Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment.

Statement on the safety of Titanium Dioxide (E171) as a Food Additive

Executive Summary

Introduction

Food grade titanium dioxide (TiO_2) was an authorised Food Additive (E171) in the EU but from the 7th of August 2022 it is no longer permitted following the publication of Commission Regulation (EU) 2022/63, amending Annexes II and III to Regulation (EC) No 1333. Since August 2022, manufacturers in Northern Ireland have not been permitted to produce goods containing titanium dioxide according to EU legislation. It currently remains authorised in Great Britain. Food grade TiO_2 comprises a mixture of micro and nanosized particles and is used in food as a colour (white pigment) to make food more visually appealing, to give colour to food that would otherwise be colourless, or to restore the original appearance of food. It is commonly used in products such as bakery products, soups, broths, sauces, salad dressings, savoury based sandwich spreads, processed nuts, confectionary, chewing gum, food supplements and cake icing. Titanium dioxide is also widely used in cosmetics and medicines.

Titanium dioxide has been the subject of multiple safety evaluations including three recent evaluations by the European Food Safety Authority (EFSA) in 2016, 2019 and 2021.

In their most recent Opinion (2021), EFSA considered that some findings regarding immunotoxicity, inflammation and neurotoxicity with respect to TiO_2 nanoparticles, which are present in food grade TiO_2, may be indicative of adverse effects. On the basis of the currently available evidence and the uncertainties, in particular a concern regarding genotoxicity which could not be resolved, the EFSA Panel concluded that E171 could no longer be considered as safe for use as a food additive.

Following this, in 2022 the COT published an interim position on titanium dioxide (COT, 2022) capturing the outcomes of discussions and outlining the next steps. A review has now been undertaken by the COT, which includes the conclusions on mutagenicity from the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), to assess the safety of TiO_2 as a food additive. This review is summarised below.

Since the EFSA and COT publications in 2021 and 2022, reviews of TiO_2 have also been carried out by Health Canada (2022), Food Standards Australia New Zealand (FSANZ) (2022) and most recently by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2024).

Characterisation and ADME considerations

Specifically in food, the primary function of TiO_2 is as an opacifier and white pigment. To achieve this function, it is critical that food grade TiO_2 (E171) exists as an aggregate of smaller primary particles with a median particle size of 200 – 300 nm. Engineered nano-TiO_2 has all (100%) of its particles less than 100 nm in diameter and is colourless and would therefore be unsuitable for use as a pigment in food applications.

The COT concluded that there is uncertainty over the toxicological effects of TiO_2 nanoparticles. The Committee therefore considered that if animals and/or humans

are exposed to test substances which contain higher levels of nanoparticles than normally found in food-grade TiO_2, that could change the toxicological profile and potentially the risk, but it is unclear by how much or in what way.

The COT concluded that the physical form of TiO_2 will affect its absorption and distribution. The focus of the COT was on food-grade TiO_2 but the wide variance of test materials (nano, micro and mixtures of nano and micro) used in experimental studies was noted. Due to this large variability, as well as the potential impact of the matrix of administration, the Committee could not ascribe a specific percentage for the absorption of food-grade TiO_2. However, the Committee considered that absorption of food grade TiO_2 (E171) is low.

Review of toxicity for the endpoints identified by the COT

The COT has reviewed toxicological studies which have been conducted using any form of TiO_2, including nanoparticles, but its conclusions are primarily based on those which have used food grade TiO_2 (E171).

The following endpoints were reviewed initially by the COT and then in more detail by a sub-group of COT Members: Aberrant crypt foci (ACF) (as a potential marker for carcinogenicity), inflammation and immunotoxicity, reproductive and developmental toxicity and neurotoxicity. The COM reviewed the genotoxicity data and reported their findings to the COT in May 2024.

The Committees gave greater weight to studies in which TiO_2 was administered orally, particularly in the diet, as they were considered to be the most relevant for human exposure to TiO_2 through consumption of food.

Reproductive and Developmental Toxicity

The COT reviewed the report of the Extended One Generation Reproductive Toxicity (EOGRT) study provided to the Food Standards Agency (FSA) by the Titanium Dioxide Manufacturers Association (TDMA) (Leuschner, 2020) as part of their safety assessment of TiO_2. This study was carried out in response to a conclusion by EFSA

regarding the uncertainty around TiO_2 (E171) and reproductive and developmental toxicity. In addition to reproductive and developmental toxicity, the EOGRT study also included cohorts for induction of aberrant crypt foci (ACF) in the colon, developmental immunotoxicity and neurotoxicity.

The COT considered the EOGRT report to be detailed and that the study was well conducted and carried out according to the relevant scientific guidelines, with no obvious deficiencies. It was noted that there were some minor effects observed in the study including focal effects on the testes and epididymides and a change in weight of the right testes. However, the COT agreed with the authors' conclusions that these changes reflected background variability (not attributable to the treatment) and were not of toxicological relevance.

The COT agreed that there was no evidence of reproductive or developmental toxicity up to and including the highest dose tested (1000 mg/kg bw per day). Therefore, this dose level was the no observed adverse effect level (NOAEL) for the study.

Analysis by the COT of the peer reviewed literature on reproductive and developmental toxicology identified two additional studies of appropriate quality (Warheit et al., 2015a; Lee et al., 2019). The COT concluded that these studies provided no significant evidence that TiO_2 is reprotoxic and the NOAELs were consistent with that from the EOGRT study.

Aberrant Crypt Foci (ACF)

The Committee considered that although small numbers of ACF were observed in some animals exposed to TiO_2 alone administered via drinking water in a single study (Bettini et al 2017), these could not necessarily be attributed to TiO_2, as ACF were also present in animals in control groups not exposed to TiO_2 in other dietary studies (e.g. Blevins et al, 2019; Leuschner, 2020). In addition, there was no evidence of the development of proliferative lesions of the colonic mucosa in any studies including the carcinogenicity study performed with Unitane 0-220 (test material comparable to the E171 specification) (NCI, 1979; TDMA, 2022). The

Committee concluded that there was no evidence that TiO_2 induced ACF and no evidence to support progression to proliferative lesions in the colon.

Inflammation and immunotoxicity

The COT noted that only three studies on inflammation or immunotoxicity, including the EOGRT, (Riedle et al., 2020; Blevins et al., 2019; and Leuschner, 2020) used E171 TiO_2 administered in the diet. These studies showed no adverse effects resulting in inflammation or immunotoxicity.

Five studies using food grade TiO_2 (E171) administered to rats or mice in water (Talamini et al., 2019; Pinget et al., 2020; Bettini et al., 2017; Han et al., 2020; and Mortensen et al., 2021) on inflammation and immunotoxicity were considered by the COT. In some of the studies, differential inflammatory cytokine and host defence gene expression was observed but this was neither consistent across studies, nor ubiquitous in terms of pathway activation, making interpretation or formulation of conclusive statements challenging.

Other potential immunotoxic effects have been reported which include, but are not limited to: immune cell mediated inflammatory foci in the spleen and gut, including in Peyer's patches; effects on broader host defence mechanisms, including antimicrobial peptides; effects in the gut microbiota; effects on gut dendritic cell populations; 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.

Overall, however, there is insufficient evidence of sufficient quality to conclude that food grade TiO_2 (E171) is of concern with regards to immunotoxicity and inflammation.

Neurotoxicity

Overall, there is no new evidence on neurotoxicity to justify a change to the COT position on this endpoint as stated in its 2021 interim position paper. The findings of

the studies on neurotoxicity were considered inconsistent by the COT. It was noted that the EOGRT study did not report any neurotoxic effects and that most of the other studies on this endpoint used titanium dioxide in the form of nanoparticles (TiO_2 NPs).

The COT noted that in the EOGRT study the routine regulatory histopathology tests would have been less sensitive than the specific neuro-histopathology tests performed in some other studies. This qualification of the COT conclusion on neurotoxicity is more conservative than that of Health Canada who considered the EOGRT to be sufficiently sensitive and relevant to conclude on the lack of neurotoxicity potential of food grade TiO_2 (E171).

The COT considered that the data from the relevant studies available indicated that TiO_2 did not induce ACF, nor were there significant effects in studies that assessed inflammation and immunotoxicity, reproductive and developmental toxicity, and neurotoxicity. On balance, the Committee considered that the NOAEL of 1,000 mg/kg bw per day, the highest dose tested, from the EOGRT study, was robust.

Review of the genotoxicity of TiO_2 by the COM

The COM reviewed a number of studies to assess the genotoxicity of TiO_2. In addition to papers reviewed by EFSA, a literature search was conducted to find papers published on “genotoxicity” and “titanium dioxide”. Most papers identified used the nano-sized fraction of TiO_2 and not the micro-sized form, nor the specific E171 form. Papers were assessed by experts and scored using a tiered approach to screen for both physico-chemical characteristics and ensure that only high-quality genotoxicity studies were included. The review therefore included papers on nano- and micro-sized TiO_2 with particular attention given to the E171-form.

The COM stated that a definitive assessment of the safety of food grade TiO_2 (E171) per se was difficult, when there were no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. It was also noted that there is a lack of high-quality data sets that are OECD compliant on any form of the compound, and

this led to conflicting data and some uncertainty in the risk assessment for TiO_2. (COM, 2024b).

The COM's opinion is that there is little evidence that TiO_2 micro- or nano-sized particles are genotoxic in vitro or in vivo based on data from the few well conducted studies that are available. There is also a lack of replication of study outcomes using the same nanoparticle in different labs. (COM, 2024a).

Overall, therefore, the COM concluded that there was little evidence in the literature to suggest that there was a health concern related to genotoxicity induction by TiO_2, particularly via the oral route and especially the micro sized TiO_2 fraction (most studies in the literature used nano-sized material). Hence, any genotoxicity risk from dietary food grade TiO_2 (E171) was considered to be low. (COM 2024b).

Following discussions of the COM report at their meeting in March 2024, the COT agreed with the conclusions of the COM.

Establishment of a Health-Based Guidance Value (HBGV)

The Committee concluded that on the basis of the available evidence, 1,000 mg/kg bw/day was a robust Point of Departure (POD). This was based on the EOGRT study findings (Leuschner, 2020) as well as studies by Warheit et al., 2015b and Lee et al., 2019 that reported no effects up to the same dose. There was variability noted in the other studies, but nothing which would alter the proposed POD for food grade TiO_2 (E171).

A standard uncertainty factor of 100 (10 for inter-species differences and 10 for inter-individual variability) was agreed by Members and applied to the POD which results in a HBGV of 10 mg/kg bw/day. There is likely to be additional conservatism in the application of this uncertainty factor to the NOAEL of E171 because 1,000 mg/kg bw per day was the highest dose of TiO_2 tested and therefore the LOAEL (lowest observed adverse effect level) could actually be appreciably higher and, because there is no metabolism of TiO_2 particles, the inter-/intra-species kinetic differences are likely to be lower than the defaults.

Exposure Assessment

Titanium dioxide (E171) can be found in a number of food categories, as well as in cosmetics and medicines. The exposures calculated and considered in this assessment are only for food and were for infants, toddlers, children, adolescents, adults, and the elderly using food consumption data from UK surveys. Maximum occurrence levels of titanium dioxide for specific food items, reported by EFSA (2021), were also used in the estimation of exposure.

The mean calculated total dietary exposures for TiO_2 ranged from 3.3 to 11 mg/kg bw per day. The 95th percentile total dietary exposures for TiO_2 ranged from 9.1 to 26 mg/kg bw per day. The 3 food groups that contribute the most to these exposures are: protein products; decorations, coatings and fillings; and sauces.

The exposure assessment took into account use levels in only sixteen food groups whereas E171 is approved in more categories (forty-eight). This may introduce underestimations for exposures. However, not all foods within the categories assessed will contain E171, which means exposure in those categories may be overestimated. In addition, the assessments are based on the assumption that all food in all categories assessed contain E171 at the maximum reported levels. This is unlikely and overall exposure is therefore more likely to be overestimated.

Risk Characterisation

Exposures for all population groups for the mean total diet are below or very close to the established HBGV of 10 mg/kg bw per day.

Exposures estimated for the 95^{th} percentile total diet range from 9.1 to 26 mg/kg bw per day. The exposures for adults (18+) and the elderly are below the established HBGV and adverse health effects would not be expected. Although estimated exposures for infants, toddlers, children and adolescents are 1.3- to 2.6-fold higher than the HBGV, actual exposures are likely to be lower than those calculated. In addition, as noted in paragraph 30, the HBGV is likely to be conservative.

Therefore, exposures to food grade TiO_2 (E171) from the diet are unlikely to present a risk to health for the UK population.

COT Overall Conclusion

The COT concludes that it is unlikely that there would be a risk to health from current UK dietary exposures of E171 TiO_2.

