Inflammation and Immunotoxicity - Statement on the safety of Titanium Dioxide (E171) as a Food Additive

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Studies using E171 or equivalent form of TiO2

EORGT study (Leuschner, 2020)

217. Details of the design on the EOGRT study are given in paragraphs 122 – 125. 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 were sensitised and the primary IgM antibody response to the sensitising antigen, in this case to keyhole limpet haemocyanin (KLH) antigen, was measured. The ability of the test compound to modulate serum anti-KLH antibody titre would be indicative of a developmental immunotoxic effect. A KLH-immunised group exposed to a known immunosuppressant (i.e., cyclophosphamide (CY)) was used as a positive control.

218. Determination of serum anti KLH-IgM antibodies was performed in F1 cohort 3 animals (10 animals per sex per group, PND 53–61) using an enzyme-linked immunosorbent assay (ELISA). The animals were terminated 5 days after intravenous bolus injection (tail vein) of KLH, blood was withdrawn and the level of anti-KLH IgM was measured in serum.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 animals per sex, PND 55) were immunised with KLH and treated with CY (single administration of 40 mg/kg bw by gavage on the same day as KLH treatment) to provide a positive control (for an inhibition of immune response). A slight, but statistically significant decrease in the antigen specific IgM level was observed at the highest dose tested (1,000 mg/kg bw per day) in males only (– 9%).

219. 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).

220. It was considered that all tested animals in the study had an immunogenic response to KLH but this was very weak and insufficient to identify any T-cell-dependent immunotoxic effect of E171. Therefore, no conclusion could be drawn on the effect of E171 on the developing immune system from these data.

221. The results from this assay should be considered in combination with additional data related to potential immunotoxic effects. In the F1 cohort 1A animals, the following may also 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, as well as the T-cell-dependent anti-KLH response (KLH assay).

222. Pathology of lymphoid organs, haematology and splenic lymphocyte subpopulations were assessed at necropsy. Populations of T cells, T helper cells, T suppressor/cytotoxic cells, natural killer (NK) cells and B cells were determined using fluorescence-activated cell sorting (FACS) flow cytometry 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 controls in both sexes. The study authors concluded that no test substance-related effect was observed on the proportion of the examined lymphocyte subtypes.

223. 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 the study authors 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 E171-treated groups, after immunisation of the animals with KLH.

224. The reason for the B-cell shift in F1 cohort 3 proposed by the study authors was KLH immunisation, supported by the fact that there was no similar shift found for the positive control animals, sensitised to KLH and treated with CY. It was also considered that KLH induced an immune reaction, which was reflected by an increase in the percentage of splenic B cells and a decrease in the percentage of splenic T cells, and that this response was influenced qualitatively by CY as

expected. The study authors noted that although the effect of CY on the antibody response to KLH, was weaker than desirable to demonstrate the sensitivity of the study. The study authors concluded that E171 had no effect on sensitisation to KLH.

Riedle et al., (2020)

225. Riedle *et al.*, (2020) dosed C57BL/6 mice with food grade (E171) TiO2 at doses of 0, 6.25, 62.5, or 625 mg TiO2/kg of diet (equivalent exposures of 0 and ≈1, 10, and 100 mg TiO2/kg body weight per day for 6, 12 or 18 weeks, at which point the GI tracts were harvested and Peyer's patches assessed. This study showed that these diets deliver TiO2 particles to the basal cells of intestinal lymphoid follicles. It also showed that with up to 18 weeks of exposure, food intake, weight gain, and Peyer's patch immune cell profiles do not differ between TiO2-fed groups or controls.

