Aberrant Crypt Foci (ACF) as a potential biomarker for carcinogenicity

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154. ACF are a potential biomarker for colorectal cancer. Genetic changes in the malignant transformation process of colorectal mucosa lead to either inactivation or activation of specific target genes. A number of biomarkers associated with genetic changes have been identified for early detection of colorectal cancer including identification of ACF as an early pre-invasive lesion and its relationship to the development of cancer (Cheng and Lai., 2003). The number of ACF were found to be significantly higher in patients with colon cancer compared to those without, but have been found in healthy patients as well (Health Canada, 2022). Most ACF do not appear to develop into neoplasms and there is evidence to suggest that dysplastic changes in ACF are a better predictor of cancer than their just their presence (Clapper et al., 2020). Most preclinical studies do not distinguish between hyperplastic and dysplastic changes in ACF.

Studies using the E171 form of TiO2 (in rodents)

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

EOGRT study (Leuschner, 2020)

156. Details of the design on this study are given in paragraphs 122 – 125. 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 E171/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 into 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.

157. The definition of ACF used by the study pathologist was ‘foci containing more than 2 aberrant crypts (ABCs)’, given by 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 4 & 5 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.

Table 4: Aberrant Crypt Foci Presence in Satellite F0 Animals.

Aberrant Crypt Foci Present.

Dosage Group	Control	Low-Dose	Mid-Dose	High-Dose
Females	1/10	0/10	1/10	2/10
Males	1/10	0/10	1/10	1/10

158. 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 5).

Table 5: Aberrant Crypt Foci Presence in the Re-Examination of Satellite F0 Animals.

Aberrant Crypt Foci Present.

Dosage Group	Control	High-Dose
Females	4/5	3/3
Males	4/4	3/5

Blevins et al., (2019)

159. The Blevins *et al*., (2019) study used test material E171, anatase, 110 – 115 nm (confirmed by SEM), 36% of the particles had a particle size 100 nm. Internal exposure was not examined. Six-week-old male Wistar Han IGS rats were exposed to E171 in a standard diet at 4 concentrations between 0 - 5,000 mg/kg diet and a control in two studies each of 7 days (n = 5/group) (equal to 1.8, 4.8, 31.4 and 374 mg/kg bw per day) and one study of 100 days (n = 15/group) (equal to 1.3, 3.5, 22.4 and 267 mg/kg bw per day). Prior to exposure to E171, animals in groups 1 - 4 were treated with one intraperitoneal injection of a sterile dose of 180 mg/kg bw 1,2-dimethylhydrazine (DMH) dihydrochloride (an inducer of GI tract tumours) in 1.5% EDTA-0.9% NaCl, pH 6.5. Animals in groups 5 - 8 were treated with a single sterile dose of 1.5% EDTA-0.9% NaCl, pH 6.5 without DMH.

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

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

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

Bettini et al., (2017)

163. In the Bettini et al study, in addition to the 7-day studies described in paragraphs 63 and 64, a second group of rats (n = 11 to 12 per group) were treated (or not) with DMH to induce colon carcinogenesis and were exposed daily to E171 at 200 µg or 10 mg/kg bw per day through drinking water for 100 days. Control animals (n = 12) received water only. Rats were used for flow cytometry and cytokine assays and for gut inflammation and ACF assessments.

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

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

166. The differential effects of TiO2 on the viability of normal or preneoplastic colonic cells were determined to assess its possible influence on growth promotion of preneoplastic lesions. This was investigated using the comparative cytotoxicity of food-grade TiO2 particles on non-mutated and pre neoplastic cells (Apc+/+ and Apc Min/+). Results showed that 24-hour exposure to TiO2 was more cytotoxic to Apc+/+ than Apc Min/+ cells.

Additional studies examining evidence of histopathological proliferation in the colon

167. No microscopic evidence of proliferative changes in the intestine have been seen in other chronic toxicity studies with TiO2 including Warheit, Brown and Donner, 2015; Akagi et al., 2023; and the NCI Unitane ® carcinogenicity 1979 study (NCI, 1979). The Akagi study was not included in the EFSA review as it was published after the EFSA Opinion, in 2023. The EFSA review also did not include the NCI 1979 study because, although the EFSA ANS Panel did conclude that the study indicated that TiO2 was not carcinogenic in rats and mice, the Panel scored the study of low reliability as they considered that the analytical information on the test item was insufficient. However, the COT notes that the Titanium Dioxide Manufacturers Association (TDMA) has more recently completed work that shows that the test item used in the NCI 1979 study was comparable to the specifications of E171 today, and so the study should be considered reliable.

