscular weakness but an increase in mean forelimb grip strength may indicate myotonia. No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in observed including in histopathological findings in brain or in peripheral nerve tissue.

196. The Panel considered that the effects on grip strength and hindlimb splay were not treatment-related but that quantitative information on peripheral nerves was not available. Overall, the Panel considered that E 171 had no adverse effects on neurofunctional endpoints at the doses used.

197. No neurotoxicity studies performed with E 171 were identified from the published literature that were considered sufficiently reliable. Some papers were identified noting effects of TiO2 NP 30 nm but these are not discussed further in

this paper.

Aberrant Crypt Foci

198. A satellite group of the EOGRT study used doses up to 1,000 mg/kg bw per day and up to this dose did not induce ACF in the colon.

Aberrant Crypt Foci Examination in Satellite F0 Animals (EOGRT Study).

Method:

199. Evaluation of ACF in the colon of a satellite group of F0 animals (10/sex per group) treated with 0, 100, 300 and 1,000 mg E 171/kg bw per day and terminated after weaning was undertaken. The colon was excised, opened longitudinally and the contents removed by rinsing with a 0.9% NaCl solution. Thereafter, the tissue was divided in parts of a suitable size for fixation by immersion in 5% buffered formalin. A blind examination of these samples stained with 0.5% (w/v) methylene blue in water was performed under a stereomicroscope at 50x magnification for presence of ACF. The Panel noted that the design of the study did not include a positive control group (e.g. treatment with a known gastrointestinal tract tumour initiator such as dimethyl hydrazine (DMH) for the development of ACF.

Results:

200. The definition of ACF used was ‘foci containing more than 2 ABCs’, taken from Shwter et al. (2016). No ACF were found in the colons of the control and the treated groups. A mildly increased 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 (See tables 1 & 2 below). These changes were assessed as inconsistent with the appearance and definition of ACF discussed above. Incidence of these single crypts observed in the mid and high doses was not significantly different from the control. The EFSA Panel agreed with this conclusion.

Table 1: Aberrant Crypt Foci Presence in Satellite F0 Animals.

Dosage Group	Control, Aberrant Crypt Foci Present	Low-Dose	Mid-Dose	High-Dose	Total
Females	1/10	0/10	1/10	2/10	4
Males	1/10	0/10	1/10	1/10	3

201. An additional submission of data included photomicrographs of mildly increased variability in crypt morphology from all seven animals. A re-examination was extended to an additional randomly selected nine control animals (4 males and 5 females) and eight high-dose group animals (3 males and 5 females). A mild increased variability in crypt morphology was observed in eight of the nine controls and six of the eight high-dose animals (see Table x). The Panel considered that oral exposure to E 171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon.

Table 2: Aberrant Crypt Foci Presence in the Re-Examination of Satellite F0 Animals.

Dosage Group	Control, Aberrant Crypt Foci Present	High-Dose	Total
Females	4/5	3/3	7
Males	4/4	3/5	7

202. With regards to the induction of aberrant crypt foci, EFSA considered additional studies available in the literature in order to form their conclusions. These studies are discussed in detail below.

Bettini et al 2017

Test materials:

203. The test materials used in this study were:

I. E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles100 nm;

II. TiO2 Nanoparticles (NM-105), anatase/rutile, 15–24 nm. Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure) of 1.

Internal exposure:

204. Qualitative measurement in tissues, methodology reliable with some limitations. Adult Wistar rats were administered by gavage 10 mg E 171/kg bw per day for one week or 100 days. In addition, a group of animals were exposed to TiO2 Nanoparticles (NM-105) 10 mg/kg bw per day for one week.

Method:

205. First experiment: 12 male rats were pre-treated with a single injection (180 mg/kg intraperitoneal in isotonic saline) of DMH. The aberrant crypts average per colon was 190 ACF and 30 large ACF after 100 days. In DMH pre-treated rats also subsequently (7 days later) exposed to either 0.2 or 10 mg/kg bw per day E 171 in drinking water (12 rats/group), there was a statistically significant increase per colon in number of aberrant crypts and large ACF and a statistically non-significant increase in total number of ACF in the high-dose group compared to DMN only controls. No statistically significant differences were observed between the low-dose and control groups. The incidence of ACF was not reported.