Blevins et al., (2019)

226. To assess gut immunopathology in the Blevins *et al.*, (2019) study as described in paragraphs 159 - 162, the number of CD103+ dendritic cells (DC) in the periphery and in Peyer's patches was quantified. CD103+ DC routinely make up less than 1% of the total leukocyte population in peripheral blood and spleen, and these percentages were not affected by the E171-containing diet following exposure to TiO2 for seven or one hundred days. However, the frequency of CD103+ DC increased modestly in the peripheral blood and spleen of animals in the 100-day study compared to the 7-day study. Pre-treatment with DMH had no effect on CD103+ DC content, with or without E171 consumption. When combining these results, there was no change in the percentage of CD103+ DC in peripheral blood, spleen or Peyer's patches following acute or chronic dietary E171 consumption.

Talamini et al., (2019)

227. Talamini et al., (2019) carried out a study with repeated oral administration to mice of an E171 suspension by an oral drip. Mice were divided into 2 groups of 22 animals/group and dosed with water (vehicle) or E171 suspension (no sonication or deagglomeration) at 5 mg/kg bw over 3 weeks, with dosing for 3 days per week (receiving an average daily dose of ~ 2 mg/kg bw per day). This study focussed on the GI tract as it had previously been suggested that this was one of the main targets of E171-induced biological effects. Deposition of

TiO2 was found in internal organs. including the intestine and liver. and in the digestive tract, as discussed in paragraph 55. Neither overt structural and morphological histological alterations or significant recruitment of monocytes/macrophages were observed in the stomach and whole intestine of E171 fed animals. Liver effects included necro-inflammatory foci with recruitment of tissue macrophages. Additional effects were seen in the stomach and intestine including increased superoxide production, compared to controls. There was no difference in the circulating levels of interleukin (IL)-1b and tumour necrosis factor (TNF)-a concentrations in the intestine with E171 dosing. However, concentrations of IL-6 and SDF-1 were increased 2.2- and 3.1-fold, respectively. Gene expression analysis of pro-inflammatory cytokines showed a significant upregulation of IL-1β in the stomach (65%) and gut (74%) and a non-significant increase in the liver, TNF-α levels were not modified in the stomach and liver but were reduced (60%) in the whole intestine, where a decrease in intercellular adhesion molecule (ICAM)-1 was also observed. Expression levels of cyclooxygenase (COX)-2 were not changed in any of the tissues, ICAM-1 showed no change in the stomach or stromal cell-derived factor (SDF)-1 in the liver. Tissue expression levels of anti-inflammatory cytokine IL-10 showed a significant change only in the liver, with a 40% reduction. (Talamini *et al.*, 2019).

Pinget et al., (2020)

228. Pinget *et al.,* (2020) investigated the impact of food grade TiO2 (E171) dosed via drinking water on gut microbiota of mice. Five- to six-week-old male C57BL/6JAusb mice were allowed access to feed and water ad libitum. The drinking water contained 0 or 2, 10, or 50 mg/kg bw per day sonicated TiO2 E171 for 3 weeks. TiO2 had a low impact on the microbiotal composition in the small intestine/colon. However, TiO2 exposure *in vivo* altered the release of bacterial metabolites and *in vitro* encouraged biofilm formation and altered the spatial distribution of commensal bacteria. Reduced expression of the colonic mucin 2 gene was found with 10 and 50 mg/kg bw per day, which the authors suggested has a detrimental impact on the mucus layer. However, no change was observed in the expression of *Tjp1,* which indicated that there was no impact of TiO2 on gut permeability. Another major mechanism of bacterial exclusion is through the release of antimicrobial peptides. Defb3 (encoding for beta-defensin-3) level was elevated at 10 and 50 mg/kg bw per day. However, expressions of other antimicrobial peptides such as granzyme B, cathelin-related antimicrobial peptide (CRAMP), regenerating islet-derived protein 3 gamma (REG3 gamma) and p-lysozyme (PLYz) were unchanged. Colonic neutrophil and dendritic cell populations were unchanged; however, the numbers of macrophages, CD8+ T

cells and Th17 cells were increased by TiO2 at 10 and 50 mg/kg bw per day. The levels of IL-6, TNF-α, interferon (IFN)-γ, IL-17A and IL-10 were upregulated in the colon of TiO2 treated mice. A significant decrease in crypt length was also observed in the colon. Conversely, neither regulatory T cells (Treg) nor TGF-beta were affected by TiO2 treatment.