Introduction

1. Titanium dioxide (TiO_2) is an inorganic compound which exists in nature in different crystalline forms - the anatase and rutile being the two most common. Titanium dioxide is comprised of particles with a range of sizes. Food grade TiO_2 (E171) is comprised of both nano- and micro-sized particles. The International Organization for Standardization (ISO) define nanoscale as a length range of approximately 1 – 100 nm. They also defined a nanomaterial as a material with any external dimension in the nanoscale (1 – 100 nm) or having internal structure or surface structure in the nanoscale (ISO, 2023). Microparticles are between 0.1 and ≥100 µm in size. EFSA have previously characterised E 171 and concluded that less than 50% of constituent particles in E171 have a minimum external dimension below 100 nm by number (EFSA, 2019).

2. Food grade titanium dioxide (TiO_2) was an authorised Food Additive (E171) in the EU but from the 7th of August 2022 it is no longer permitted following the publication of Commission Regulation (EU) 2022/63, amending Annexes II and III to Regulation (EC) No 1333. Since August 2022, manufacturers in Northern Ireland have not been permitted to produce goods containing titanium dioxide according to EU legislation. It currently remains authorised in Great Britain in both anatase and rutile forms.

3. Food grade TiO_2 (E171) is used in food as a colour to make food more visually appealing, to give colour to food that would otherwise be colourless, or to restore the original appearance of food. It is commonly used in products such as bakery products, soups, broths, sauces, salad dressings, savoury based sandwich spreads,

processed nuts, confectionary, chewing gum, food supplements and cake icing. It has also been widely used in cosmetics and medicines (EFSA, 2016).

4. Where stated, titanium dioxide/TiO_2 refers to both E171 and non-E171 forms and undefined specifications, E171 refers specifically to titanium dioxide which complies with the updated EFSA specification for titanium dioxide E171 as a food additive, which have been transferred over to assimilated Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council (Text with EEA relevance). (EC, 2012: Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council (Text with EEA relevance) (legislation.gov.uk).

5. In 1969, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) allocated an acceptable daily intake (ADI) 'not limited except for good manufacturing practice'. In 1975, the Scientific Committee on Food (SCF) did not establish an ADI for titanium dioxide, whereas in 1977, the SCF included titanium dioxide in the category 'colours for which an ADI was not established but which could be used in food'.

6. Since 1975 TiO_2 has been the subject of multiple safety evaluations which are detailed below.

Evaluations by EFSA prior to the 2021 Opinion

7. The EFSA Food Additives and Nutrient Sources (ANS) Panel (2016) did not establish an ADI, or other health-based guidance value (HBGV), during their review. This was due to the lack of an extended 90-day toxicity study, a multi-generation or an extended one generation reproductive toxicity (EOGRT) study with E171. These data were considered necessary due to possible adverse effects which were identified in the reproductive system from studies published in the peer-reviewed

literature, using test substances that were non-food grade, or which contained inadequately characterised nanomaterial.

8. The EFSA 2016 review determined that E171 TiO_2 consisted mainly of micro-sized TiO_2 particles, with a nano-sized (< 100 nm) fraction which was less than 3.2% by mass. Uncertainties around the identity and characterisation of E171 were highlighted, noting that no limits for the particle size of E171 had been set. EFSA requested further data in the form of an EOGRT study, as no HBGV could be established due to the evidence gaps. These gaps included the lack of consistency and characterisation of the form of TiO_2 in the test materials and in the test animal during absorption and metabolism, and considerations around the exposure to TiO_2 test substances which contain higher levels of nanoparticles (NPs) that could change the toxicological profile. However, it is unclear by how much the toxicological profile could be changed, or at which point during the exposure. There was no comparison of results to food-grade TiO_2; an ideal study had not been conducted.

9. The specifications of E171 TiO_2 were reviewed again by EFSA in 2019. Based on the fraction of nanoparticles present in E171, it was determined that the food additive fell under the scope of the EFSA guidance on nanotechnology for “a material that is not engineered as nanomaterial but contains a fraction of particles, less than 50% in the number–size distribution, with one or more external dimensions in the size range 1–100 nm”. (EFSA, 2019).

Other Evaluations by Regulatory bodies prior to the EFSA 2021 Opinion

10. Following a report by the French Authorities in 2016 and a proposal for evaluation of titanium dioxide, the Committee for Risk Assessment (RAC) of the European Chemicals Agency (ECHA) concluded, in June 2017, that titanium dioxide met the criteria to be classified as a substance suspected of causing cancer (category 2) if inhaled. The main mechanism thought to explain the effects induced by titanium dioxide, in common with effects seen with other particulate substances, was inflammation and an indirect genotoxic effect through production of reactive oxygen species (ROS) arising from the biopersistence and insolubility of all forms of titanium dioxide particles. However, a direct interaction with DNA could not be

excluded, since titanium dioxide had been found in the cell nucleus in various in vitro and in vivo studies. This was in line with the International Agency for Research on Cancer (IARC) evaluation which concluded that in relation to exposure via inhalation "titanium dioxide is possibly carcinogenic to humans (Group 2B) based on sufficient evidence in experimental animals and inadequate evidence from epidemiological studies." (IARC 2010). However, the 2016 report by the French Authorities the Agency for Food, Environmental and Occupational Health and Safety (ANSES) concluded that there was no carcinogenic concern after oral or dermal administration.

11. In 2018, the Dutch Office for Risk Assessment and Research held a workshop on the "potential health effects of the food additive titanium dioxide (E171)". The results were published in 2019, which overall stated the need for further studies to investigate the effects of titanium dioxide exposure, particularly for the endpoints of colon tumours and immunotoxicology based on the data gaps. Study limitations of the available database at the time were also highlighted. The need to better characterise the composition of E171 was noted. In 2020, a review was published (Bischoff et al., 2020) that summarised the outcomes of this workshop and additionally aimed to identify and evaluate recent toxicological studies on food-grade titanium dioxide and nano-sized titanium dioxide in ex-vivo, in-vitro, and in-vivo experiments along the gastrointestinal route, and to postulate an adverse outcome pathway (AOP) following ingestion. Adverse effects were identified including the generation of ROS, alterations of the gut microbiota, persistent inflammation, and other effects on the immune system. It was noted that the findings were inconsistent between the different species and independent research groups. With regards to the animal studies that reported positive effects on precancerous lesions/tumour formation, it was noted that those were mainly used as research models and a proper investigation of a dose-response relationship was not performed. Based on the available information, it was not possible to carry out a risk assessment. When considering the mode of action, it was postulated that it was closely related to the ability of titanium dioxide to induce ROS formation and promote inflammation. The potential key events were considered to be persistent inflammation and ROS generation (that can result in oxidative stress) as well as persistent epithelial cell injury which could potentially lead to DNA damage and exert a tumour-promoting

effect of E171 which was seen in some of the studies. Finally, it was noted that it is generally assumed that the round and spherical crystal forms of TiO_2 contribute to the induction of adverse effects to a lesser extent when ingested than nano-sized titanium dioxide particles which are suspected to induce more adverse effects than other particle sizes. However, a study by Proquin et al., (2017) was also mentioned that demonstrated that a mixture of nano- and micro-sized TiO_2 particles, as present in E171, induced more adverse effects than the single fractions alone. The authors further expanded on possible interactions of E171 with its direct environment as well as other factors that could potentially affect agglomeration, for example and discussed how these might directly affect the properties of titanium dioxide. Bischoff et al. therefore, considered that "it is important to carefully examine and analyze the physicochemical characteristics of TiO_2 particles in its vehicle, as well as in its surrounding matrix as their final milieu, to guarantee a profound assessment of potential adverse health effects of E171 and to adequately compare different studies in the process of risk assessment." (Bischoff et al.,2020).

12. In their most recent evaluation, the EU Scientific Committee on Consumer Safety (SCCS) assessed titanium dioxide used in cosmetic products that lead to exposure by inhalation. The SCCS considered both micro and nano-sized particles. With regards to mutagenicity and genotoxicity, the SCCS noted that in the 2010 evaluation, IARC concluded that most of the in vitro genotoxicity studies with titanium dioxide exposure were negative despite the high rate of false positives and that the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) Panel in 2016 considered that the positive genotoxicity results may have been due to experimental conditions associated with the induction of oxidative stress. The ANS Panel (2016) had concluded that orally ingested TiO2 particles (micro- and nanosized) are unlikely to represent a genotoxic hazard in vivo. The SCCS also noted that studies showing a positive association between exposure to the so-called group of Poorly Soluble Low Toxicity (PSLT) particles and genotoxicity are generally consistent with the mechanism that sub-toxic concentrations of PSLT particles can cause inflammation and oxidative stress, which may lead to mutations. Oxidative stress is considered the underlying mechanism of the proliferation and genotoxic responses to PSLT particles including titanium dioxide and thus there is a large body of evidence that titanium dioxide has no direct genotoxic potential. The SCCS was of

the opinion that “The genotoxic effects of titanium dioxide most probably manifest through an indirect mechanism (oxidative stress), or secondary mechanisms (e.g. oxidative stress and inflammation caused by immune cells). The SCCS therefore considers it plausible that there is a practical threshold for this mode of action and therefore a risk assessment could be carried out for its use in cosmetic products.” They concluded that when used in cosmetic products, titanium dioxide does not pose a genotoxic risk (SCCS, 2020).

Opinion of the EFSA Panel on Food Additives and Flavourings (FAF), (2021)

13. In their 2021 Opinion, the EFSA FAF Panel had considered that some potential immunotoxicity and inflammation findings specifically with TiO_2 food additive E171, as well as potential neurotoxicity with TiO_2 nanoparticles may be indicative of adverse effects. They also concluded that there were indications of the induction of aberrant crypt foci (ACF) with E171 and that there were no studies which had been appropriately designed and conducted to investigate the potential carcinogenicity of TiO_2 nanoparticles. On the basis of the available evidence and the uncertainties, in particular a concern regarding genotoxicity that could not be resolved, the EFSA Panel concluded that E171 could no longer be considered as safe when used as a food additive. (EFSA, 2021).

COT and COM considerations of the 2021 EFSA Review

14. The COT and Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) conducted an initial review of the updated EFSA opinion on E171 (EFSA, 2021), following a request from the Food Standards Agency, and disagreed with EFSA’s conclusion. It was considered that the conclusions were not robust, and it was decided that the UK should undertake its own evaluation. This would be undertaken by the COM for genotoxicity and the COT for inflammation, immunotoxicity, allergenicity, aberrant crypt foci (as a biomarker for colon cancer), reproductive and developmental toxicity and neurotoxicity.

15. The COT produced an interim position paper in 2022 (TiO_2 COT Interim position paper (food.gov.uk) (COT, 2022)) outlining the views of the COM and the

COT, on the EFSA Opinion. The available data have since been evaluated by both committees.

Evaluations by Authorities after the 2021 EFSA Opinion

16. Health Canada (2022) have published a document summarising the state of the science concerning the safety of TiO_2 as a food additive. TiO_2 is insoluble, poorly bioavailable and generally considered toxicologically inert via the oral route. However, Health Canada recognised that its safety has recently been questioned due to the presence of particles in the nanoscale range (<100 nm), (although not engineered as a nanomaterial). TiO_2 particles in the nanoscale, as well as food-grade TiO_2 containing nanoparticles, may produce toxic effects in various test systems when dispersed and stabilized in simple matrices such as water. However, it is not certain what the relevance is for human health, as TiO_2 used in food preparations is of agglomerated particles that are not dispersed to the same degree. Health Canada noted that in vivo studies that administered TiO_2 in the diet tended not to replicate findings from studies using dispersed TiO_2 in simple matrices and also that the particles used in studies are often poorly described which makes it difficult to establish relationships between particle characteristics (e.g., size, agglomeration state, surface area, particle number, etc.) and toxicity. It is also difficult to determine the relevance to human exposure and the forms of TiO_2 used in food.

17. Health Canada also noted that TiO_2 is not metabolized to any significant degree and the majority is excreted unchanged in faeces. Studies in animals and human volunteers indicate that approximately 0.001%, may be systemically available via the oral route, and that where TiO_2 particles have been found this was predominantly in Peyer's Patches, the liver and spleen. They further noted that in a study by Bettini et al. (2017) with TiO_2 administered in drinking water at 10 mg/kg bw per day for 100 days, large aberrant crypt foci (ACF) were observed in exposed animals. However, similar findings have not been found, even in other studies with concentrations orders of magnitude higher. In addition, there are three significant studies which assessed the effects of dietary exposure to TiO_2 in food in rodents. A chronic rodent bioassay carried out by the United States National Cancer Institute

(NCI, 1979) showed no evidence of intestinal tumours or other intestinal lesions, including inflammation, in rats or mice exposed to TiO_2 incorporated into feed for two years. A study designed to replicate the findings of Bettini et al. (2017) (Blevins et al, 2019) found no evidence of ACF in the colon following 100 days exposure to food-grade TiO_2 up to the highest concentration tested (236 – 300 mg/kg bw/day). In a recent extended one-generation reproductive toxicity study performed according to the Organisation for Economic Co-operation and Development (OECD) guideline No. 443 (Leuschner, 2020), no adverse effects were observed up to the highest dose tested (1,000 mg/kg bw per day) administered via the diet when rats were continuously exposed from pre-conception through to adulthood. Health Canada also concluded that the available evidence indicates that food-grade TiO_2 is not genotoxic in vivo, although the number of studies available is limited and more research is recommended to confirm these findings.