Studies using E171 or equivalent form of TiO2

Han (2020)

168. A 90-day oral repeated dose toxicity study of E171 was carried out in five-week-old male and female Sprague-Dawley rats (40 male and 40 female) according to OECD TG 408 and GLP. Ten rats per sex and per dosage group were randomly assigned to one of four groups (0, 10, 100, or 1,000 mg/kg dosage of E171) administered daily by oral gavage for 13 consecutive weeks (90 days). The control group was provided with the equivalent volume of water. Forty-two tissues were collected at necropsy for histopathological evaluation. An in vitro study was conducted, in which AGS cells (human stomach-derived epithelial cell line) (70 - 80 % confluence) were incubated with E171 (0, 10, 20, 40 µg/mL) for 24 h. Colon and stomach sections, together with AGS cells treated with 40 µg/mL E-171 were fixed, sectioned and images were produced for analysis using a TEM. Samples of stomach, colon, kidney, and spleen were analysed for Ti using ICP-MS.

169. There were no dose-related changes in any OECD test guideline endpoints. E171 deeply penetrated cells lining the stomach tissues at the maximum dosage (1,000 mg/kg), The concentration of Ti increased (but with coinsidrable variability) in the colon of both sexes in high dose animals, but not in the kidneys or spleen. TEM revealed that E171 accumulated in the cytosol and

nuclei of several cell types in the colon of high dose animals and formed lamella-like structures. There was lower blood IgM (male and female) and GM-CSF (female) levels in the high dose E171-treated animals compared to control animals (unclear whether these changes were statistically significant). Colonic superoxide dismutase (SOD)-1 protein levels decreased in males and females and SOD-2 protein levels decreased in females in high dose animals (only dose investigated).

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

171. The authors concluded that the NOAEL for E171 was less than 1,000 mg/kg for both male and female rats in this 90-day study, based on TiO2-induced formation of lamella-like structures in the colon, potentially indicative of impairment of defence against foreign bodies. The authors proposed that further chronic toxicity studies be conducted due to the potential for E171 to reduce a host's immune defence function by decreasing antioxidant capacity.

NCI (1979)

172. The Study on Unitane ® 0-220 (NCI, 1979), a compound previously manufactured in the US for use as food-grade TiO2, was considered relevant by the sub-group as recent work by the TDMA had demonstrated that the test item used in the study was comparable to the updated specification of E171. In a preliminary 13-week study, male and female B6C3F1 mice and Fischer 344 rats (n = 10 per sex per group) were fed doses of Unitane ® 0-220 (TiO2, anatase, 98% purity). A further cohort (n = 50 per sex per group) were fed Unitane ® 0-220 via the diet for 103 weeks at concentrations of 0, 25,000 or 50,000 ppm (equivalent to 0, 3,250 or 6,500 mg/kg bw/day and 0, 4,175 or 8,350 mg TiO2/kg bw/day for male and female mice (respectively) and doses of 0, 1,125 or 2,250 mg/kg bw/day and 0, 1,450 or 2,900 mg/kg bw/day for male and female rats (respectively)) and then observed for 1 additional week. Fifty untreated rats of each sex and 50 untreated mice of each sex were used as controls. All remaining rodents were euthanised at 104 weeks. No evidence was observed (in either species) of lesions in the gastrointestinal tract e.g., proliferative non-neoplastic or neoplastic findings. It was also noted following examination of the pathology data that nematodes were recorded in the intestine which introduces an additional potential for GI inflammation which could potentially induce inflammatory and

proliferative changes, however none were observed in the study.

Warheit, Brown and Donner (2015)

173. Warheit, Brown and Donner (2015) summarised three OECD-type studies of TiO2 in rats of varying particle sizes and surface coatings. Study 1 was a subchronic 90-day study (OECD TG 408) in which adult male and female rats were fed rutile, surface-coated pigment-grade TiO2 test particles (d50 ¼ 145 nm, 21% nanoparticles) by oral gavage for 90 days with a NOAEL of 1000 mg/kg bw/day. Study 2 was a 28-day repeated-dose oral toxicity study (OECD TG 407) in adult male rats fed two rutile-type, uncoated, pigment-grade TiO2 test particles (d50 ¼ 173nm) by oral gavage at a dose of 24,000 mg/kg bw/day with no test item-related effect. Study 3 was an acute oral toxicity study (OECD TG 425) with female rats fed surface-treated rutile/anatase nanoscale TiO2 particles (d50 ¼ 73 nm) up to 5,000 mg/kg. The oral LD50 for the test substance was >5,000 mg/kg bw. Collectively, no test item-related adverse effects were found including during microscopic examination of the colon.