Bettini et al., (2017)

229. Bettini et al. dosed rats with E171 TiO2, TiO2 NPs (NM-105) or water via gavage for 7 days (paragraph 63 - 64) or through their drinking water for 100 days (paragraphs 163 - 166). In a third series of experiments, untreated rats (n= 4) were used for ex vivo cytotoxicity and proliferative assays on isolated immune cells.

230. The authors noted that “after 7 days of oral exposure, both NM-105 and E171 induced a significant increase in the dendritic cell frequency in Peyer’s Patches without affecting the spleen at the systemic level”. These early effects on the dendritic cells in the Peyer’s Patches were found to be transient, as they were not detected in rats exposed to E171 for 100 days through drinking water. NM-105 nanomaterial had no effect on Treg cells in the Peyer’s patches after 7 days of oral exposure. However, after 7 days of E171 exposure, a significant decrease in Treg cells was measured. This effect was still observed in Peyer’s patches after 100 days of E171 exposure. The authors suggested that the decreased levels of Tregs observed, together with a decrease in CD4+ CD25+ T helper (Th) cells, indicated a failure of Th cell expansion. No changes were detected in the content of basal cytokines TNF-α, IFN-γ, and IL-17 in the mucosa of the small and large intestine compared to control rats, after 7 days of oral NM-105 and E171 TiO2 dosing.

Han et al., (2020)

231. Male and female SD rats were randomly assigned (10 rats/sex/dose) to one of four groups (0, 10, 100, or 1,000 mg/kg). E171 was administered daily by oral gavage for 90 days in accordance with OECD test guideline 408. The control group received equal volumes of drinking water. From a personal communication, Health Canada reported “particles were dispersed in distilled water by at least 10 minutes of sonication and dose formulations were prepared at least once per week, with homogeneity determined by sampling from the top, middle and bottom of all preparations” (Health Canada, 2022).

232. Following dosing, there was no mortality, and no dose-related changes in body weight, clinical chemistry parameters, organ weights or histopathological endpoints. There was a slight and statistically significant decrease in lymphocyte count of males for the 10 and 1,000 mg/kg bw per day dose groups compared to controls. Compared to control animals, there was a reduction in the levels of GM-CSF in female rats dosed with 1,000 mg/kg bw per day and a decrease in IgM concentrations of both male and female rats (1,000 mg/kg bw per day). However, there is variability in these parameters and no dose response.

Urrutia-Ortega et al., (2016)

233. A study by Urrutia-Ortega et al. (2016) had also been reviewed by the EFSA ANS Panel in 2016, the EFSA FAF Panel in 2021 and Health Canada (2022). Due to the nature of the study, a carcinogen-induced colitis-associated cancer (CAC) model, it was deemed not suitable for use in the risk assessment of TiO2 as a food additive. The COT agreed with this conclusion and this study will therefore not be included in this assessment.

Mortensen et al., (2021)

234. Mortensen et al., (2021) dosed standardised litters of male and female Sprague Dawley rat pups (n = 5 per sex) between PND 7-10. The pups received a daily dose of either 10 mg/kg bw per day of E171 dispersed and dispersed in water, or an equivalent volume of water by oral gavage. Dosing solutions were prepared fresh each day and dispersed using ultrasonication. Four hours after the fourth and last dose had been given on PND 10, one male and one female from each group was terminated and the duodenum, jejunum, ileum, and colon were harvested for histopathology. The remaining pups were terminated on PND 21 and the liver and brain were collected.