206. Second experiment: Male rats received either drinking water (12 controls) or 10 mg E 171/kg bw per day in drinking water (n=11) for 100 days. No ACF were observed in the colons of controls. Four rats in the treated group developed one to three ACF per colon (which in three rats consisted of 1–3 aberrant crypts/ACF with the remaining rat having 12 aberrant crypts in an ACF). The increase in the incidence of rats with ACF (4/11 vs. 0/12 in the control group) was statistically significant.

207. The Panel considered that these data indicate that E 171 has pro-inflammatory potential at the systemic level, paralleled by the development of an inflammatory microenvironment in the intestinal mucosa.

208. The Panel considered that E 171 alone at a dose of 10 mg/kg bw per day may induce development of ACF in male rats. The Panel also noted that E 171 at a dose of 10 mg/kg bw per day increased the number of ACF initiated by a

genotoxic carcinogen (not considered further in this paper).

Results:

209. Titanium was detected in the immune cells of Peyer's patches in which patches dendritic cell percentage were increased. Effects were not noted in the spleen. It was noted that this effect was transient (observed at 7 days but not at 100 days). The percentage of regulatory T cells and T-helper (Th) cells were significantly decreased at both time points in E 171 exposed animals. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T-helper (Th)-1 IFN-c secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated in-vitro with anti CD3/CD28 antibodies.

Intestinal mucosal inflammation:

E 171 treatment:

210. One week = no intestinal inflammation, 100-day = colon microinflammation evidenced by significantly increased IL-1b, IL-8 and TNF-a expression in the colon in addition to increased IL-10. Aberrant crypts were examined in the colon after staining with methylene blue. Data on the effects of TiO2 Nanoparticles on intestinal mucosa were not presented.

211. ACF Definition: The authors did not explicitly give their definition of an aberrant crypt foci (ACF) but the Panel presumed it was 1-or more aberrant crypts/ACF. The authors defined a 'large ACF' as consisting of more than three aberrant crypts per ACF.

Blevins et al. 2019

212. The test material used was E 171, anatase, 110–115 nm (SEM), 36% particles 100 nm. Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure): 3.

213. Internal exposure was not examined.

Method:

214. Six-week-old male Wistar Han IGS rats were exposed to E 171 in a standard diet at 4 concentrations between 0-5,000 mg/kg diet concentration in

two studies of 7 days (n=5/group) and 100 days (n=15/group) (equal to 1.8, 4.8, 31.4, 374 mg/kg bw per day) for 7 and (equal to 1.3, 3.5, 22.4 or 267 mg/kg bw per day) for 100 days. The two studies were performed at different Institutions, with the 7-day study performed twice whereas the 100 day study was performed once.

215. The objectives of the study were to evaluate the acute (7 days) and sub-chronic (100 days) effects of dietary E 171 exposure on the immune system of the GI tract and periphery as well as to evaluate effects of the sub-chronic exposure either alone or after pre-administration of a known intestinal genotoxic carcinogen, DMH and an examination of colon for presence of aberrant crypt foci and of aberrant crypt was included.

216. Immune system metrics: Phenotyping of immune cells (i.e. CD103+DC, total and activated T helper cells, total and activated Treg cells) and inflammatory cytokines [(IL-1a,IL-1b, IL-6, interferonc (IFNc), IL-12p70, IL-17A, IL-18, IL-33. CCL2/MCP-1, CXCL1/KC (IL-8), GM-CSF and tumour necrosis factor a(TNF-a).