18. Health Canada concluded that “In summary, the adverse effects associated with oral exposure to TiO_2 are largely derived from non-standard studies that administered stable, homogenized suspensions of ultrasonically dispersed particles. While these intensive sample preparation steps are necessary and appropriate for particle characterization and hazard identification for nanoscale materials in general, in the opinion of Health Canada’s Food Directorate they do not fully represent exposure to TiO_2 as a constituent of food”. Overall, Health Canada’s Food Directorate did not identify any compelling health concerns for the use of TiO_2 as a food additive in the course of this review. While some uncertainties in the database were identified that would benefit from further research, the weight of available evidence suggested that these data gaps were not significant enough to warrant a more precautionary approach at this time. As is the case for food additives generally, Health Canada’s Food Directorate will continue to monitor the emerging science concerning the safety of TiO_2 used as a food additive and this conclusion may be revisited should new scientific information become available.” Health Canada (2022).

19. The Food Standards Australia New Zealand (FSANZ) published a report (FSANZ, 2022) “Titanium Dioxide as a Food Additive” which had reviewed the key evidence relating to the safety of TiO_2 as a food additive, following the assessment by EFSA in 2021. It was noted that most of the concerns around the safety of TiO_2 as

a food additive were based on studies with TiO_2 in the nanoscale range, or in which food-grade TiO_2 had been sonicated. These are not reflective of the human dietary exposure scenario. FSANZ noted that there is a lack of *in vivo* genotoxicity studies with food-grade TiO_2 in the feed but there was no evidence that exposure through oral gavage or intraperitoneal administration was genotoxic *in vivo*. Their report supported the findings of Health Canada (2022) noting that ACF had been observed in a drinking water study (10 mg/kg bw per day) however this was not replicated in two studies using TiO_2 administered via the diet at much higher concentrations (up to 267 or 1,000 mg/kg bw per day). This was also inconsistent with results from a 2-year bioassay conducted by the United States National Cancer Institute (NCI) which showed no evidence of toxicity or carcinogenicity at dietary concentrations of up to 50,000 ppm TiO_2, (equivalent to doses of 2,250 to 8,350 mg/kg bw per day, depending on sex and species). It was also noted that there was no evidence of systemic toxicity, developmental or reproductive toxicity, developmental neurotoxicity or developmental immunotoxicity in the EOGRT undertaken by Leuschner (2020). Overall FSANZ concluded that “there is no evidence to suggest that dietary exposures to food-grade TiO_2 are of concern for human health”. (FSANZ, 2022).

20. More recently, JECFA (2024) have published the report of their risk assessment of titanium dioxide International Numbering System for Food Additives (INS) 171 (E171). It was noted that the absorption of TiO_2 was very poor and that the bioavailability in humans was very low. Although there were uncertainties in the genotoxicity data, JECFA noted that INS 171 was not carcinogenic in adequately conducted 2-year studies at doses of up to 7,500 mg/kg bw per day for mice and 2,500 mg/kg bw per day for rats, the highest doses tested. JECFA also considered that there was no evidence for reproductive or developmental toxicity effects in animals. There were no epidemiological studies that allowed conclusions to be drawn with respect to associations between dietary exposure to INS 171 and human health effects. JECFA concluded that “Considering the very low oral absorption of INS 171, and in the absence of any identifiable hazard associated with INS 171 in the diet, the Committee reaffirmed the ADI “not specified” established at the Thirteenth meeting.” (FAO/WHO, 2024).

Methodology of the COT review

21. This statement on the safety of TiO_2 E171 as a food additive (considered at the request of the Food Standards Agency following the recent (2021) review of TiO_2 E171 by EFSA) constitutes the views of the COT, on the safety of E171. This assessment has been conducted jointly by the COT and the COM. The COM statements are summarised and referenced in the genotoxicity section. The COM have assessed the available data on the genotoxicity of TiO_2 in vitro and in vivo and the COM statements will be available on their website. The COT had previously considered and commented on the EFSA 2016 Opinion on TiO_2, whilst the UK was part of the EU.

22. In 2023, the COT agreed that a small group of Members would be set up to undertake a more detailed critical evaluation on the additional endpoints (ACF, reproductive and developmental toxicology, neurotoxicity and immunotoxicity).

23. For the purposes of this assessment, the COT mainly considered the updated evidence provided in the 2021 EFSA opinion, with a focus on the EOGRT study commissioned after the 2016 EFSA review, together with several additional studies from 2015 to 2023. In this statement, references to 'The Committee' will be the COT unless otherwise stated.

24. The report by Health Canada, "State of the Science of Titanium Dioxide (TiO_2) as a Food Additive" (2022) had previously been reviewed and commented on by COT Members prior to its publication. Therefore, some of the discussion and conclusions from this document have also been included in the endpoints discussed below. Conclusions and discussion from EFSA (2021), FSANZ (2022) and JECFA (2024) have also been included in the sections discussed below.

25. The EFSA updated opinion (2021) and the EFSA conclusions on the papers that were considered most relevant were reviewed by the COT with an additional literature search undertaken for the COT to consider relevant literature and opinions published between 2021 and 2023, including the Health Canada report on Titanium Dioxide.

26. For the literature search, the database Lit-fetch was used to search the terms listed in Annex A between the dates 2021-01-01 to 2023-04-28. Lit-fetch searches used EBSCO, Scopus, Springer and Pubmed. For the first 2 search strings, the numbers in brackets denote (number of hits; number of relevant hits), the 3rd search string just denotes number of hits. An updated search was also carried out for the first 2 search strings for 2023-04-28 to 2024-03-01, but only for in vivo studies. The search terms can be found in Annex A.

27. Paper titles and abstracts were manually screened, and papers deemed not relevant were disregarded. The methodology used by the COM is described in detail in their statements (COM, 2024a; COM, 2024b).

28. The ADME and toxicity studies used in the assessment are summarised in the relevant sections. Summaries of the conclusions from the assessments by EFSA (2021), Health Canada (2022) FSANZ (2022) and JECFA (2024) are included at the end of each section. The COT review and conclusions for that endpoint are included at the end of the section. A summary table of all of the studies considered by the COT is available in Annex B.

29. The methodology for the exposure assessment is described in detail in the relevant section with additional information provided in Annex C.

30. Following the publication of the COT Interim Position Paper (COT, 2022), the COT have continued to review aspects of the safety of TiO_2 as a food additive and have prepared the following additional papers:

- TOX/2023/16: Introduction - Review of EFSA Opinion | Committee on Toxicity (food.gov.uk),
- TOX/2023/32: Review of Titanium Dioxide: Discussion Paper for Additional Endpoints | Committee on Toxicity (food.gov.uk),
- TOX/2023/33 First draft statement on the safety of Titanium Dioxide (E171) as a Food Additive | Committee on Toxicity,

- TOX/2023/44 Exposure to Titanium Dioxide in the UK population | Committee on Toxicity (food.gov.uk),
- TOX/2023/56 Review of Titanium Dioxide – second draft statement,
- TOX/2024/02 Cover Paper and Tables - Third draft statement on the safety of Titanium Dioxide (E171) as a Food Additive | Committee on Toxicity,
- TOX/2024/14: Fourth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive,
- TOX/2024/18: Fifth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive- Introduction | Committee on Toxicity.

Titanium Dioxide

General information

31. Titanium dioxide (TiO_2) is an inorganic compound which exists in nature in different crystalline forms, the anatase and rutile being the two most important for its use as a food additive. Food grade TiO_2 (E171) is comprised of a mixture of both nano- and micro-sized particles.

32. The Chemical Abstracts Service (CAS) Registry number for TiO_2 is 13463-67-7 and the European Inventory of Existing Commercial Chemical Substances (EINECS) number is 236-675-5. The Colour Index (C.I.) number is 77891.

33. Food grade titanium dioxide (TiO_2) was an authorised Food Additive (E171) in the EU but from the 7th of August 2022 it is no longer permitted following the publication of Commission Regulation (EU) 2022/63, amending Annexes II and III to Regulation (EC) No 1333. Since August 2022, manufacturers in Northern Ireland have not been permitted to produce goods containing titanium dioxide according to EU legislation. It currently remains authorised in Great Britain. TiO_2 is identified by JECFA as INS 171 and is a food additive included in table 3 of the General Standard for Food Additives (GSFA).

34. The uses of TiO_2 as a food additive include:

- as a colour to make food more visually appealing,
- to give colour to food that would otherwise be colourless, and
- to restore the original appearance of food.

35. It is found in a wide variety of food items such as pastries, chocolate and sweets, sauces and chewing gum. It is also widely used in cosmetics and medicines (EFSA, 2016).

Physicochemical Characterisation of food grade TiO_2

36. The characterisation of TiO_2 has been described in detail by EFSA (2016) and in the Health Canada State of the Science Report (2022). This section provides a summary of the TiO_2 characterisation to provide background information for the better understanding of this statement on the safety of TiO_2 as a food additive.

37. TiO_2 is a white powder used as a pigment in various industries and is valued for its high refractive index. Specifically in food, the primary function of TiO_2 is as an opacifier and white pigment. To achieve this function, it is critical that food grade TiO_2 exists as an aggregate of smaller primary particles with a median particle size of 200 – 300 nm, which corresponds to roughly half the wavelength of visible light and provides optimal light scattering, thus producing the desired whitening effect (Winkler et al. 2018).

38. TiO_2 can also be in the form of engineered nano-TiO_2. One form of nano-TiO_2 that is generally sold for catalytic applications and is also widely used in toxicity studies is P25. Food-grade and P25 TiO_2 are engineered products, frequently synthesized from purified titanium precursors, and not milled from bulk scale minerals (Yang et al., 2014). Engineered nano-TiO_2 (e.g., P25, NM-101, NM-102, NM-103, NM-104 and NM-105, see Table 1 below) has 100% particles less than 100 nm in diameter, and is colourless and therefore unsuitable for use as a pigment/opacifier in food and pharmaceutical applications (Farrell and Magnuson, 2017).

39. Pure TiO_2 assembles in several crystal structures although only anatase, rutile or a mixture of the two are used in foods. Food- and pigment-grade TiO_2 are engineered products synthesized from purified Ti precursors. The anatase form can only be made using the sulfate process, whereas the rutile form can be made using both the sulfate and chloride processes (Ropers et al., 2017). The majority of food-grade TiO_2 is produced by the sulfate process in the anatase crystal structure. For many applications, including cosmetics and some therapeutic products, surface coatings are applied to TiO_2 particles that alter their physicochemical properties. Therefore, toxicity and ADME studies using these materials may not be relevant to TiO_2 used as a food additive, which normally undergoes no surface treatment and is uncoated (EFSA, 2016). Due to differences in the manufacturing processes, food-grade TiO_2 differs in surface composition compared to other TiO_2 materials, such as TiO_2-NPs. The surface of food-grade TiO_2 particles is covered by superficial phosphate groups, while materials manufactured with alternative methods may have different surface functional groups (e.g., hydroxyl groups) (Dudefoi et al., 2017a; Yang et al., 2014). The surface properties of TiO_2 materials have a significant impact on their behaviour in various environments, including biological media. Therefore, TiO_2 materials with surface properties that differ from food-grade TiO_2 may not be relevant models when studying the fate of dietary TiO_2 (Dudefoi et al. 2017a).

40. Suspended TiO_2 particles tend to aggregate/agglomerate to form larger clusters. The term “aggregate” designates an assembly of particles held together by covalent or metallic bonds. Instead, “agglomerates” result from weak forces like van der Waals interactions, hydrogen bonding, electrostatic attractions or adhesion by surface tensions. Due to this aggregation and agglomeration, it is important not to equate the nanoparticle fraction measured by number with the same value by mass (Winkler et al., 2018).

41. However, despite having a mean particle size in the desired range (200 - 300 nm), primary particles in food-grade TiO_2 form a broad size distribution that invariably contains particles below 100 nm, i.e. nanomaterial. A number of studies have measured particle size in food grade TiO_2 or E171 and found a range of 17 – 36% by number of TiO_2 NPs with a diameter less than 100 nm (Verleysen et al. 2020 and 2021; Yang et al, 2014; Dudefoi et al. 2017; Weir et al., 2012). The percentage by

number of particles with a diameter less than 30 nm is of the order of 1% or less (EFSA 2021a).

42. When calculating exposure to TiO_2 NPs the equivalent mass of NPs is more relevant than particle number. Several studies reviewed by Ropers et al estimated the mass (weight %) of nanoparticles present in E171 across a range from 0.31 to 12.5% (EFSA, 2016; Bachler et al., 2014; Rompleberg et al., 2016; and Dudefoi et al., 2017). This variation explains some differences in the estimated exposures to TiO_2 nanoparticles in the literature including between the study by Rompelberg et al. who used a value of 0.31% of NPs and the evaluation of EFSA who used a weight ratio of 3.2%. (Ropers et al., 2017).