235. Enhanced darkfield microscopy with hyperspectral imaging (EDM-HSI) was used to evaluate intestinal uptake of particles. NPs were detected in the lumen, and the gastric mucus and low levels in the underlying epithelial tissue. The majority of NPs were in the lumen. Overall, the percentage of E171 in all areas of intestinal tissues was higher in female pups than in male pups. Analysis with ICP-MS showed no increase in liver Ti concentration.

226. Histopathological analyses included an evaluation of changes in the number of intraepithelial lymphocytes (IEL) and granulocytes in the duodenum and colon. The number of IEL was significantly increased following TiO2 E171 administration in duodenum in both sexes, but not in the colon. However, the

number of granulocytes significantly increased in both duodenum and colon for male and female pups. No sign of active inflammation was observed. The authors concluded that this increase in IEL and granulocytes demonstrates that NPs leads to the recruitment of immune cells in young rats, with the strongest effect observed in the small intestine.

237. A three-phase (bland, transitional and acidic) in vitro digestion model was used to investigate the stability of E171 during simulated digestion. DLS measurements of E171 showed increased hydrodynamic diameter and polydispersity index during simulated digestion, which was confirmed by SEM.

Warheit, Brown and Donner, (2015)

238. Warheit, Brown and Donner (2015) reported the outcomes of two repeated-dose studies of TiO2 in rats as described in paragraph 173. In the 90-day study, TiO2 particles were detected within the digestive tract, draining lymph tissues and the nose, although there was no evidence of an adverse tissue response. In the 28-day study TiO2 particles were observed in intestinal lymphoid tissue. However, no treatment-related adverse effects on any endpoints were observed.

Studies using the nanoparticle form of TiO2

Huang et al., (2017)

239. Huang *et al.*, (2017) treated Raw264.7 and J774a.1 cell lines and primary mouse macrophages with TiO2 NPs with primary diameters of 10 nm (NP10) or 50 nm (NP50) at concentrations of 0.1, 1, or 10 µg/mL and incubated for 48 h. C57BL/6 wild type and C57BL/6 TLR4-knockout ($TLR4^{-/-}$) mice were fed standard laboratory diet or diet containing 0.1% TiO2 NP10 or NP50 for 1 month. Experiments for macrophage chemotaxis, phagocytosis and bactericidal activity, nitric oxide production and mouse cytokine analysis were carried out in both the macrophage cell lines and macrophages from the mice. A lipopolysaccharide (LPS) septic shock model in wildtype and TLR4-knockout mice and cecal ligation and puncture procedure to induce TLR4-unspecific septic shock, in wildtype mice, were also used.

240. After a month of dietary exposure to TiO2 NPs in wildtype mice, no changes in bodyweight were observed. NP10 and NP50 TiO2 led to an elevation in the levels of pro-inflammatory IL-1β, IL-6, IL-12a, TNF-α, and nitric oxide

synthase-2 (Nos2). Conversely, anti-inflammatory factors IL-4 and IL-10 were reduced in groups exposed to TiO2 NPs. These mice showed an M1-like pro-inflammatory activation state. Mice exposed to NP10 or NP50 showed a reduction in TG4-induced macrophages compared to the control group. The data also suggest that the phagocytic activity of the macrophages exposed to TiO2 NPs was inhibited. These findings indicate that exposure to TiO2 NPs could disrupt immune homeostasis and aggravate the inflammatory responses to an external stimulus such as LPS. After a month of dietary exposure to low doses of TiO2 NPs, septic shock following LPS challenge was aggravated with increased levels of inflammatory cytokines in serum and reduced overall survival. TiO2 had little or no effect in TLR4-knockout mice, nor did it affect that lack of response to LPS in these animals. TiO2 did not exacerbate non- TLR4-dependent septic shock induced in wildtype mice.

241. Following exposure *in vitro* to TiO2 NPs (10 and 50 nm), the expressions of pro-inflammatory genes in macrophages were increased, and the expressions of anti-inflammatory genes were decreased. In addition, for macrophages exposed to TiO2 NPs in vitro and in vivo, their chemotactic, phagocytic, and bactericidal activities were lower.