Studies using the nanoparticle form of TiO2

Akagi et al., (2023)

174. In the Akagi *et al.*, 2023 study, TiO2 NPs (6 nm) were examined in male and female rats by repeated oral administration of 10, 100, and 1,000 mg/kg bw/day for 28 days (5 individuals per sex per dosage group) and of 100, 300, and 1,000 mg/kg bw/day for 90 days (10 individuals per sex per group). The authors reported that no mortality was observed over either study length with no treatment-related effects observed in body weight, urinalysis, hematology, serum biochemistry or organ weight. Histopathological examination of the 28-day study specimens found TiO2 particle deposition in the gastro-intestinal lumen, nasal cavity, epithelium and stromal tissue as well as, in the 90-day study, in Peyer’s patches in the ileum, cervical lymph nodes, mediastinal lymph nodes, bronchus-associated lymphoid tissue, and trachea. Despite this, no inflammation or tissue damage were observed. Little evidence was found of TiO2 absorption or of accumulation in the liver, kidneys, and spleen. Immunohistochemical analysis showed no extension in colonic crypts of the proliferative cell zone or preneoplastic cytoplasmic/nuclear translocation of β-catenin up to 1000 mg/kg bw/day in males or females. With regard to genotoxic endpoints, no observed increase in micro-nucleated or γ-H2AX positive hepatocytes was found. No effects

were observed after repeated oral administration of TiO2 (6 nm particle size) up to 1,000 mg/kg bw/day.

EFSA review and conclusions on induction of ACF by TiO2

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

Health Canada review and conclusions on induction of ACF by TiO2

176. As mentioned in paragraph 24, the Health Canada State of the Science report on titanium dioxide had previously been reviewed (pre-publication) by the Committee and its conclusions endorsed. The Committee agreed that the report was comprehensive and included further discussions with pathologists from the Blevins *et al.* study to evaluate the colon findings.

177. The Health Canada report concluded that while there were some uncertainties identified which may require further studies, they did not identify consistent evidence of preneoplastic lesions in the colons of rodents exposed to food-grade TiO2 via the oral route. A single non-guideline study in which rats were exposed to food-grade TiO2 dispersed in drinking water at doses of ~10 mg/kg bw per day for 100 days (Bettini et al., 2017) resulted in formation of ACF, but these results were not replicated in subsequent dietary studies when higher doses were administered, up to ~236 - 300 mg/kg bw per day for 100 days in a non-guideline study (Blevins et al., 2019) and up to 1000 mg/kg bw per day for ~18 - 19 weeks in a GLP- and OECD guideline-compliant EOGRT study (Leuschner, 2020). An OECD guideline-compliant study that administered food-grade TiO2

dispersed in water to rats via oral gavage at doses up to 1,000 mg/kg bw per day for 90 days showed no histopathological changes in the gastrointestinal tract (GIT) (or any other tissues) (Han et al., 2020). The report concluded that there was no evidence of, and therefore that there was a low level of concern for, carcinogenicity, chronic toxicity, or other non-neoplastic lesions of the GIT. A weight of evidence approach did not identify data gaps of such significance to require a more precautionary approach currently.

FSANZ review and conclusions on induction of ACF by TiO2

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

179. FSANZ noted EFSA's concerns from the 2021 opinion around the risk of induction for ACF in the colon identified in Bettini et al. (2017). FSANZ's previous assessment of Bettini et al. (2017) concluded that this study had limited relevance in humans due to design limitations including the use of sonicated test item delivered via drinking water and its applicability to the use of TiO2 as a food additive. This concern was raised by ANSES in 2017 in which it was concluded that the Bettini et al. (2017) study results could not be used for risk assessment without a confirmatory study, which included dietary administration of the test item. It was also noted that in their 2016 opinion on TiO2, EFSA agreed that these data "were not sufficient to raise a concern on the potential initiation or promotion properties of TiO2 on colon carcinogenesis".