43. Since E171 is a particulate material containing a fraction of nanoparticles, and toxicology studies mainly focus on the systemic absorption of this nanofraction after ingestion, EFSA (2016) noted the need for more data; for example, information related to the particle size distribution of the titanium dioxide when used as a food additive. Moreover, it was noted that the EU specifications for E171 should also include full characterisation of the particle size, distribution of the materials as well as an indication on the percentage (in number and by mass) of the particles in the nanoscale, together with the information on the analytical methods/techniques used for detection and quantification of the nanosized particles. In this respect, the European Commission called in early 2017 for data addressing those EFSA recommendations. (Verleysen et al., 2020; Geiss et al., 2020).

Physicochemical Characterisation of nano grade TiO_2

44. The consensus within the risk assessment community on the need for standardised and validated dispersion methods for nanoparticles to ensure reliable and reproducible results to help enable the advancement of their risk assessment is well known. Validated approaches for the preparation of TiO_2 nanoparticles solutions either monodispersed or protein stabilised dispersions in relevant biological media, are regularly used for acute *in vitro* or *in vivo* toxicity assessment. Dispersions in biological media, that remain stable for at least 48 h (acute testing timeframe) under typical incubation conditions, are considered useful. The initial objective of most

studies is to determine whether monomodal nanoscale dispersions, in the tested media, can be achieved by mixing aqueous nanoparticle stock with a biological media without prior stabilisation with addition of fetal bovine serum (FBS) or bovine serum albumin (BSA), or other dispersants more relevant to the test system. To achieve this mono-dispersion surface modifications of the NPs, during synthesis, is often undertaken. Most academic studies are based on assessment of dispersed particle solutions, to help answer specific questions, which may not fully represent specific products or properties of products on the market.

45. The OECD Working Party on Manufactured Nanomaterials (WPMN) 'Guidance Manual for Sponsors of the OECD Sponsorship Programme for the Testing of Manufacture Nanomaterials" has collaborated with numerous international projects to develop an understanding of nanomaterials. The Joint Action, NANOGENOTOX, nanogenotox.eu, co-financed by the Executive Agency of the Directorate General for Health and Consumers of the European Commission and 11 EU member states was established to determine characterisation and testing of TiO_2, with a focus on reference materials produced by the Joint Research Centre laboratories. Examples of EU projects testing the materials from the Repository are MARINA (marina-fp) and NANoREG (nanoreg.eu). The NANOGENOTOX sample preparation protocol developed by Commissariat à l'énergie atomique et aux énergies alternatives (CEA), Institut National de la Recherche Sciéntifiqué (INRS) and The National Research Centre for the Working Environment (NRCWE) and the final dispersion protocol is published on the project's web page. To highlight the interventions required to achieve dispersion "2.56 mg/mL of material sterile-filtered 0.05 % w/v BSA-ultrapure water are sonicated (probe sonicator) for 16 minutes, placed in an ice bath, at 400 W and 10 % amplitude while controlling that the sonication probe does not touch the walls of the scintillation vial. Use of different sonication conditions (power and amplitude) require different sonication times. The energy input should be calibrated to be in the order of 3,136 MJ/m3." This highlights the upstream processes required prior to material assessment to investigate the possible relevance of the nano component in toxicology studies.

46. The physico-chemical characterisation of the titanium dioxide series from the JRC repository: NM-100, NM-101, NM-102, NM-103, NM-104 and NM-105 (NM-100

is included in the series as a bulk comparator) is summarised in Table 1. Each material is available as a 2,000 mg white powder kept in amber coloured vials under argon atmosphere. NM-103 and NM-104, have 2% of dimethicone as an external organic coating. The coatings of NM-103 and NM-104 may be unstable under in vitro test conditions. NM-105 is the purest form of the material with others showing trace background elements of C, O, K, Ca and Al.

Table 1. Properties and indicative content of TiO_2 for the JRC titanium dioxide series

NM code	Label name	Properties	Indicative content of TiO_2 (%Wt)
NM100	Titanium Dioxide	Bulk (non nano)	97.7
NM101	Titanium Dioxide	Anatase	98.1
NM102	Titanium Dioxide, Anatase	Anatase	99.6
NM103	Titanium Dioxide thermal, hydrophobic	Rutile	91.3
NM104	Titanium Dioxide thermal, hydrophilic	Rutile	92.7
NM105	Titanium Dioxide, Anatase-Rutile	Anatase-Rutile	99.8

47. Transmission electron microscopy (TEM) micrographs indicate that the TiO_2 NMs, detailed above, have a polydisperse particle size distribution; the average value of the primary particle size was estimated to be below 26 nm for NM-103, NM-104 and NM-105, below 10 nm for NM-102, and above 100 nm for NM-100; for NM-100 primary particle sizes ranging from 20 nm up to 300 nm were detected. The shape of the particles also varied with those for NM-103 and NM-104 given in Table 2.

Table 2. Particle characteristics of selected JRC titanium dioxide materials

Material	Sphericity	Shape Factor	General Morphology
NM-103	Low sphericity.	Very angular to sub-angular.	Angular, low sphericity.
NM-104	Low sphericity.	Angular to sub-rounded.	Sub-angular, low sphericity.

48. It should be noted that the variation of these test material physicochemical characteristics and the nature of their preparation, required for study, should be considered, when attempting potential read-across activities.

Physicochemical Characterisation considerations for this assessment

49. Due to the differences in the characteristics of food grade TiO_2 and engineered nano-TiO_2 this assessment has focussed on the data from studies using food grade TiO_2 or E171. Consideration has been given to studies that used nano-TiO_2 or food-grade TiO_2 which was de-agglomerated prior to testing. However, as these are likely to provide limited information for the evidence base for food grade TiO_2 or E171 when tested as consumed, these are usually only summarised in the text, but details of the studies can be found in the study summary tables in Annex B.

50. The majority of studies tend to use percentage of NPs by particle number rather than mass.

Absorption, Distribution, Metabolism and Excretion (ADME)

51. The COT reviewed a number of studies to assess the ADME of TiO_2, which had also been reviewed by EFSA, Health Canada, JECFA and/or FSANZ. These studies are described below and a brief overview of the review and conclusions of EFSA, Health Canada, FSANZ, and JECFA are included. The COT review and conclusions are presented at the end of each section.

Studies used to review the toxicokinetics of the E171 form of TiO_2

52. The toxicokinetics of E171 has been addressed in a few studies. The COT have assessed 8 studies in total in their review, 6 studies in mice and rats and 2 studies in humans.

E171 studies in rats and mice

Talamini et al (2019)

53. Groups of NRF mice (n = 22/group) were treated with 5 mg/kg bw per day of E171 (99.3% pure anatase, 35% nanoparticles, 201.2 ± 8.5 nm) dispersed in water (no sonication or deagglomeration). The animals were treated for 3 days/week for 3 weeks receiving a total of 9 treatments in 21 days (average daily dose of 2 mg/kg bw) with E171 or water (control) which was slowly dripped with a pipette into the mouths of the mice.

54. The animals were weighed at the beginning of the experiment and observed daily. On day 21, the animals were terminated and the lungs, liver, stomach, spleen, kidney, brain, testes and whole intestine were removed. The concentrations of titanium were determined in 4 animals.

55. No signs of general toxicity were observed. In the brain, kidney and testes, titanium levels were <0.03 µg/g, the limit of quantification (LOQ) of the analytical method for solid tissue samples. In lungs and spleen, the levels were low, with a not statistically significant, but slightly higher deposition in spleen of E171 treated animals compared to the controls. The authors reported that “Titanium concentration was one order of magnitude greater in the small intestine compared to the above tissues and distinctly higher in the stomach, large intestine and liver”. The concentrations of titanium in treated animals were: 1.07 ± 0.38 µg Ti/g tissue in the large intestine and 0.94 ± 0.57 µg Ti/g tissue in the liver. These levels were 1.8 and 3.6 times higher compared to the controls, respectively.

Comera et al (2020)

56. In the first round of experiments adult C57BL/6 mice (12 – 18 weeks) (4 animals/group) were treated with a single gavage dose of 40 mg/kg of E171 (>95% anatase, 20 – 340 nm TEM; 44.7% nanoparticles) suspended in water and sonicated or water only as a control. In the second round of experiments, 300 µg/mL of E171 was suspended in buffer and used to fill a closed mid-jejunal loop of 10 cm, pre-treated with inhibitors of tight junctions, micropinocytosis, clathrin-mediated endocytosis or raft-dependent endocytosis.

57. Animals were terminated at 2-, 4-, 8- and 24-hours following treatment. Confocal microscopy and micro x-ray fluorescence imaging were used to analyse the presence of particles in both the first and second round of experiments, whilst inductively coupled plasma mass spectrometry (ICP-MS) was used to determine titanium concentration in blood and tissues (jejunum, ileum, colon). The jejunal or colonic intraluminal contents were recovered by gentle scraping.

58. In mice treated with a single dose of E171, the number of titanium dioxide reflective particles in the lumen of the small intestine was significantly increased, with analysis of the particles suggesting that no further agglomeration of titanium dioxide occurred during its transit through the intestinal tract, and that as it moved in the distal intestine, there was a decrease in its agglomeration state (as indicated by smaller particle size in the colonic versus the jejunal lumen).

59. An increase in the reflective particle content was observed in the jejunal and ileal villi, Peyer's Patches and colon crypts. The overall particle content in jejunal villi increased from 2 hours after gavage, peaked at 4 hours, and returned to basal values at 8 hours. A statistically significant increase ($p < 0.001$) 4 hours after E171 administration was observed in the titanium dioxide particle density in the jejunal mucosa (increased by 3.4-fold over the controls). A lower and non-significant trend of increased particle content was also observed at 4 hours in the ileum and colon, with the values decreasing close to control levels at the 8-hour timepoint in all three intestinal sections. In the jejunum, the reflective TiO_2 particle spots displayed a mean diameter of 700 ± 59 nm ($n = 70$). From the ICP-MS determined concentrations of TiO_2 in the intestines and the weight of the mice tissues, the authors calculated that approximately 0.007% of the titanium administered was present in the entire intestine at the 4-hour timepoint. The authors concluded that TiO_2 was absorbed predominantly in the ileum, partly in the jejunum and a small amount was absorbed in the colon. Based on the information on uptake capacity per unit of surface area it was concluded that titanium dioxide is predominantly absorbed by the small intestinal villi and to a lesser extent through Peyer's patches.

Riedle et al., (2020)

60. C57BL/6 mice (6 males and 6 females per treatment group) were fed diets with food grade TiO_2 (anatase, with a diameter of 119 nm) at doses of 0, 6.25, 62.5 and 625 mg/kg of diet. These were equal to 0 and approximately 1, 10 and 100 mg food grade TiO_2/kg bw per day.

61. The animals were terminated at 6, 12 and 18 weeks. Animals terminated at 18 weeks were also used to validate that the diet permitted uptake in the intestinal lumen. The basal regions of the Peyer's patches were surveyed, and reflectance confocal microscopy was used to determine the presence of TiO_2.

62. Reflectant foci, indicative of TiO_2 presence, were found at the base of the Peyer's patches in all dose groups. Scanning electron microscopy (SEM) coupled to energy-dispersive X-ray (EDX) confirmed that the tissue contained subsurface particles rich in titanium. In the low and mid dose groups, weak signals were detected in the impacted cells at the base of the Peyer's patches, whereas higher signals were observed at the highest dose group.

Bettini et al., (2017)

63. The COT noted that there was evidence of significant clearance through organs, including the liver, in the Bettini *et al*. (2017) study which examined the fate of TiO_2 along the gut-liver axis in adult male rats. Firstly, rats (10 per group) were dosed daily by gavage with food-grade TiO_2 (E171) or TiO_2 NM-105 (10 mg/kg bw/day) for 7 days (the control group was given water). These animals were used for tissue imaging, flow cytometry and cytokine assays and to carry out tissue inflammation and gut permeability measurements.

64. It was found that TiO_2 particles did not re-agglomerate in vivo during transit through the gut. Following absorption, light-diffracting TiO_2 particles were found along the small intestine, in the colonic (large intestine) mucosa and liver of rats dosed orally with E171 for 7 days, but not in the controls. Using NanoSIMS analyses of subcellular TiO_2 distribution in the immune cells of Peyer's patches after 7 days of oral exposure, Ti was detected in the gut lumen and in the colonic mucosa. TiO_2 was also found in the liver, with the highest density found close to the portal vein sinus. There was no significant change in epithelial paracellular permeability to 51Cr-

ethylenediaminetetraacetic acid (EDTA) observed in the E171 group when compared with control animals. This indicates that oral exposure to E171 did not affect gut permeability in vivo.