242. The authors concluded that “These results demonstrate that TiO2 NPs induce an abnormal state of macrophages characterized by excessive inflammation and suppressed innate immune function in a TLR4-dependent manner, which may suggest a potential health risk, particularly for those with additional complications, such as bacterial infections”. Huang et al., (2017).

Kampfer et al., (2021)

243. Kampfer et al., (2021) exposed male and female mice C57BL6/J to P25 TiO2-NPs via the diet. The particles were incorporated into pelleted diet at either 0.2% or 1% TiO2 by mass, (estimated doses of 400 or 2000 mg/kg bw per day, respectively). Mice (n = 10 per sex per group) were exposed to a control diet or P25 TiO2-enriched diets for 28 days. Following exposure, intestinal tissue was harvested and analysed for DNA damage via the alkaline comet assay using freshly isolated colonocytes, as well as for markers of inflammation and gene expression analysis. None of the investigated genes related to DNA repair and oxidative stress or inflammation were significantly changed in their expression. The authors concluded there is an absence of major local adverse effects in the intestines of mice following repeated exposure to TiO2-NPs via the diet.

Akagi et al., (2023)

244. The COT also reviewed a paper by Akagi *et al*. (2023) (methods described in paragraph 174) which was not included in the review by Health Canada. In a 28-day subacute oral toxicity study, the authors stated that "hematological analysis… showed that a significant decrease in white blood cell count (WBC) was observed in the 10 mg/kg bw per day group of males" (Akagi et al., 2023).

245. In the 90-day subchronic oral toxicity study also reported by these authors, it was stated that "Hematological analysis showed significant changes in leukocyte fractions (increase in neutrophils, monocytes, and basophils, and a decrease in lymphocytes) in the male 1000 mg/kg bw per day group. However, no differences were observed in the WBC count and absolute numbers of each leukocyte type. A significant decrease in mean corpuscular hemoglobin and an increase in the absolute number of eosinophils were observed in the female 1000 mg/kg bw per day group. A significant decrease in the fraction and absolute number of basophils was observed".

246. However, in the discussion it was noted "The significant decrease in MCH observed in the 1000 mg/kg bw per day group of females in both the 28-and 90-day studies was considered of little toxicological significance because the change was minor, and there were no changes in the other erythrocyte markers suggestive of anemia in red blood cell count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC)" (Akagi *et al.*, 2023).

247. It was also noted "In the 90-day study the significant changes in leukocyte fractions in the 1000 mg/kg bw per day group of males were of little toxicological significance because no significant differences in total leukocyte counts and absolute numbers of each leukocyte type were observed. The significant increase in the absolute number of eosinophils observed in the 1000 mg/kg bw per day group of females was considered to have no toxicological significance, as it was a minor change and no fractional changes were observed." (Akagi *et al.*, 2023).

248. With respect to induction of inflammation, in the discussion it was noted that "Notably, no reactive changes such as inflammatory reactions or tissue injury were observed at the site of deposition, which is consistent with previous findings, suggesting that there were no adverse effects on the organs." (Akagi *et al.*, 2023).

EFSA (2021) review and conclusions on the immunotoxicity of TiO2

249. EFSA did not review immunotoxicity, other than in the EOGRT study, in detail in 2021. The EFSA FAF Panel had noted that in the KLH assay, the treatment with CY was not performed at the same time as the rest of the cohort, and there was no separate control for the CY response conducted at the same time. They therefore considered the positive results invalid and the sensitivity of the test, therefore not demonstrated. The EFSA FAF Panel did not agree with the conclusion of the study authors (Leuschner, 2020) 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.”

250. The study authors suggested that even if the positive CY control did not perform as expected, the data still indicated there was no effect of E 171 on sensitisation to KLH. However, the Panel did not agree with this conclusion. Overall, the EFSA Panel considered that the data did not allow them to conclude on developmental immunotoxicity with respect to E171.

