Fifth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive- COM review and conclusions

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COM review and conclusions

186. The COM have provided conclusions on the in vitro and the in vivo studies separately and are presented below.

COM Opinion of the in vitro genotoxicity studies reviewed

187.“After reviewing the in vitro genotoxicity studies performed to date on TiO2, we note the following points:

i: There were four in vitro studies of the highest quality (labelled “green” here) that used TiO2 nanoparticles of different sizes and forms in the micronucleus assay. Only one study tested micro-sized (anatase)TiO2 that was more representative of E171 (Demir et al. 2015) which was negative in the micronucleus assay. All four “green” studies that used anatase TiO2 nanoparticles reported negative results for the MN endpoint. Of the two green studies that used rutile TiO2 nanoparticles, one was negative and the other was weakly positive for MN induction in a non-standard cell line but only at the two lowest doses used (1 and 5 mg/ml) (Di Bucchianico et al 2017). Two green studies used TiO2 nanoparticles of mixed anatase/rutile form and both were negative for MN induction.

ii: There were two green studies that both used anatase/rutile TiO2 nanoparticles in either the hprt gene mutation assay or CA assay. The TiO2 nanoparticles were negative in the hprt assay. In the CA assay, the TiO2 nanoparticles were positive, but the CA frequency decreased with increasing TiO2 concentration, and despite

the significant induction of CA, this study was negative with the micronucleus assay.

iii: There were eight amber studies (i.e., ones that contained some suboptimal aspects) that used TiO2 nanoparticles of different sizes and forms in the micronucleus assay. Four studies used anatase TiO2 nanoparticles and three of these were negative for micronuclei induction. The one positive study reported a dose-dependent increase in micronuclei induction in lymphocytes from healthy individuals. All three studies that used nanoparticles of mixed anatase/rutile TiO2 were negative for micronuclei induction. Two studies that used anatase/brookite TiO2 nanoparticles reported positive results for micronuclei induction.

iv: The one amber study on hprt mutations was positive at low anatase TiO2 nanoparticle doses but not at higher doses (Vital et al. 2022).

v: Some “green” studies included other assays (e.g. Comet assay) to provide mechanistic information but results were inconsistent, showing either no increase (Demir et al., 2015), or an increase in oxidative DNA damage (Di Bucchianico et al., 2017) but only at the highest dose (Unal et al., 2021). Andreoli et al., 2018 and Stoccoro et al., 2017 showed ROS involvement.

188. Overall, the COM opinion is that there is little evidence that TiO2 micro-sized or nanoparticles are genotoxic in vitro based on data from well conducted studies. The limited number of positive studies all report no dose-response effects, with significant effects being observed at the lowest doses used, although it is acknowledged this may be due to differences in dispersion and agglomeration at low and high doses. There is also a lack of replication of study outcomes using the same nanoparticle in different labs.

189. Currently a definitive assessment of the safety of food grade E171 is difficult when there are no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. With the exception of one study, the studies identified in this report are not representative of E171, where the fraction of nanoparticulate is 50% and according to the recent "Guidance on the implementation of the Commission Recommendation 2022/C 229/01 on the definition of nanomaterial" (https://data.europa.eu/doi/10.2760/143118), E171 would not fall under the definition of a NM, hence we need GLP studies with E171 that also include robust physicochemical characterisation and nano-specific adaptations to the TG protocol to definitively assess the hazard. Nanoparticles of TiO2 are considered worst-case scenario for E171, as E171 is anticipated to be

less reactive.

190. We also note that there is a dearth of high-quality datasets available with well documented nanomaterial characteristics where the relevant OECD test guidelines (using suitably adapted protocol designs for the testing of nanomaterials) have been followed." (COM, 2024a).