Farrell and Magnuson (2017)

65. A Good Laboratory Practice (GLP)-compliant study of food-grade TiO_2 (anatase) was carried out in rats and performed in accordance with OECD test guideline (TG) 417 to assess the absorption, distribution and excretion routes in rats after oral exposure to TiO_2. The TiO_2 form of interest from this study was the anatase form (D50 = 133 – 146 nm) and was incorporated into the feed at a dose equivalent to 30 mg/kg bw per day of male and female SD rats (n = 10 per sex per group). This is based on 200 ppm in feed and assumes daily consumption of 30 g diet per day by a 200 g rat. Rats received either TiO_2 or a control diet (no TiO_2 added, background concentration 7 – 9 ppm) for 7 days. Following the 7 days treatment, the TiO_2 was withdrawn from the diet and 3 rats per sex were terminated at 1, 24 and 72 hours. After the TiO_2 was removed from the diet, the animals were housed individually and those allocated to the 72 hours group were housed in metabolic cages and all excreta including the cage washings were kept. The Ti content of excreta, whole blood, liver, kidneys and muscle was measured using inductively coupled plasma – atomic emission spectrometry (ICP-AES).

66. TEM micrographs indicated that the anatase TiO_2 test material exists primarily as aggregated particles up to approximately 1 µm in diameter and approximately 36% of the discrete but aggregated particles are <100 nm in diameter. However, it is not anticipated that these will de-aggregate during the manufacture of pharmaceutical or food products or in the gastrointestinal (GI) tract. Aggregation of the anatase TiO_2 was also assessed using dynamic light scattering technique. The median particle size (d50) was determined as 336 nm. There was no measurable quantity of TiO_2 that had a particle size <100 nm.

67. No adverse effects were found during clinical observations related to the consumption of TiO_2. Ti levels in liver, kidney and muscle samples were below the limit of detection (LOD) (0.1 to 0.2 mg/kg wet weight) in most rats at most time points; of a total of 270 tissue samples (90 each from liver, kidney and muscle), 176

(65%) were below the LOD. Sporadic observations of levels above the LOD were found, in the range of 0.1 to 0.3 mg/kg wet weight and occurred at similar levels and frequency in TiO_2-exposed rats as those in the control group.

68. The majority of Ti was excreted in faeces with concentrations in urine below the LOQ (<0.04 mg/L; equivalent to < 2% daily dose/L) for all samples except the 0-to-24-hour urine sample for one rat (0.05 mg/L); collection of urine and faeces began after withdrawal of the test diet and therefore no mass balance recovery was estimated. Whole blood Ti concentrations were also below the LOD in both treatment and control groups. The authors concluded that food-grade TiO_2 administered in diet is not appreciably absorbed and distributed in mammalian tissues, and there is no evidence of accumulation in liver, kidney and muscle following repeated oral exposure for 7 days.

EBRC (2022)

69. An unpublished summary report of a GLP-compliant, multi-site toxicokinetics study of 5 different grades of TiO_2 carried out in accordance with OECD 417 test guidelines was submitted to the FSA by the TDMA (EBRC, 2022). In this study, male and female CD rats received either a vehicle control or a single dose of 1,000 mg/kg bw of TiO_2 administered by oral gavage and the total Ti content of whole blood was measured for up to 96 hours post-dosing. A soluble Ti reference substance (Titanium (IV) bis(ammonium lactato)dihydroxide solution (50 wt. % in H_2O)) was administered orally (100 mg/kg bw) or intravenously (10 mg/kg bw) to assess the relative bioavailability of the various grades of TiO_2.

70. The test articles included E171-E which had a median particle diameter (SD) of 99.9 ± 2.0 nm and contained approximately 50-51% by number of constituent particles in the nanoscale (LNE, 2020). The other four particles were identified as G6-3 (a rutile TiO_2-NP coated with alumina and hydrophobic organic, D50 = 9.2 ± 2.0 nm), G2-5 (uncoated anatase TiO_2-NP, D50 = 5.5 ± 2.0 nm), G3-1 (uncoated pigmentary rutile TiO_2, D50 = 146.9 ± 5.9 nm) and G4-19 (pigmentary rutile TiO_2 coated with alumina and polyol, D50 = 177.5 ± 3.9 nm). (Health Canada, 2022).

71. Blood was collected over a period of up to 96-hours post-dosing and Ti concentrations measured by ICP-MS/MS following microwave-assisted acid digestion of whole-blood samples using H_2SO_4. The use of this analytical method and of whole blood includes the bioavailability of particulate TiO_2 as well as any dissolved Ti.

72. Blood Ti concentrations of vehicle-control treated male rats were variable with several males considered statistical outliers and a decision was made to exclude these from data processing.

73. After oral administration of all five TiO_2 test articles, the mean blood Ti concentrations of male and female rats was below 0.2 µg Ti/g blood. After i.v. and oral administration of the soluble titanium reference standard, substantially higher blood concentrations of 90 µg Ti/g blood and 0.9 µg Ti/g blood, respectively were measured.

74. The group that had received the food-grade TiO_2 test item E171-E had the highest blood Ti concentrations following oral dosing. Areas under the curve (AUC) were plotted for rats that had been dosed with the reference substance orally or by i.v. and that had received E171-E by oral administration. The maximum relative oral bioavailability of E171-E was determined to be 0.0013%.

75. The measured Ti blood levels from rats exposed to the other four forms of TiO_2 were below the LOD after background correction. The authors concluded that the oral bioavailability of all five of the TiO_2 forms tested was very close to the LOD of the analytical system. It was also noted that most reagents used in the process contained low but measurable Ti background concentrations, Therefore the analysis of low levels is made challenging. The background levels of Ti measured in the blood of control animals, especially in the males, shows some variability, which is consistent with time zero levels measured during dietary studies." (EBRC, 2022).

E171 studies in humans

Pele et al., (2015)

76. Eight healthy volunteers with normal intestinal permeability were given a permeability solution. At 7am, following an overnight fast, baseline urine samples were collected. After consumption of the solution, urine samples were collected for 5 hours.

77. Baseline blood samples were also taken at 9 am. Following that, the subjects received two tablets, each containing 50 mg of pharmaceutical/food grade TiO_2 (anatase, d50 of 260nm) (total dose 100 mg). Blood samples were collected at: 30 minutes, 1-, 1.5-, 2-, 3-, 6-, 8- and 10-hours post TiO_2 ingestion. Of the 8 volunteers, only 7 completed the study as blood could not be withdrawn from the cannula of 1 subject.

78. Dark field microscopy was used to identify titanium dioxide in the blood. Random areas were visualised and the estimation of particles within each field was based on four reflective grades: 0 (<5 particles/field); 1 (5-10 particles/field); 2 (10-20 particles/field); and 3 (>20 particles/field). This analysis was only performed in 5/7 subjects due to blood clotting in two subjects. ICP-MS was used to quantify titanium in the blood for 0 - 10 hours, except in two subjects for whom samples could not be collected at 8 hours (2 subjects) and 10 hours (1 subject).

79. Based on the results of the dark field microscopy, it was determined that some of the ingested titanium dioxide was absorbed directly into the blood. A significant increase in positive signals was observed from 2 hours onwards and both dark field microscopy and ICP-MS demonstrated a peak in absorption at 6 hours, reaching up to 11 ng/mL and decreasing to around 5 ng/mL by 10 hours post exposure. Only the titanium levels from 6 hours post exposure onwards were significantly different than the baseline. A positive correlation between reflective grades and total titanium levels was observed.

80. The authors hypothesised that two routes of uptake in the gut were involved: one proximal (in the duodenum/jejunum) and one distal (Peyer’s patches in the ileum). This was because at two hours the uptake was visible in the dark field microscopy and the levels peaked at 6 hours as determined by ICP-MS (i.e., early absorption and late peak).

Guillard et al., (2020)

81. The test material used was titanium dioxide (E171) particles with a mean particle size of 104.9 ± 44.9 nm and a particle size distribution ranging from 20 to 440 nm, with 55% of NPs by number.

82. Human placentae were collected at term from normal pregnancies. Transplacental passage of titanium dioxide (E171) (15 µg/mL) was determined using an ex vivo placental transfusion model with a perfusion time of 90 minutes. The samples were analysed using ICP-MS and scanning transmission electron microscopy (STEM) coupled to EDX spectroscopy for content analysis of titanium and analysis of titanium dioxide particle deposition, respectively.

83. All term placental samples (n=22) contained titanium with the total content ranging from 0.01 to 0.48 mg/kg of tissue. STEM-EDX confirmed the presence of titanium and oxygen in the particle deposits seen by TEM, as well as aluminium, silicon, iron, zinc and tin trace elements. Most of the analysed titanium dioxide particles were below 100nm. Size particle analysis of all particles indicated that 50% were below 100nm in diameter.

84. Meconium samples were also collected from nappies. In 50% of the meconium samples (total of 18 samples), titanium was detected (0.02-1.5 mg/kg). TEM-EDX analysis confirmed the presence of titanium and oxygen elements in the particle deposits, alongside silicon, aluminium, iron and zinc. Analysis of all particles indicated a diameter of 5-194 nm, with 26/33 (80%) samples in the nano range.

85. In the transplacental passage experiment, of the 7 ex vivo isolated perfused placentae, round shaped or small particle aggregates of titanium dioxide were observed. Titanium dioxide particles were recovered in the syncytiotrophoblast microvilli and had translocated in deeper areas of the placental chorionic mesenchyme surrounding foetal vessels. The particles had a diameter of below 250 nm, with 17 of them in the nano range.

86. The authors concluded that the results indicated the passage of titanium dioxide particles across the human placenta with potential local accumulation during

pregnancy, depending on the individual. The findings of the perfused placenta experiment indicated, according to the authors, that the human placental barrier is unable to completely prevent the passage of titanium dioxide from dietary sources and protect the fetus.

87. Based on both experiments (results of perfused placenta study and the titanium levels in the placenta and meconium), the authors noted that there was a need to assess the risk of titanium dioxide nanoparticle exposure in pregnant women and warranted specific attention for oral exposure to the nanosized fraction of the E171 food additive.

Studies used to review the toxicokinetics of the nanoparticle form of TiO_2

Studies in rats and mice

88. Three studies investigated the kinetics of TiO_2 nanoparticles using intravenous injection (Geraets et al., 2014; Disdier et al., 2015; and Kreyling et al., 2017a). In these, just under 100% of the administered dose was biodistributed to the organs. However, when administered by gavage Kreyling et al (2017b) estimated absorption from the GI tract was 0.6% of the administered dose. Therefore Kreyling et al. (2017b) concluded that the kinetic pattern following the intravenous (i.v.) route were very different from those of the oral route and that “i.v. injection appears not to be an adequate surrogate for assessing the biodistribution and potential health effects occurring after oral exposure to TiO_2 nanoparticles”. The focus of the COT assessment is TiO_2 as a food additive, where the relevant route of administration is oral, therefore, studies using i.v. administration have not been considered further in this Statement, but the study details are included in the study summaries in Annex D.

89. Of the remainder of the studies that assessed TiO_2-NP kinetics, none involved dietary administration. Seven studies dosed the animals using gavage (Kreyling et al., 2017b; Geraets et al., 2014; Ammendolia et al., 2017; Chen et al., 2020; Warheit, Boatman and Brown, 2015; Tassinari et al., 2014; and Bettini et al., 2017). The study by Hendrickson et al., (2016) used intragastric administration, and the study by Hendrickson et al., (2020) used an isolated intestinal loop technique.

90. The studies that used nanoparticles (not classed as food-grade or E171 by the authors) involved a range of sizes. The majority of the studies measured individual nanoparticles (engineered nanoparticles such as NM-101 at 5-10 nm up to about 60 nm in one dimension) and the size of agglomerates/aggregates (which ranged from about 80 nm up to approximately 2 µm) predominantly using TEM, SEM and dynamic light scattering (DLS) techniques. A few studies also assessed the percentage (by number) of the particles. For example, in Ammendolia et al. (2017) the average diameter of NPs in MilliQ water ranged from 70 – 1,200 nm with a peak at 60 – 90 nm, average diameter of 76 nm. This corresponded to an abundance of 11% of the particles. Only 13% of the NPs had dimensions less than 100 nm. In the study by Tassinari et al (2014) TEM showed individual NPs of 20 – 60 nm and analysis using SEM showed 87% of particles were 30 – 900 nm and 48% of those were 100 – 300 nm, with an average diameter of size distribution of 284 ± 43 nm. Thirteen percent of particles had dimensions <100 nm whereas the size distribution was dominated by agglomerates up to 1.6 µm in diameter.

91. The study by Kreyling et al (2017b) which looked at the distribution of [^{48}V]TiO_2NP in rats after 1 hour and up to 7 days following intra-esophageal instillation estimated only approximately 0.6% of the dose was absorbed across the intestinal barrier in the first hour after gavage. After 7 days, only about 0.05% was retained in the body. The rest of the material is excreted in the faeces. Other studies agreed that the absorption of TiO_2 across the gut epithelium was very low following dosing via gavage (oral route).