251. The EFSA FAF Panel noted that there were methodological shortcomings in the design of this part (developmental immunotoxicity) of the EOGRT study. Therefore, the Panel could not conclude on immunotoxicity.

252. The Panel concluded overall that “observations of potential immunotoxicity and inflammation with E 171…. may indicate adverse effects”.

Health Canada review and conclusions on the immunotoxicity of TiO2

253. The summary provided by Health Canada in their report on the State of the Science of Titanium Dioxide as a Food Additive states, “The evidence suggests that TiO2 particles when well dispersed in simple matrices can produce inflammation and immunological perturbations and may alter gut microbiota and metabolism in ways that may potentially be adverse. In studies using food-grade

TiO2 specifically, some effects on inflammation and immune dysregulation were observed when the substance was administered in water in stable dispersions; however, these findings could not be replicated when TiO2 was administered via the dietary route. Therefore, it is concluded that concerns that food-grade TiO2 may produce inflammation or immunotoxicity appear to a great extent contingent on the oral dosing paradigm. Liu *et al.*, (2020) have pointed out that specific absorption of biomolecules (i.e., the particle corona) can also alter the immunological identity of particles and may either enhance or diminish their immunogenicity. Studies in humans have consistently demonstrated accumulation of pigment including TiO2 in macrophages in the base of Peyer's patches of the terminal ileum but not elsewhere in the GIT. However, no association between the presence of particles and immune activation or pathological state has been observed. Further information is required in order to investigate the potential mitigating effects of the food matrix on local toxicity in the GIT to determine whether studies involving stably dispersed food-grade TiO2 in simple matrices are relevant to the hazard characterization of this substance when used as a food additive."

254. In their overall summary the Health Canada conclusions on inflammation and immunotoxicity stated "No consistent evidence of inflammation or immunotoxicity in the GIT of rodents exposed to food-grade TiO2 via the oral route. While a few non-guideline studies suggest food-grade TiO2 when administered in water may produce inflammation or immune dysregulation in male mice and rats at doses up to 50 mg/kg bw/d (e.g. Pinget et al., 2020; Bettini et al., 2017, Talamini et al., 2019), these findings were not observed when food-grade TiO2 was administered in the diet in a non-guideline study in male rats at doses up to ~236 - 300 mg/kg bw/d (Blevins et al., 2019), in male and female mice at doses up to 100 mg/kg bw/d for 18 weeks (Riedle et al., 2020), in a GLP- and OECD-guideline-compliant EOGRT study in male and female rats at doses up to 1000 mg/kg bw/d (Leuschner, 2020), or a two-year chronic bioassay with a form of TiO2 highly comparable to the form of TiO2 added to food at concentrations up to 5% w/w in male and female mice and rats (NCI, 1979). In addition, no treatment-related histopathological abnormalities were observed in the spleen, thymus, lymph nodes and bone marrow and no abnormal hematological findings were reported for any immune-related parameters in the EOGRT (Leuschner, 2020) or chronic bioassay (NCI, 1979). Similarly, no treatment-related changes in hematology or gross or histopathological abnormalities in lymphoid organs were observed in rats following the gavage administration of food-grade TiO2 dispersed in water at doses up to 1000 mg/kg for 90 days in another OECD guideline-compliant study (Han et al. 2020)."

FSANZ review and conclusions on the immunotoxicity of TiO2

255. FSANZ identified three studies which fulfilled the criteria for assessing immunotoxicity (two used food-grade TiO2 administered by the diet and one sonicated food-grade TiO2 by gavage, at doses up to 1000 mg/kg bw per day for 90 days (Blevins et al., 2019 and Riedle et al., 2020, and the EOGRT study (Leuschner 2020)). These studies found no adverse immunotoxic effects in rodents (mice or rats).