COM Opinion of the in vivo genotoxicity studies reviewed

191. "After reviewing the *in vivo* genotoxicity studies performed to date (up to 2023) on TiO2, we note the following points:

i: The highest quality *in vivo* studies labelled here as "green" (n=2), both show negative results for the micronucleus endpoint (Donner et al., 2016; Sadiq et al., 2012). There were no "green" studies for other endpoints.

ii: Only Donner et al., (2016) used pigment grade TiO2 (including micro-sized anatase that was most similar to E171) and therefore was most relevant to the concern for human health in this case. This study showed no micronucleus induction.

iii: The Donner et al., (2016) paper also used a physiologically relevant oral route, which is most appropriate for the assessment of dietary exposure of food grade TiO2.The authors acknowledge that absorption from the GI tract is low, meaning poor bone marrow exposure. This is important for risk assessment purposes where the oral bioavailability of E171 in humans is very low (≤0.0013% - refer to COT opinion/)

iv: The Sadiq et al., (2012) study, that used an i.v. route (a route that is most likely to achieve bone marrow exposure), also showed a negative micronucleus response and confirmed bone marrow exposure to titanium.

v: The studies labelled as "amber" (i.e., contained some suboptimal aspects) showed a mixture of positive (4/9) and negative (5/9) results for the genotoxicity endpoints studied.

vi: The positive studies included chromosomal and DNA damage endpoints and were all associated with cytotoxicity and/or indirect mechanisms of genotoxicity, such as oxidative damage and inflammation. There was no evidence of gene mutations, however no definitive conclusion can be made due to the deficiencies

in the study designs and limited number of available studies.

vii: The route of administration of nano-sized TiO2 in these “amber” studies was often not via the most relevant oral route (only 2/9 studies) when considering the use of E171 as a food grade material. The less relevant endotracheal route was employed in 3/9 studies and the i.v. route and i.p. route were employed in 3/9 and 1/9 studies, respectively. Often the dosing regimens employed in these studies were suboptimal and did not follow the recommendations of the OECD test guidelines, which also makes interpretation difficult.

viii: All these “amber” studies used a nano-sized TiO2 material which is less relevant to the E171 material.

192. Overall, we conclude that there is little evidence in the literature to suggest that there is a health concern related to genotoxicity induction by TiO2, particularly via the oral route and especially the micro sized TiO2 fraction (most studies used the nano-sized material).

193. Currently a definitive assessment of the safety of food grade E171 is difficult when there are no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. We also note that there is a dearth of high-quality data sets that are OECD compliant and this has led to a lot of conflicting data and uncertainty in the risk assessment for TiO2.” (COM, 2024b).

COT review and conclusions

194. The COT agree with the COM conclusions.

Inflammation and Immunotoxicity

Studies using E171 or equivalent form of TiO2

LPT (2020)

195. The immunotoxicology results of the EOGRT (LPT, 2020) are described in the EOGRT summary section (paragraphs 123 – 133).

Riedle et al., (2020)

196. 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 the TiO2 particles to the basal cells of intestinal lymphoid follicles. It also showed that 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)

197. To assess gut immunopathology in the Blevins *et al.*, (2019) study as described in paragraphs 149 - 152, 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 due to acute or chronic dietary E171 consumption.

Talamini et al., (2019)

198. 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 dosed for 3 days per week (receiving an average daily dose of ~ 2 mg/kg bw/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 50. 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 tissue macrophages recruitment. 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 IL-1b and 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 ICAM-1 was also observed. Expression levels of COX-2 were not changed in any of the tissues, ICAM-1 showed no change in the stomach and SDF-1 in the liver. Tissue expression levels of anti-inflammatory cytokine IL-10 was only significantly modified in the liver with a 40% reduction. (Talamini *et al.*, 2019).

Pinget et al., (2020)

199. 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/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 for 10 and 50 mg/kg bw/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) was elevated at 10 and 50 mg/kg bw/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/day. The levels of IL-6, TNF-α, 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)

200. Bettini et al. dosed rats with E171 TiO2, TiO2 NPs (NM-105) or water via gavage for 7 days (paragraph 58 - 59) or through their drinking water for 100 days (paragraphs 153 - 156). In a third series of experiments, untreated rats (n= 4) were used for ex vivo cytotoxicity and proliferative assays on isolated immune cells.

201. 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 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, E171 led to a significant decrease in this cell subset. This effect was still observed in Peyer's patches after 100 days of exposure. The authors noted that decreased levels of Tregs appeared along with a decrease in CD4+ CD25+ T helper (Th) cells, which the authors suggest indicates 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 TiO_2 dosing.