92. Kreyling et al (2017b) and others (Geraets et al., 2014; Ammendolia et al., 2017; Chen et al., 2020; Warheit, Boatman and Brown, 2015; Tassinari et al., 2014; and Bettini et al., 2017) also looked at deposition of TiO_2 NPs in various tissues. The tissues in which measurable levels were detected across all studies include small intestine, liver, spleen, kidneys, lungs, heart and brain. However, the studies used different nanomaterials, different dosing duration and evaluation timepoints, and different methods of analysis.

Studies in humans

Heringa et al., (2018)

93. Titanium was measured using high resolution ICP-MS in liver and spleen from 15 deceased human subjects (nine women and six men) who had donated their bodies for research and educational purposes. The donors had died at the age of 56 to 104 years. Samples were fixed in 4% formaldehyde after collection. Two of the subjects had received titanium implants. The LOD of the method was 10 ng/g tissue. TiO_2 particles were measured using single particle ICP-high resolution MS (spICP-HRMS). The smallest particle size that could be detected was 85 nm.

94. Total Ti content in the liver ranged from 0.02 to 0.09 mg Ti/kg tissue with an average value of 0.04 ± 0.02 mg Ti/kg tissue. In spleen, total Ti content ranged from 0.02 to 0.4 mg Ti/kg tissue with an average value of 0.08 ± 0.1 mg Ti/kg tissue. Levels in the two subjects who had received titanium implants were comparable to those in the other 13 subjects. TiO_2 particles were detected in 7/15 liver and 13/15 spleen samples. The number-based TiO_2 particle size distributions in liver and spleen were comparable and had a size range of 85–550nm and 85–720nm, respectively. In the tissues, 24% of the TiO_2 particles in the number-based size distribution was <100 nm, but this fraction may be underestimated, given the LOD_{size} was 85 nm.

95. The particle mass concentration in liver ranged from 0.01 to 0.3 mg titanium/kg tissue. In the spleen, the concentration ranged from 0.01 to 0.4 mg titanium/kg tissue. The average concentration in samples where titanium could be determined was 40 ng/g in the liver and 80 ng/g in the spleen.

96. Small tissue grains of liver and spleen from two subjects were analysed using SEM-EDX to visualize the titanium dioxide particles. The observed particles were composed of titanium and oxygen and were present as an aggregate or agglomerate, consisting of smaller primary particles of 75–150 nm. Presence of titanium was also confirmed semi-quantitatively by EDX analysis in dry-ashed liver and spleen samples.

Peters et al (2020)

97. Post-mortem human liver, spleen, kidney, jejunum and ileum samples were analysed from 15 human subjects, 7 male and 8 female, who died at the age of 64–98 years. Only 12 kidneys, jejunum and ileum were obtained. From these persons, written informed consent was obtained during life that allowed the use of their entire bodies for educational and research purposes. Samples were fixed in 4% formaldehyde after collection. Titanium was measured by ICP-HRMS, with an LOD of 10 ng/g tissue. TiO_2 NPs were quantified using spICP-HRMS. The size limit for detection was 50 nm. The presence of TiO_2 NPs was confirmed by SEM-EDX.

98. The total titanium concentration in the organs ranged from 0.01 to 2.0 mg titanium/kg tissue with an average value of 0.17 mg titanium/kg tissue and a standard deviation of 0.33 mg/kg. The authors considered that this was an indication of large differences between subjects and organs. The highest concentrations were detected in the jejunum and ileum (average of 0.34 and 0.43 mg titanium/kg respectively), followed by the kidney, spleen and liver (0.08, 0.06 and 0.03 mg titanium/kg respectively).

99. The particle sizes ranged between 50 and 500 nm in the different tissues and were comparable. 17% of particles were <100 nm. TiO_2 particle mass concentrations ranged from 0.01 - 1.8 mg Ti/kg tissue with an average of 0.14 ± 0.30 mg Ti/kg tissue. The titanium dioxide particle concentrations were considered by the authors to represent about 80% of the total titanium present.

EFSA review and conclusions on ADME of TiO_2

100. On the basis of the data available, the EFSA ANS Panel concluded that the absorption and oral bioavailability of titanium dioxide was low, independent of size. EFSA had reviewed the toxicokinetics of E 171 in three mouse studies and two human studies. The studies by Comera et al., (2020) and Talamini et al., (2019) allowed the derivation of estimates of internal exposure of 0.01 and 0.1% of the external dose, respectively. (EFSA, 2021).

101. In humans, the Panel considered that after oral administration of 100 mg E171 (Pele et al., 2015), blood Ti increased ca. 5- to 10-fold from 6 to 10 h post-dosing,

demonstrating some oral systemic availability. A study by Guillard et al., (2020), indicated that TiO_2 is systemically available after ingestion and can distribute to the placenta. The Panel noted that the extent of transfer across placental membranes was small. (EFSA, 2021).

102. The Panel noted that materials other than E 171, mainly TiO_2 NPs, were also investigated, in rats and humans. In rats, two intravenous studies (Disdier et al., 2015; Kreyling et al., 2017a) demonstrated long half-lives and, hence the potential for accumulation. Out of five oral rat studies, one provided an estimate for oral systemic availability of 0.0002% based on a limited number of organs (Hendrickson et al., 2016) and another study provided an estimate of 0.6% (Kreyling et al., 2017b). The Panel noted that in a study by Hendrickson et al., (2020), the authors provided data indicating the presence of TiO_2 NPs either as single particles or as smaller and larger agglomerates in intestine, liver and spleen.

103. "The Panel considered that from the two studies analysing tissues from deceased subjects (Heringa et al., 2018 and Peters et al., 2020), the presence of Ti-containing nanoparticles was observed in liver, spleen and kidney as well as in the intestine. Quantification of the Ti in the organs and comparison with the estimated mean daily intake of E 171, allowed the Panel to conclude that the oral systemic availability of TiO_2 NP would be low (less than 1% by mass). (EFSA, 2021).

104. Overall, the FAF Panel considered that E 171 has a low oral systemic availability, probably not greater than 0.5%. It may pass the placenta and may be transferred to the fetus. The Panel had also considered that rat studies with TiO_2 NPs, with primary particle sizes between 7 and 90 nm, showed long half-lives, a potential for accumulation and long time to reach steady state (Geraets et al., 2014; Disdier et al., 2015). The oral systemic availability of these materials was low (most probably <1%) but higher than for E 171. In tissues from deceased subjects, TiO_2 particles were identified in liver and spleen, the low Ti amount of the investigated organs indicating low oral systemic availability of E171 TiO_2. (EFSA, 2021).

Health Canada review and conclusions on ADME of TiO2

105. Engineered TiO_2-NPs are often used as surrogates in toxicity tests to represent the fraction of particles in the nanoscale in food-grade TiO_2. However, unlike food-grade TiO_2, these particles have a distribution wholly in the nanoscale. (Health Canada, 2022).

106. There is evidence of very low and size-dependent oral absorption of TiO_2 particles in rodents and humans that may occur primarily via the GALT, with the absorbed material mainly being retained in the intestines, liver, spleen, and kidneys (Bettini et al. 2017; Coméra et al. 2020; Farrell and Magnuson 2017; Heringa et al. 2018; Hummel et al. 2014; Peters et al. 2020; Riedle et al. 2020; Talamini et al. 2019; EBRC 2022). Only one GLP- and OECD guideline-compliant toxicokinetics study with food-grade TiO_2 was identified in the literature (Farrell and Magnuson 2017). In this study, repeated exposure to food-grade TiO_2 in the diet at concentrations of 200 ppm (equivalent to 30 mg/kg bw/d) for 7 days resulted in no appreciable absorption or distribution to tissues or organs and no evidence of accumulation in the liver, kidney, or muscle of male or female rats. A second unpublished multi-site toxicokinetics study conducted according to OECD and GLP guidelines was submitted to Health Canada by industry (EBRC 2022). In this study, the maximum relative bioavailability of 5 different TiO_2 grades was approximately 0.001% following a single oral dose of 1000 mg/kg bw in CD rats. (Health Canada, 2022).

107. Taken together, evidence from rodent and human studies indicates very low and size-dependent oral absorption of TiO_2 particles may occur primarily via the GALT, with the absorbed material mainly being retained in the intestines, liver and spleen (Winkler et al. 2018; Heringa et al. 2018). (Health Canada, 2022).

108. It should be noted that the majority of studies that investigated the toxicokinetics of TiO_2-NPs in rodents administered particles by oral gavage. Gavage studies conducted with insoluble particles have several issues that may complicate interpretation of the results, including disruption of the gastric mucus layer, which may enhance systemic bioavailability of the administered dose. In addition, concentrated bolus doses used in gavage may produce artifactual changes in particle size distribution which would not be reflective of what humans are exposed to

through the diet; for example, higher bolus doses of TiO_2 may lead to greater agglomeration of particles and paradoxically lower exposure to primary particles. The lack of exposure to a food matrix prior to ingestion may also potentially affect toxicokinetic properties. (Health Canada, 2022).

FSANZ review and conclusions on ADME of TiO_2

109. Studies submitted to FSANZ with food grade TiO_2 indicated that the relative oral bioavailability in rats is ≤0.0013%. They noted that the majority of the material that is absorbed is retained in the Peyer's patches of the intestine. There is some distribution to the liver, spleen and kidneys. Data from human cadavers shows relatively low bioavailability and with the age of the subjects the TiO_2 would be expected to have reached steady state. FSANZ concluded that, considering the animal and human study data, the absorption of TiO_2 following oral exposure is very limited. (FSANZ, 2022).

JECFA 2024 conclusion on ADME of TiO_2

110. The extent of absorption of TiO_2 is difficult to measure because of the variability in background concentrations of Ti in tissue, and concentrations of Ti in blood and tissue that are close to the detection limits. It is also difficult to compare the extent of absorption between studies due to the different vehicles used to suspend the TiO_2 (which may affect the size of aggregates), the methods of preparation (e.g. sonication) and the methods of detection.

111. JECFA reviewed and summarised the animal studies (Riedle et al, 2020; Talamini et al, 2019; Farrell and Magnuson, 2017; Leuschner, 2020; Bettini et al, 2017; Vignard et al, 2023) and absorption studies in humans (Bockmann et al, 2000; Balcaen et al, 2014; Jones et al, 2015; and Pele et al, 2015).

112. JECFA concluded that when TiO_2 is administered to animals, (oral) absorption is very low (e.g. < 0.00075%). Absorption in humans is also very low.

COT review and conclusions on ADME of TiO_2

113. It should be noted that various chemical forms of TiO_2 were used in the different toxicokinetic studies. The COT concluded that the size and shape of TiO_2 particles in test materials can affect absorption and particle agglomeration. The modes of delivery varied, with some studies administering TiO_2 in water rather than via complex food groups (Bettini *et al.*, 2017; Talamini *et al*., 2019; and Comera *et al*., 2019). The COT raised concerns that TiO_2 may not fully disperse in water, which could affect absorption, however the effect remains unclear. Some studies where the TiO_2 was prepared suspended in water included the addition of stabilisers to force deagglomeration, while other studies utilised long sonication steps, which can cause structural changes to TiO_2.

114. The COT noted further concerns around absorption, especially relating to nanoparticles/nanofractions, as these made a variable contribution to the test substances and hence their absorption varied in the studies described above. The COT could not separate the impact of the TiO_2 form from the effects of the matrix of administration to conclude on the causes of the observed variability in absorption.

115. The COT noted that TiO_2 particles can cross the placenta. However, because gastrointestinal absorption is low, this would be a very small number from dietary exposure. It was unclear whether this was by passive diffusion or active uptake and, what form the TiO_2 was in by the time it got to these barriers. Once intracellular, materials can change in response to differences in pH and be transferred elsewhere.

116. The COT opinion with regard to absorption was that there was no reason to believe that titanium dioxide particles behaved differently to other particles in the gastrointestinal tract.

117. The COT noted that the Heringa et al. (2018) study, in samples from human subjects after many decades of exposure, would have reflected steady state levels of TiO_2. Due to the nature of the study, it was possible to identify the sources of the TiO_2 but these would not have included just the diet and hence the values reported are likely to be an overestimation of the quantities that can be accumulated over a lifetime from food.

118. Overall, the COT concluded that the form of TiO_2 may affect the likelihood and quantity of the TiO_2 material crossing barriers into organs. The wide variance of test materials used in each study between nano, micro and a mixture in sizes of TiO_2 was noted. Due to the large variance in test materials (form and size) as well as the potential impact of the matrix of administration on the absorption of TiO_2, the Committee could not ascribe a specific percentage for the absorption of TiO_2. However, the Committee considered that, on balance, absorption is low.

Review of toxicity for endpoints identified by the COT

119. The following endpoints were reviewed, initially by the COT and then in more detail by a sub-group of COT Members: aberrant crypt foci (as a potential marker for carcinogenicity), genotoxicity (also reviewed by COM), inflammation and immunotoxicity, allergenicity, reproductive and developmental toxicity and neurotoxicity. The COT's reviews are discussed in detail in each of these sections for the endpoints.