JECFA 2024 review and conclusions on the immunotoxicity of TiO2

256. JECFA had reviewed studies by Talamini et al (2019), Bettini et al (2017) and Blevins et al (2019). They noted that for the Talamini study some inflammatory biomarkers were altered but were probably the result of an adaptive change rather than a toxic response. No changes were noted in myeloperoxidase activity or in the content of basal cytokines in the mucosa of the small and large intestine, relative to control animals in the Bettini et al study. In the study by Blevins et al, E171 caused no adverse effects on any tissue histopathology or immune parameters up to the highest doses for 7 and 100 days.

COT review and conclusions on the immunotoxicity of TiO2

257. The COT noted that with regard to the dietary dosing paradigm, only three studies (Riedle et al., 2020; Blevins et al., 2019; and Leuschner, 2020) use E171 TiO2. These studies showed that no adverse effects on inflammation or immunotoxicity were observed when E171 was administered by this route.

258. Five studies using food grade TiO2 in water (Talamini et al., 2019; Pinget et al., 2020; Bettini et al., 2017; Han et al., 2020; and Mortensen et al., 2021) were considered by the COT. These studies in mice and rats yielded mixed findings. In several studies, differential cytokine and host defence gene expression was observed but was neither consistent across studies, nor ubiquitous in terms of pathway activation (for example, one antimicrobial defence gene *Defb3* was increased, but others including cathelin-related antimicrobial peptide (CRAMP)

and lysozyme were not). Non-dose dependent increases in expression of IL-10, TNF and IL-6 were observed by Pinget et al, but in Talamini et al., a significant increase in proinflammatory cytokine IL-1β mRNA transcripts was reported in stomach (~65%) and whole intestine tissues (~75%), but not liver tissues. TNF-α levels were significantly different (a reduction of 60%) only in the intestine. A statistically significant reduction in liver anti-inflammatory cytokine IL-10 mRNA transcript expression was observed (~40%). It was also noted that Bettini et al. did not observe any changes in the content of basal cytokines TNF-α, IFN-γ, and IL-17 in the mucosa of the small and large intestine compared to control rats, after 7 days of TiO2 dosing. It is suggested therefore that tissue specificity and differential cytokine expression may play a role in the effects of TiO2.

259. It is important to note that cytokine mRNA or protein measurement was performed in a variety of tissues or cell types that was not consistent across studies and thus considering this as an additional variable will likely be of value in the context of assessing immunotoxicity.

260. The available data suggest that food grade E171 and TiO2-NPs, delivered either in water or by gavage, could have the potential to induce inflammogenic responses. This is likely species, tissue- and cell-dependent, and it is suggested that ROS or oxidative stress may be key in the initiation of inflammation. The oral dosing regimen is also a key factor. However, the Committee highlighted the inconsistency in findings across the studies in this area, making interpretation or formulation of conclusive statements challenging.

261. Various immune targets have been identified in the context of TiO2 immunotoxicity. These include, but are not limited to: induction of immune cell mediated inflammation in the gut, including in Peyer’s patches, as well as in the spleen and via peripheral blood mononuclear cells; effects on broader host defence mechanisms, including antimicrobial peptides; effects in the gut microbiota; effects on dendritic cell populations in the gut; effects on T cell subpopulations and macrophage populations in the gut; effects on plasma lymphocyte counts and proportions; and disruption of the mucus layer in the gut.

262. Broadly, there are a number of studies, in which immune cell activation, alteration, change in number or suppression of normal function has been observed, although this is highly inconsistent between studies, and appears to be affected by the nature of exposure and the form of TiO2 used. The quality of data available and the use of appropriate controls in some studies therefore impacts consensus interpretation of the data and the toxicological significance of some of the findings.

263. Overall, the weight of evidence is that food grade TiO2 in the diet is not of concern with regards to immunotoxicity and inflammation. However, this is based on limited evidence, much of it of poor quality, and hence there is appreciable uncertainty in this conclusion..