Han et al., (2020)

202. "The 90-day oral repeated dose toxicity study of E171 was also investigated in male and female SD rats in a study conducted according to OECD TG 408 (Han et al. 2020). Animals (10 per sex per group) received E171 by oral gavage at doses of 0, 10, 100 or 1000 mg/kg bw/d for 90 d. 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 (Dr. Seokjoo Yoon, pers. comm. 09 September 2021). No mortality or effects on body weight, clinical chemistry, urinalysis, organ weights or gross or histopathological endpoints were observed. Gene profile analysis indicated that changes in immune-response associated microRNAs were associated with E171 exposure. A slight but statistically significant decrease in relative lymphocyte count (~8%) was observed in high and low dose males and alterations in granulocyte-macrophage colony-stimulating factor (GM-CSF) in females and plasma IgM (both sexes) were observed at the highest dose tested, but given the lack of a doseresponse and the natural variability in these parameters, it is unclear if these changes can be considered adverse; notably, the EFSA FAF Panel (2021a) considered the highest dose tested in this study to be the NOAEL." (HC, 2020)

Urrutia-Ortega et al., (2016) (Study summary to be deleted)

203. 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) and 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 TiO_2 as

a food additive. The COT agreed with these conclusions and this study will therefore not be included in this assessment.

Mortensen et al., (2021)

204. “The effects of oral exposure to food-grade TiO2 on inflammation and immunity in the GIT of male and female rat pups was investigated by Mortensen et al. (2021). Lactating Sprague Dawley rats with standardized litters of five male and five female pups at PND 2-3 were acclimated for 4-5 days prior to dosing. Between PND 7-10, pups received a daily dose of either 10 mg/kg bw/d of E171 (purchased from Pronto Foods Co., Chicago, IL, USA) dispersed in water or an equivalent volume of water by oral gavage (three litters per dose group for a total of 15 male and 15 female pups per group). Particles were dispersed by ultrasonication in deionized water and DLS was used to measure the hydrodynamic diameter every 2- 5 minutes until the change in diameter was less than 5% (the critical delivered energy was determined to be 1690 J/ml); dosing solutions were prepared fresh each day. One male and one female from each group was sacrificed 4 h after the fourth and last dose on PND 10 and the duodenum, jejunum, ileum, and colon were harvested for histopathology. The remaining pups were sacrificed on PND 21 and the liver and brain were collected. The study also included a three-phase in vitro digestion model 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. Enhanced darkfield microscopy with hyperspectral imaging (EDM-HSI) was used to evaluate intestinal uptake of particles. The majority of particles were present in the lumen, although some particles were also detected in the gastric mucus and a smaller number still in the underlying epithelial tissue; the percentage of E171 in all areas of intestinal tissues was higher in female pups than in male pups. There was no increase in liver Ti concentration based on ICP-MS. Histopathological analyses included an evaluation of changes in the number of intraepithelial lymphocytes (IEL) and granulocytes in the duodenum and colon. Following E171 administration, a significant increase in the number of IEL was observed in the duodenum but not the colon in both sexes, while the number of granulocytes increased in both the duodenum and the colon. No sign of active inflammation was observed. The authors concluded that oral exposure to E171 well dispersed in water leads to the recruitment of immune cells in young rats, with the strongest effect observed in the small intestine, although whether this early life exposure is associated with long-term effects on intestinal homeostasis is unknown.” (HC, 2022).

Warheit, Brown and Donner, (2015)

205. Warheit, Brown and Donner (2015) reported the outcomes of two repeated-dose studies of TiO2 in rats as described in paragraph 165. 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.