217. 7-Day Studies: Rats were randomised into 4 groups of 5 animals and the data from the two studies were pooled. Total food and water consumption were determined at the end of each study. Body weights were determined at the start and end of the 7-day. As calculated by the Panel and taking the mean of the two periods given in the paper: 1.5, 3.9, 25.5, 294 or 1.1, 3, 19, 236 mg/kg bw per day for weeks 1-10 or 11-15, respectively (groups 1–4) and 1.5, 4.1, 25.7, 300 or 1.1, 3.1, 19.2, 237 mg/kg/bw per day for weeks 1–10 or 11–15, respectively (groups 5–8) exposure period at the time of euthanasia.

218. 100-day Study: Animals were randomised into 8 groups of 15 animals each. At the start of the study, animals in groups 5–8 were treated with a single intraperitoneal injection of a sterile dose of 180 mg/kg bw dimethylhydrazine (DMH) dihydrochloride while groups 1–4 were treated with vehicle only. Seven days after intraperitoneal injection, dietary administration of 0, 40, 400 or 5,000 mg/kg diet E 171 was started and continued for 100 days. Bodyweights were determined weekly beginning on day 0 of the study and just prior to euthanasia. Food consumption was determined weekly beginning with administration of the E 171 supplemented diets. Water consumption was determined during weeks 3, 8 and 13 of the study.

Results:

219. No significant changes in food intakes or body weights or liver and spleen weights were found and no mortality was observed. A trend towards increased food consumption in rats of the high E 171 group was observed. Dietary E 171 produced no general signs of overt toxicity at the highest dose tested, over 100 days.

220. Following the 7- and 100-day feeding periods, rats were euthanised and measurements of inflammatory cytokines (using the LEGEND plex rat inflammation Panel) and phenotyping of immune cells (by-flow cytometry) in the periphery and GI tract were performed. Peyer's patches, peripheral blood mononuclear cells (PBMC) and spleen cells were analysed for inflammatory and regulatory T-cell responses directly ex-vivo or after in-vitro stimulation with anti-ratCD3 (5lg/ml) and anti-rat CD28 (5lg/ml) for 4 days. Histopathology, ACF, ABC and goblet cell evaluations were performed on rats in the 100-day study.

221. All tissues were collected from well-defined areas, and measurements, procedures and evaluations were performed in a standardised and blinded manner. CD103+dendritic cells (DC) were evaluated in the gut, Peyer's patches, spleen and in peripheral blood over time period. No change in the percentage of CD103+DC in peripheral blood, spleen or Peyer's patches due to acute or sub-chronic dietary E 171 consumption alone was observed. The total percentage of CD4+T helper cells, the percentage of T helper cells expressing CD25, an indicator of T helper activation, and the percentage of Treg cells (CD4+FoxP3+) and activated Treg (CD4+CD25+FoxP3+) which could lead to a low level inflammatory response in the absence of increased inflammatory cells, were quantified in peripheral blood, spleen and Peyer's patches. Dietary E 171 exposure did not change the frequency of CD4+T helper cells systemically or in intestinal Peyer's patches. In addition, there was no detectable impact on the percentage of activated CD4+T helper cells or on the percentage of Treg cells either peripherally or locally in the Peyer's patches of treated rats fed for 7 or 100 days. Collectively, these results suggest that E 171 consumption does not alter T-cell-mediated mechanisms of immune control, either promoting inflammatory CD4+T helper cell activation or in reducing the percentage of anti-inflammatory Treg cells.

222. Regarding the effects on cytokines, the data presented suggest that dietary E 171 does not induce inflammation peripherally or in the GI tract at both time points. In addition, studies were conducted to explore the possibility that E 171 might alter the effector cytokine profile of T helper cells in lymphoid tissue or circulation, which may not be manifest without T cell-specific stimuli.

Lymphocytes were isolated from peripheral blood, spleen and Peyer's patches and activated ex-vivo with anti-CD3/anti-CD28 for 4 days to induce T helper cell cytokine production. No effects of E 171 exposure on any of the induced cytokines produced from ex-vivo stimulated T helper cells were observed.