Reproductive and Developmental Toxicity

Studies using E171 or equivalent form of TiO_2

EOGRT study (Leuschner, 2020)

120. Following a call for data by EFSA to address several data gaps for TiO_2, an extended one generation reproductive toxicity (EOGRT) study was conducted (Leuschner, 2020). Because the data from this study covered a number of endpoints in addition to reproductive and developmental toxicity, i.e. aberrant crypt foci, immunotoxicity, and neurotoxicity, the study methodology is summarised in the following paragraphs and the results discussed in the relevant endpoint sections below. No other reproductive or developmental toxicity studies of E171 were identified.

121. The COT reviewed the EOGRT study in TOX/2023/16. The EOGRT study section below uses the information provided in that paper.

Methodology

122. The EOGRT study was commissioned by interested business operators to address the data gaps identified in the EFSA 2016 Opinion. The protocol was later amended to include a satellite group per dose at the F0 generation to accommodate the investigation of additional parameters related to the occurrence of titanium dioxide-related induction of aberrant crypt foci (ACF) in the colon (preneoplastic lesions that had been reported by Bettini et al. (2017)). The study was carried out according to OECD TG 443 and good laboratory practice (GLP) compliance.

123. The test material: Titanium dioxide E171-E, Particle size (ECD); (number measurement, primary particle size) x10 = 0.070 µm x50 = 0.110 µm x90 = 0.180 µm via the diet. The doses used were: Group 1: 0 mg/kg b.w./day; Group 2: 100 mg/kg b.w./day; Group 3: 300 mg/kg b.w./day; 4: 1000 mg/kg b.w./day. Twenty male and 20 female rats from each dose group were evaluated. The concentration of the test item in the diet was adjusted based on the mean group food consumption per sex. The concentration was adjusted weekly using the food consumption values from the previous week.

124. The test item was administered from 10 weeks prior to mating, during mating and until weaning of the F1 and F2 generations. The F1 generation was dosed in the same way as the F0 generation after weaning. Until weaning, the exposure of the F1 pups to the test item was indirectly through the breast milk, however the pups additionally received the test item directly when commencing feeding by themselves during the last week of the lactation period. The duration of dosing depended on the requested endpoints for the different cohorts of the F1 generation. Cohort 1B animals were maintained on treatment beyond postnatal day (PND) 90 and bred to obtain an F2 generation. Detailed examination of key developmental endpoints, such as offspring viability, neonatal health, developmental status at birth, and physical and functional development until adulthood, was performed to identify specific target

organs in the offspring. Possible endocrine disruptor effects of the test item were also examined.

125. Table 3 describes the parameters considered in the EOGRT study with the corresponding generation, cohort and number of animals assessed for each.

Table 3. Parameters considered in the EOGRT study.

Generation	**F0**	**F0 satellite**	**F1 pups**	**F1 1A**	**F1 1B**	**F1 2A**	**F1 2B**	**F1 3**	**F2**
Number of animals /sex per dose group	20	30	N/A	20	20	10	10	10	N/A
Mortality	X	N/A	X	X	X	X	N/A	X	N/A
Clinical signs	X	N/A	N/A	X	X	X	N/A	X	N/A
Body weight	X	N/A	X	X	X	X	N/A	X	X
Food consumption	X	N/A	N/A	X	X	X	N/A	X	N/A
Water consumption	X	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A
Haematology	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Clinical biochemistry	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Lymphocyte typing (spleen)	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Urinalysis	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Sexual hormone levels	X	N/A	N/A	X	X	X	X	N/A	N/A
Thyroid hormone levels	X	N/A	X	X	N/A	N/A	N/A	N/A	N/A
Sexual maturation	N/A	N/A	N/A	X	X	X	N/A	X	N/A
Oestrous cycle data	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Sperm parameters	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Necropsy	X	N/A	X	X	X	X	N/A	X	X

Histopathology	X	N/A	N/A	X	N/A	X	X	N/A	N/A
Reproductive parameters	X	N/A	X	N/A	X	N/A	N/A	N/A	N/A
Pre-postnatal development	N/A	N/A	X	N/A	X	N/A	N/A	N/A	X
Functional neurotoxicity observations	N/A	N/A	N/A	N/A	N/A	X	N/A	N/A	N/A
Neurohistopathology	N/A	N/A	N/A	N/A	N/A	X	X	N/A	N/A
Lymphocyte typing (spleen) after KLH immunistaion	N/A	N/A	N/A	X	N/A	N/A	N/A	X	N/A
Anti-KLH IgM levels after KLH immunisation	N/A	N/A	N/A	N/A	N/A	N/A	N/A	X	N/A
Aberrant crypt foci (ACF) scoring	N/A	X	N/A	N/A	N/A	N/A	N/A	N/A	N/A

EOGRT: extended one-generation reproductive toxicity; KLH: keyhole limpet haemocyanin; IgM: immunoglobulin M.

This table is taken from EFSA (2021).

Results

Evaluation of Sexual Function and Fertility

Male fertility

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

Female fertility

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

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

after failing to litter. Live litter sizes and litter weights were comparable to control values in all dose groups in the F0 and the F1 generation.

Developmental Toxicity

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

130. Growth and sexual development: No treatment-related effects were observed in birth weights and growth of the pups. There were no indications for any androgenic and/or oestrogenic effects from the male and female anogenital distance (AGD) and the retention of nipples in males.

131. The mean age at vaginal opening was comparable between control and treated groups. The statistically significant lower body weight on the day of vaginal opening in cohort 1A at 300 mg/kg bw per day was not considered to be biologically relevant due to the slightly higher litter sizes in all treated groups. There was no effect of treatment on the age at balanopreputial gland cleavage. It was noted that there was a deviation from OECD TG 443 in that balanopreputial gland cleavage, rather than balanopreputial separation, was examined.

Reproductive and developmental toxicity studies using the nanoparticle form of TiO_2

Lee et al., (2019)

132. Mated female Sprague–Dawley rats (12 females per group) were treated with TiO_2 NPs (nominally 21 nm) daily by gavage at dose levels of 0, 100, 300 and 1,000 mg/kg bw per day from GDs 6 to 19. The physicochemical characterization of TiO_2 nanoparticles, included analyses of primary shape, primary size, purity and hydrodynamic size. The majority of the TiO_2 nanoparticles had spherical and anatase crystal shapes with a purity of 100%. The mean primary size of the TiO_2 NPs was

17.8 ± 5.46 nm. The hydrodynamic size of the TiO_2 NPs was 341.5 nm, which indicated that TiO_2 NPs were prone to aggregation.

133. In the Lee *et al*., (2019) study, no statistically significant differences were noted in general clinical signs, bodyweight, organ weights (absolute and relative to body weight), or macroscopic findings. There were no significant differences in parameters measured after Caesarean section, fetal external or visceral examinations. The COT considered that no adverse maternal and developmental effects were observed with TiO_2 NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.

Warheit, Boatman and Brown, (2015)

134. Warheit, Boatman and Brown (2015) evaluated the potential maternal or developmental toxicity of six different forms of predominantly NP form of TiO_2 in pregnant rats (22 or 23 rats per group). The dose levels for each form of TiO_2 were 0, 100, 300, and 1000 mg/kg bw per day. The TiO_2 formulations were in sterile water and delivered via oral gavage. This study noted that in a set of companion analyses, one was of the pigment-grade (pg) TiO_2 and the other was of the ultrafine (uf) TiO_2 test material (BET (Brunauer-Emmett-Teller) and the surface areas of pg and uf samples ranged from 7-17 m^2/g and 50-82 m^2/g respectively). Each material was evaluated for potential systemic exposure/uptake from the GI tract to the blood for circulation. Studies were performed according to OECD TG 414.

135. In three studies, Warheit, Boatman and Brown (2015) dosed time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22 per group), daily with TiO_2 (uf-1, uf-3, & pg-1) by gavage on Gestational Days 6 to 20. Three additional studies included pregnant Wistar rats (n = 22 – 23 per group) exposed daily to TiO_2 (uf-2, and pg-2, pg-3) by gavage from Gestational Days 5 to 19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day with the TiO_2 specifications detailed below:

i. uf-1: 89% anatase/11% rutile (100% nanomaterial), d50 = 43 nm (by XSDC), d50 = 23 nm (by TEM), irregular shape (TEM).

ii. uf-2: 100% anatase (100% nanomaterial), d50 = 42 nm (by XSDC), d50 = 19 nm (by TEM), irregular shape (TEM).
iii. uf-3: 100% rutile (100% nanomaterial), d50 = 47 nm (by XSDC),d50 = 22 nm (by TEM), rod-like shape (TEM).
iv. pg-1: 100% anatase (27% nanomaterial), d50 = 153 nm (by XSDC), d50 = 120 nm by (TEM), irregular shape (TEM).
v. pg-2: 100% rutile (11% nanomaterial), d50 = 195 nm (by XSDC), d50 = 165 nm (by TEM), irregular shape (TEM).
vi. pg-3: 100% rutile (26% nanomaterial), d50 = 213 nm (by XSDC), d50 = 132 nm (by TEM), irregular shape (TEM).

136. No dose-dependent increase in TiO_2 was observed in the rat blood at 48 or 72 hours, or in the liver at 72 hours following a single exposure (2000 mg/kg bw TiO_2). These results suggested that there was little to no absorption of particles from the GI tract into the blood, which conflicted results from a previous study by Tassinari *et al*., 2014.

137. Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to identify any abnormalities. Among all six forms of TiO_2, effects were seen only with uf-1. At 1,000 mg uf-1/kg bw per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. The mean number of male fetuses was 7.2 compared with 5.5 male fetuses for the concurrent control group; the test facility historical control group data ranged at that time from 5.2 to 7.4. The mean number of female fetuses was 4.8 compared with 6.7 for the concurrent control group; the test facility historical control group data ranged at that time from 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mg uf-1/kg bw per day group was 60% (males/females) compared with a sex ratio of 46% in the concurrent control group; the test facility historical control group data ranged at that time from 43% to 53%. The authors did not consider the change in sex ratio to be test substance related, because fetal sex is determined shortly after conception and well before the onset of dosing with TiO_2 on GD 6.

138. Apart from some incidental changes in body weight and feed intake, no other changes were observed in the dams or the fetuses in these studies. The authors concluded that there were no significant toxicological or developmental effects in females or fetuses at any of the dose levels or compounds tested and considered the NOAEL for each compound to be 1,000 mg/kg bw per day, the highest dose tested.

EFSA review and conclusions on reproductive and developmental toxicity of TiO_2

139. EFSA based its review of the development and reproductive toxicity of E171 only on the results of the EOGRT study as no other reliable studies were found in the literature addressing these effects with E171.

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

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

142. Developmental Toxicity: EFSA concluded that “No effects of E 171 on pre- and postnatal development were observed. Data on the attainment of puberty in males (i.e. an appropriate assessment of the timing of the balanopreputial separation) were missing; however, given the lack of any other treatment-related effects on other parameters, the (FAF) Panel does not consider this to be critical in this case”.

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

144. EFSA concluded that the NOAEL for reproductive and developmental toxicity was 1,000 mg/kg bw per day, the highest dose tested, of a commercially available E171 in the EOGRT study.

Health Canada review and conclusions on reproductive and developmental toxicity of TiO_2

145. Health Canada concluded that in the EOGRT study "There was no evidence of adverse effects on reproduction or development up to the highest dose tested, and a NOAEL of 1,000 mg/kg bw per day can be established for reproductive and developmental effects on the basis of this study. The recent EOGRT study is considered the most reliable study found in the literature that addresses reproductive and developmental effects of E171 exposure".

FSANZ review and conclusions on reproductive and developmental toxicity of TiO_2

146. FSANZ noted the 2016 EFSA request for an additional developmental and reproductive toxicity study in the form of the subsequent EORGT study. FSANZ stated that the EORGT study (Leuschner, 2020) using food-grade TiO_2 showed no evidence of developmental or reproductive toxicity with an upper test dosage and NOAEL of 1,000 mg/kg bw/day.

147. FSANZ noted an additional study (food-grade TiO_2 in the diet for 7 weeks and by oral gavage for 10 weeks, 4 per group) which described germ cell sloughing in mouse testes in animals receiving the highest dose of 1,379 mg/kg bw per day in the diet and 5 mg/kg bw er day by gavage (Rodríguez-Escamilla et al., 2019). However, these changes were not observed in the follow-up study, with 9 mice per group and administration for 16 weeks. FSANZ also noted the lack of histopathological changes observed in the testes in three additional studies with up to 1,000 mg/kg bw/day (Leuschner, 2020; Han et al., 2021; and Blevins et al., 2019).

JECFA 2024 review and conclusions on reproductive and developmental toxicity of TiO_2

148. JECFA reviewed the EOGRT study (Leuschner, 2020) and noted that the EOGRT study had not been able to establish whether there were effects on developmental immunotoxicity resulting from exposure to E171 due to a weak immune response against the administered keyhole limpet haemocyanin (KLH) antigen, and that the study authors stated that this part of the study will be repeated. The Committee concluded that, apart from the lack of conclusive results on developmental immunotoxicity, the NOAEL for the study was 1,000 mg/kg bw per day, the highest dose tested.