Duan et al., (2021)

206. “Duan et al. (2021) exposed male ICR mice (n = 6 per group) to two different forms of TiO2 via the diet for 1, 3 or 6 months. The particles are described as “food-grade” and were incorporated into a commercial pelleted diet at a concentration of 1% by mass; control animals received the basal diet (Ti content not reported). Based on JECFA conversion factors (JECFA 2000), a concentration of 1% w/w in food is equivalent to a dose of 1500 mg/kg bw/d in mice. The first particle type was a nano-sized anatase form with an average primary particle diameter of 38.3 ± 9.3 nm. The second particle type was a micro-sized rutile form with an average primary particle diameter of 128.0 ± 33.4 nm and both particle types were reported to be nearly spherical in shape based on TEM imaging. After receiving control or TiO2-mixed feed for one, three or six months, animals were euthanized and blood and organs (liver, spleen, and kidney) were collected for histological and elemental analyses. Ti content in blood was higher in animals fed a TiO2-containing diet for 6 months than those fed for 1 or 3 months, although the Ti content of organs was not significantly different from controls. The authors evaluated the influence of TiO2 exposure on the homeostasis of 22 trace elements in blood, liver, kidney and spleen (17 of which were above the LOD). The results are reported as ratios of these elements relative to controls and a number of minor alterations were observed that are of unclear toxicological significance. Moreover, the number of statistical comparisons was large (4 tissues x 17 elements x 3 dosing conditions x 3 time points = 612 comparisons) and the authors do not appear to have controlled for the familywise error rate (i.e., Bonferroni correction or similar); with an alpha level for significance of 0.05, the approximately 18 statistically significant observations reported does not exceed that which would be expected based on chance alone. Histopathological examination revealed that after six months of feeding, mice exposed to the micro-sized particles showed occasional protein casts in kidney canaliculus but no significant effects in liver or spleen. Mice exposed to the nanoform showed some liver pathology, described as punctate cell necrosis and infiltration of

mononuclear lymphocytes, as well as macrophages engulfing particle aggregates in the spleen, the latter of which could be viewed as an adaptive response. It should be noted that the equivalent dose on a mass basis would result in far greater exposure on a particle number basis to those animals receiving the nanoform. As only one dose was tested, no indications of a dose-response could be gleaned from this study." (HC, 2022).

Studies using the nanoparticle form of TiO2

Huang et al., (2017)

207. 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 ($TLR4^{-/-}$) 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 and cecal ligation and puncture procedure was performed on the mice.

208. After a month of dietary exposure to TiO2 NPs in 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 and following LPS challenge, an aggravated septic shock occurred which caused increased levels of inflammatory cytokines in serum and reduced overall survival. The authors stated 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).

209. For 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. In mice, after a month of dietary exposure to low doses of TiO2 NPs, an aggravated septic shock occurred in response to lipopolysaccharide challenge, leading to elevated levels of inflammatory cytokines in serum and reduced overall survival. Moreover, TLR4-deficient mice and primary macrophages, or TLR4-independent stimuli, showed less response to TiO2 NPs.

Kampfer et al., (2021)

210. “Kampfer and colleagues (2021) also exposed male and female mice C57BL6/J to TiO2-NPs via the diet in order to evaluate effects on intestinal tissue. The particles were P25 (a mixed phase catalytic material consisting of spherical particles ~85% anatase/15% rutile, average primary diameter 21 nm) and were incorporated into pelleted diet at either 0.2% or 1% TiO2 by mass, corresponding to an estimated dose of 400 or 2000 mg/kg bw/d. Mice (n = 10 per sex per group) were exposed to a control diet (basal Ti content not stated) or one of the two TiO2-enriched diets for 28 days. Following exposure, intestinal tissue was harvested and analyzed for DNA damage via the alkaline comet assay in freshly isolated colonocytes as well as for markers of inflammation and gene expression analysis. No increase in DNA damage was observed in animals exposed to TiO2-NPs, there was no enhancement in proinflammatory cytokines and none of the investigated genes related to DNA repair, oxidative stress or inflammation were differentially expressed. 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. They note that incorporating TiO2-NPs in the feed allows for a highly realistic manner of exposure that mimics human exposure via the diet, although the results may not be consistent with studies that employ bolus dosing by gavage.” (HC, 2022).