223. In the 100-day study, all animals were treated with E 171, some groups were initiated with 180mg/kg bw DMH before the start of the dietary exposure to E 171 and an additional control initiated with DMH was also included. The same parameters as described above were evaluated, with some differences observed. A modest increase in the relative spleen weight in 22.4 mg E 171/kg bw per day + DMH compared to not initiated animals, an increase in IL-17A in colon (22.4 mg E 171/kg bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects, were observed. There were no changes in spleen cellularity across any of the treated groups. No changes were observed in the percentage of CD103+DC, CD4+T helper cells or total or activated.

224. Safety assessment of the food additive titanium dioxide in peripheral blood, spleen or Peyer's patches in animals exposed to E 171+DMH compared to animals treated with only DMH. No treatment related histopathological changes in the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E 171 were found. Rats that were initiated with DMH only and those which received 171 in the diet after the initiation displayed several histopathological abnormalities. There were two invasive adenocarcinomas in one animal in the 1.3 mg E 171/kg bw per day + DMH group, and single adenomas in one animal in the 3.5 mg E 171/kg bw per day E 171+DMH group and in one animal in the 22.4 mg E 171/kg bw per day + DMH group.

225. There were no other histopathological changes in the large intestines of the other animals treated with DMH. One rat in the 1.3 mg E 171/kg bw per day +DMH group and one rat in the 22.4mg E 171/kg bw per day +DMH group had subpleural lymphocytes in the lung, but without any evidence of acute inflammatory changes or hyperplasia.

226. Limitations: A significant amount of the epithelial surface of the sampled colon (proximal, middle and distal) was obscured when observed by light microscopy and so were unable to examine the entire surface of the colon samples. The results for the areas of epithelium that were examined indicated an increase in ACF/cm^2 and ABC/cm^2 in groups initiated with DMH compared to the groups that were not initiated with DMH. E 171 treatment administered after DMH did not result in statistically significant increases in aberrant crypt foci or aberrant crypts or any change in the number of aberrant crypt foci or aberrant crypt were

observed due to E 171 exposure alone.

227. The Panel noted a 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).

228. Dietary E 171, with or without treatment with DMH, had no effect on the length of the colonic glands examined or the number of goblet cells/unit. The Panel considered that this study indicates that acute and sub-chronic dietary intake of E 171 resulted in no significant effects on either peripheral or GI tract immune homeostasis as evidenced by immune cell phenotyping or inflammatory cytokine analysis. Limitations in the pathological examination for ABC and ACF preclude a conclusion on potential for ABC and ACF formation.

Overall EFSA conclusion on ACF

229. The Panel considered that there was uncertainty regarding the extent of the internal exposure to E171 TiO2 nanoparticles across the range of tested doses. The Panel considered that the effect of E 171 in producing ACF reported by Bettini et al. (2017) was not replicated in later investigations (EOGRT study and Blevins et al., 2019).

230. 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 E 171 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.

EFSA’s Concluding remarks

231. No reproductive or developmental toxicity studies performed with E 171 and considered sufficiently reliable with respect to their internal validity were identified from the published literature.

232. 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 TiO2 materials, TiO2 Nanoparticles or TiO2 containing a

fraction of nanoparticles (Warheit et al., 2015a) (Score: 4 for NSC).

233. In mice, the effects of TiO2 nanoparticles 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 TiO2 Nanoparticles /kg bw per day. The lowest dose at which the effects were observed was 50 mg TiO2 Nanoparticles /kg bw per day (Karimi et al., 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 (Score: 4 for NSC). In rats, administration of TiO2 Nanoparticles (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, Score: 3 for NSC).

Literature Search

234. A number of studies available in the literature were assessed by the Panel in addition to the extended one-generation reproduction toxicity (EOGRT) study. No E171 studies were identified in the literature search with reliability scores of 1 or 2. No effects were reported up to a dose of 1,000 mg/kg bw per day for titanium dioxide containing a fraction of nanoparticles, the highest dose tested in the EOGRT study. Several studies using TiO2 Nanoparticles 30 nm were reported. These are detailed below and summarised in Annex B. These were included for additional information and may be relevant with respect to whether a minimum limit for particle size should be included in the EU specifications for E 171 however EFSA considered these of limited relevance to the safety of E171.