180. FSANZ noted that while Bettini et al. (2017) reported evidence suggesting an increased incidence of ACF related to oral exposure to TiO2, two additional studies using sonicated food-grade TiO2 administered to rats by gavage for up to 90 days (Blevins et al. 2019 and the unpublished EOGRT Study (Leuschner, 2020)) found no evidence of treatment-related histopathological changes in the gastrointestinal tract up to doses of 1000 mg/kg bw per day. In addition, FSANZ also noted that the NCI 2-year bioassay results "found no evidence of carcinogenicity of a test item comparable to food-grade TiO2 in the colon or other tissues in rats and mice at dietary concentrations up to 50,000

ppm, equivalent to doses of 2,250 – 2,900 and 6,500 – 8,350 mg/kg bw per day, respectively", a finding which was also inconsistent with the Bettini et al. (2017) results.

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

JECFA 2024 review and conclusions on induction of ACF by TiO2

182. JECFA considered Unitane 0-220 to be representative of INS 171 (E171). They reviewed the NCI Unitane study (1979) and concluded that under the conditions of the bioassay, Unitane 0–220 was not toxic or carcinogenic by the oral route for both mice and rats. The Committee also concluded that the NOAELs from this study were 7,500 mg/kg bw per day in mice and 2500 mg/kg bw per day in rats. These were the highest doses tested. JECFA reviewed the 90-day study by Han et al (2021) in which no treatment-related adverse effects were observed but noted that the Ti concentration was increased in the colon compared to controls at the maximum dose tested (1,000 mg/kg bw per day). JECFA concluded that the NOAEL for this study was 1,000 mg/kg bw per day.

183. JECFA had reviewed the studies by Bettini et al (2017), Blevins et al (2019) and Leuschner (2020) in detail under the heading of special studies. They also considered additional studies which reported gastrointestinal effects (Proquin et al, 2018; Pinget et al, 2019; Perez et al, 2021; Talamini et al, 2019; and Mortensen et al 2021). These studies dosed E171 either in the drinking water or via gavage and used sonication before dosing. JECFA considered the relevance of these to be questionable for the safety assessment of E171 due to study design deficiencies, limitations with dosing methodologies, small animal numbers and/or variability in the results. It was considered that adverse effects associated with E171 oral exposure mostly came from studies which had used homogenised suspensions or ultrasonically dispersed material and was not representative of dietary exposure.

184. JECFA had reviewed three epidemiological studies (Lomer et al, 2005; Ruiz et al, 2017; and Trakman et al, 2022) and considered that potential associations with inflammatory bowel disease are discussed without allowing any conclusions to be made.

COT review and conclusions on induction of ACF by TiO2

185. The Committee questioned whether results from studies in which TiO2 was administered by methods other than through the diet were relevant to exposure via consumption of food. In one study that assessed ACF (Bettini *et al.*, 2017) the test item was administered via water whereas in the EOGRT study and Blevins *et al.*, 2019 exposure was dietary. In studies of TiO2 administered in water, BSA was added to provide protein to stabilise the suspension, and the mixture was sonicated for up to 1 hour to disperse the particles. Such preparations may not be representative of that when used in food, due to effects of sonication on the structure of TiO2.

186. The results from the Bettini et al study where ACF were identified in rats pre-treated with DMH and in a small subset of animals exposed only to food-grade TiO2 dispersed in drinking water at doses of ~10 mg/kg bw per day for 100 days, were not replicated in the Blevins et al or EOGRT dietary studies. In addition, there was no evidence of further proliferative changes in the colon mucosa in Bettini et al, nor in the two dietary studies or in other studies of repeat-dose administration of TiO2, where histopathology of the colon was performed. The Committee concluded that there was no convincing evidence that TiO2 induced ACF and no evidence to support progression to proliferative lesions in the colon.

187. The Committee considered that although small numbers of ACF were observed in some animals exposed to TiO2 alone in Bettini *et al.*, (2017), these could not necessarily be attributed to TiO2, as ACF were also observed in control animals without exposure to TiO2 in other studies (Blevins et al., 2019, and Leuschner, 2020). Additionally, none of the studies distinguished between hyperplastic and dysplastic ACF in any groups of control or treated animals. The Committee also concluded that there was very little evidence that the effects of TiO2 were systemic at the doses tested but also that TiO2 measurements were not accurate in the analysis of TiO2 in the tissues. One explanation for why there is little systemic toxicity of TiO2 administered via the diet, with a NOAEL is 1,000 mg/kg bw per day, the highest dose tested, is because there is very little gastrointestinal absorption.