Gao et al., (2020)

211. Gao and colleagues (2020) exposed male Sprague Dawley rats (n = 10 per group) to two types of uncoated anatase TiO2, a nano-sized form or a micro-sized form, intended to represent the two fractions of food-grade TiO2. The nano-sized particles had a mean primary particle diameter of 24 ± 5 nm, whereas the micro-sized particles averaged 120 ± 30 nm in diameter. The particles were dispersed in water by ultrasonication and administered via oral gavage at doses of 2, 10, or 50 mg/kg bw/d for 30 consecutive days; control rats were treated with an equal

volume of ultrapure water. Following exposure for 30 days, rats were fasted overnight and anesthetized in order to examine the influence of TiO2 particles on nutrient absorption and metabolism through an in situ intestinal loop experiment. Blood samples were collected at baseline as well as at 1 and 2 h following the introduction of a mixed nutrient solution to the closed intestinal loop. Following the in situ closed-loop experiment, animals were euthanized for histopathological examination of the small intestine and the ultrastructure of the small intestine mucosa was further analyzed by TEM. Sparse and short microvilli and inflammation of the small intestine was observed in the 50 mg/kg bw/d groups exposed to either nano- or micro-sized particles, although the effects were more pronounced in the nano-sized group. In the intestinal absorption experiment, six amino acids (Thr, Met, Val, His, Lys, and Trp), three metal elements (Mg, Mn, Zn), and glucose were injected into the in situ intestinal loop. Some inhibition of added histidine absorption was observed, particularly in the groups exposed to TiO2-NPs, although there was no clear indication of a dose-response relationship. There was no influence on the remaining amino acids, glucose levels or metal elements. Ti levels were elevated in blood after exposure to TiO2-NPs but not microscale particles.

Akagi et al., (2023)

212. The COT sub-group also reviewed a paper by Akagi *et al*. (2023) (methods described in paragraph 164) 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/day group of males” (Akagi et al., 2023).

213. 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/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/day group. A significant decrease in the fraction and absolute number of basophils was observed”.

214. However, in the discussion it was noted “The significant decrease in MCH observed in the 1000 mg/kg bw/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).

215. It was also noted "In the 90-day study the significant changes in leukocyte fractions in the 1000 mg/kg bw/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/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).

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

216. With regards to other endpoints the EFSA Panel discussed findings observing immunotoxicity and inflammation with E171 as well as neurotoxicity with TiO2 NPs may be indicative of adverse effects.

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

Health Canada review and conclusions

218. 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."

219. 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 (LPT 2020 as cited in EFSA 2021a), 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 (LPT 2020 as cited in EFSA 2021a) 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

220. FSANZ identified three studies which fulfilled the criteria for assessing immuntoxicity (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/day for 90 days (Blevins et al., 2019 and Riedle et al., 2020, and the EOGRT study (LPT 2020). These studies found no adverse developmental immunotoxic effects in rodents (mice or rats).

COT review and conclusions

221. The COT sub-group noted that in reflection of the dietary dosing paradigm, only the three studies (Riedle et al., 2020; Blevins et al., 2019; and LPT, 2020) use E171 TiO2. These studies showed that no adverse effects on inflammation or immunotoxicity were observed when using an E171 dietary dosing approach.

222. 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 exposures of mice and rats to food grade TiO2 in water has 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 were reported in stomach (~65%) and whole intestine tissues (~75%), but not liver tissues. TNF-α levels were only significantly different (a reduction of 60%) 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.

223. It is important to note that cytokine mRNA or protein measurement can take place in a variety of tissues or cell types that are not consistent across studies and thus considering these as an additional variable will likely be of value in the context of immunotoxicity.

224. The available data suggest that food grade E171 and TiO2-NPs, delivered either by dietary exposure, or in water, or dispersant, 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 paradigm, is also a key factor. However, the sub-group highlighted an inconsistency in the findings of the studies in this area, making interpretation or formulation of conclusive statements challenging.

225.Various immune toxicity targets have been identified in the context of TiO2 immunotoxicity, that are distinct from the induction or modulation of inflammation. 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.

226. 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.

227.Overall the weight of evidence for the food grade TiO2 is not sufficient or of sufficient quality to arrive at a consensus that food grade TiO2 is of concern with regards to immunotoxicity and inflammation.

Reproductive and Developmental Toxicity

Studies using E171 or equivalent form of TiO2

LPT., (2020)

228. The Extended One Generation Reproductive Toxicity (EOGRT) study is described in paragraphs 113 - 122. The reproductive and developmental effects from this study are described within those paragraphs.