149. JECFA reviewed the developmental toxicity study in rats by Warheit, Boatman and Brown (2015) and noted that the NOAELs for both maternal and developmental toxicity were 1,000 mg/kg bw per day, the highest dose tested in all the studies.

COT review and conclusions on reproductive and developmental toxicity of TiO_2

150. The COT considered that the EOGRT study was carried out according to the relevant scientific guidelines with no obvious deficiencies. No other reliable studies with food-grade TiO_2 were identified in the literature. The Committee concluded that no toxicological effects of TiO_2 on the reproductive and developmental system were observed in the EOGRT study. A range of endpoints, including body weight, haematology, urinalysis, sex hormone levels, thyroid hormone levels, circadian rhythm and sperm parameters, were investigated, in both the F0 and F1 generations.

151. It was noted that there was a suggestion of focal effects on the testes and epididymides in one male in the highest dose group, which were considered to be spontaneous and not test item-related, and abnormalities in sperm. Data from the EOGRT study showed test item-related differences for the examined absolute and relative testis weights between the control and the treatment groups (low = 100 mg, intermediate = 300 mg or high = 1,000 mg TiO_2 E171/kg bw per day). These slight differences included a small but statistically significantly reduced absolute right testes weight at the intermediate and the high dose level. These were only noted for the right testes, not the left testes and it was concluded that changes were spontaneous

and not of toxicological relevance. The COT agreed with the authors' conclusions. Overall, no adverse effects on reproductive and developmental toxicity were observed, up to the highest dose tested.

152. The best overall quality additional studies TiO_2, which used forms other than food grade, were determined by the COT to be those by Warheit, Boatman and Brown (2015) and & Lee *et al.*, (2019). The COT considered the 1,000 mg/kg bw per day NOAEL identified in the EOGRT study may reflect low absorption. Other studies indicate NOAELs consistent with that from the EOGRT study. The COT noted variations in methods and endpoints used to characterise TiO_2 among several of the studies and as a consequence the physicochemical differences between the test items were unclear.

153. The COT concluded that there was no good evidence that TiO_2 was reprotoxic.

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

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 TiO_2 (in rodents)

155. With regards to ACF, the effects of TiO_2 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/cm^2 and ABC/cm^2 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 TiO_2 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 TiO_2 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 TiO_2 particles on non-mutated and pre neoplastic cells (Apc+/+ and Apc Min/+). Results showed that 24-hour exposure to TiO_2 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 TiO_2 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 TiO_2 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 TiO_2

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 considerable 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 TiO_2-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 TiO_2, 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 (TiO_2, 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 TiO_2/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 TiO_2 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 TiO_2 test particles (d50 ¼ 145 nm, 21% nanoparticles) by oral gavage for 90 days with a NOAEL of 1,000 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 TiO_2 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 TiO_2 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 TiO_2

Akagi *et al.*, (2023)

174. In the Akagi *et al.*, 2023 study, TiO_2 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 TiO_2 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 TiO_2 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 TiO_2 (6 nm particle size) up to 1,000 mg/kg bw/day.

EFSA review and conclusions on induction of ACF by TiO_2

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 TiO_2 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 TiO_2

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 TiO_2 via the oral route. A single non-guideline study in which rats were exposed to food-grade TiO_2 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 TiO_2 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 TiO_2

178. FSANZ noted that there were no chronic toxicity or carcinogenicity studies available in the literature in which food-grade TiO_2 had been administered orally. The NCI 1979 Unitane study was previously considered suitable for assessing the carcinogenicity of food-grade TiO_2, however FSANZ noted that the EFSA 2021 opinion on TiO_2 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 TiO_2 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 TiO_2, EFSA agreed that these data "were not sufficient to raise a concern on the potential initiation or promotion properties of TiO_2 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 TiO_2, two additional studies using sonicated food-grade TiO_2 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 TiO_2 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 TiO_2 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 TiO_2 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 TiO_2

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 TiO_2

185. The Committee questioned whether results from studies in which TiO_2 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 TiO_2 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 TiO_2.

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 TiO_2 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 TiO_2, where histopathology of the colon was performed. The Committee concluded that there was no convincing evidence that TiO_2 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 TiO_2 alone in Bettini *et al.*, (2017), these could not necessarily be attributed to TiO_2, as ACF were also observed in control animals without exposure to TiO_2 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 TiO_2 were systemic at the doses tested but also that TiO_2 measurements were not accurate in the analysis of TiO_2 in

the tissues. One explanation for why there is little systemic toxicity of TiO_2 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.

Genotoxicity

188. The COM reviewed a number of studies to assess the genotoxicity of TiO_2. In addition to papers reviewed by EFSA, a literature search was conducted to find papers published on “genotoxicity” and “titanium dioxide”. Most papers identified used the nano-sized fraction of TiO_2 and not the micro-sized form, nor the specific E171 form.

189. All papers were screened against a series of criteria to assess the characteristics of the material used in the study and the generic study design (tier 1); and the generic experimental details of the genotoxicity study including adherence to OECD test guidelines (tier 2). These criteria were assessed by several members of the Committee through an iterative process. Finally, the experimental details of the study were thoroughly evaluated using expert judgement (tier 3). A number of exclusion criteria were used in the assessment of these studies. Further details of the scoring methodology are described in the COM statements (COM 2024a, b). A red, amber, green (RAG) rating was used to rank the studies. Papers which achieved a red RAG rating were not assessed any further.

In vitro genotoxicity assessment

190. Overall, for the in vitro assessment, from a total of 191 papers that were initially assessed, 15 papers detailing 16 assays, were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the *in vitro* genotoxicity assessment of TiO_2.

191. Six assays were rated as ‘green’ for the in vitro assessment. These included: 4 assays for the mincronucleus (MN); 1 assay of the mammalian cell hypoxanthine phosphorybosyl transferase (*hprt)*; and 1 assay for chromosome aberration (CA).

192. Ten assays were rated amber and of those: 9 were MN assays; and 1 was an *hprt* assay.

193. All of the assays assessed are described in detail in the 'Assessment of in vitro studies of TiO_2 genotoxicity' (COM, 2024a).

In vivo genotoxicity assessment

194. The in vivo assessment initially assessed 53 papers, six papers (detailing 12 assays) were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the *in vivo* genotoxicity assessment of TiO_2.

195. Two assays were rated as 'green' for the in vivo assessment. These papers were both for the in vivo MN assay.

196. Ten assays were rated amber and of those: 3 were for the in vivo MN; 1 was for CA; 1 was for the in vivo comet assay; 3 were *Pig-a* mutation studies; 1 was a gpt mutation assay; and 1 was a γ-H2AX assay

197. All of the assays assessed are described in detail in the 'Assessment of in vivo studies of TiO2 genotoxicity' (COM, 2024b).

EFSA review and conclusions on the genotoxicity of TiO_2

198. In EFSA's review of the genotoxicity of TiO_2 in 2016, "The (ANS) Panel concluded that, based on the available genotoxicity database and the Panel's evaluation of the data on absorption, distribution and excretion of micro- and nanosized TiO_2 particles, orally ingested TiO_2 particles (micro- and nanosized) are unlikely to represent a genotoxic hazard in vivo" In their assessment in 2021, EFSA focused particularly on the genotoxicity of TiO_2 nanoparticles, and the vast majority of studies were on this form of the substance.

199. Gene mutation: EFSA considered that some in vitro studies demonstrated that TiO_2 NPs can induce gene mutations in cultured mammalian cells although others did not. Six in vivo studies were considered relevant, one of which indicated the induction of large DNA deletions (also assessed in 2016), however the remaining five studies, that could identify point mutations and small deletions, gave consistently negative results. EFSA concluded that the available experimental data do not confirm the potential of TiO_2 NPs (< 30 nm) to induce gene mutations in vivo.

200. Induction of MN/CA: EFSA noted that the majority of in vitro MN or CA tests gave negative results, independent of particle size. 60% of the tests used NPs as the test material. The majority of in vivo MN and CA tests in vivo were considered positive. All of these studies were with partciles < 60 nm. There were very few studies on larger particles. Taking into account the available evidence, EFSA considered that - on balance - TiO_2 NPs have the potential to induce MN/ CA. They noted “that a significant portion of the studies was performed using TiO_2 NPs < 30 nm, however some positive results were observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in MN/CA assay was observed” (EFSA, 2021).

201. In vitro and in vivo Comet assay: EFSA concluded that based on the results of the in vitro and in vivo comet assays, TiO_2 particles have the potential to induce DNA damage. As noted for MN and CA effects, “a significant portion of the studies were performed using TiO_2 NPs < 30 nm, however some positive results were also observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in Comet assay was observed” (EFSA, 2021).

202. DNA Binding: EFSA also concluded that there is evidence, from both in vitro and in vivo studies, for interaction(s) of TiO_2 NPs with DNA. However, due to the techniques employed, it was not possible to determine whether these interactions involved covalent or non-covalent binding.

203. Overall, the FAF Panel concluded that “a concern for genotoxicity of TiO_2 particles that may be present in E 171 cannot be ruled out. A cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified.”

Health Canada review and conclusions on the genotoxicity of TiO_2

204. Health Canada's Food Directorate noted that, based on the currently available studies including the EOGRT study (Leuschner, 2020), food grade TiO_2 is not genotoxic in-vivo. However, they recognise that the studies available on this endpoint are limited and additional OECD guideline-compliant studies are recommended to confirm this. They conclude that any adverse effects found related to oral exposure were based on findings from non-standard studies in which the form of TiO_2 administered was homogenized suspensions of particles with ultrasonic dispersion and that these do not fully represent exposure to food grade TiO_2.

FSANZ review and conclusions on the genotoxicity of TiO_2

205. No in-vivo genotoxicity studies in which food-grade TiO_2 with dietary administration was used could be identified. FSANZ identified four in-vivo genotoxicity studies, two studies using food-grade TiO_2 administered by oral gavage (Bettini et al., 2017 and Jensen et al., 2019) and two studies using intraperitoneal administration of Unitane (0-220) (Shelby et al., 1993 and Shelby and Witt, 1995). These latter two studies assessing genotoxicity by the NCI/NTP were identified by the US National Toxicology Program and were assumed to use Unitane 0-220, as the test item was not described (Shelby et al., 1993 and Shelby and Witt, 1995)

206. FSANZ noted that DNA damage was not observed either in the two comet assays (food-grade TiO_2, oral gavage) or the micronucleus and chromosomal aberration studies (Unitane 0-220, intraperitoneal injection). In vitro studies (GLP- and OECD test guideline-compliant, food-grade TiO_2) found no evidence of gene mutation in mammalian cells nor clastogenicity or aneugenicity or cellular uptake (micronucleus assay using human peripheral blood lymphocytes). There was some evidence of uptake and internalisation by A549 cells however no particles were detected in the nucleus. FSANZ noted that "the absence of confirmed cellular uptake in the in vitro genotoxicity studies may limit confidence in the negative results, although alternatively the absence of direct exposure of the nucleus to food-grade

TiO_2 in these studies may indicate a low intrinsic hazard from a direct genotoxicity perspective (OECD, 2014)".

JECFA 2024 review and conclusions on the genotoxicity of TiO_2

207. JECFA noted that overall, the available data did not provide convincing evidence of genotoxicity for food grade TiO_2 E171 (INS 171). However, the Committee recognised the limitations of the current methodology with respect to the testing of poorly soluble particulate materials.

COM review and conclusions on the genotoxicity of TiO_2

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

COM Opinion of the in vitro genotoxicity studies reviewed

209. "After reviewing the in vitro genotoxicity studies performed to date on TiO_2, we note the following points:

i. There were four in vitro studies of the highest quality (labelled "green" here) that used TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. Only one study tested micro-sized (anatase)TiO_2 that was more representative of E171 (Demir et al. 2015) which was negative in the micronucleus assay. All four "green" studies that used anatase TiO_2 nanoparticles reported negative results for the MN endpoint. Of the two green studies that used rutile TiO_2 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 TiO_2 nanoparticles of mixed anatase/rutile form and both were negative for MN induction.

ii. There were two green studies that both used anatase/rutile TiO_2 nanoparticles in either the hprt gene mutation assay or CA assay. The TiO_2 nanoparticles were negative in the hprt assay. In the CA assay, the TiO_2 nanoparticles were positive, but the CA frequency decreased with increasing TiO_2 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 TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. Four studies used anatase TiO_2 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 TiO_2 were negative for micronuclei induction. Two studies that used anatase/brookite TiO_2 nanoparticles reported positive results for micronuclei induction.

iv. The one amber study on hprt mutations was positive at low anatase TiO_2 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.

210. Overall, the COM opinion is that there is little evidence that TiO_2 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.

211. 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 TiO_2 are considered worst-case scenario for E171, as E171 is anticipated to be less reactive.

212. 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. Not yet published)

COM Opinion of the in vivo genotoxicity studies reviewed

213. "After reviewing the *in vivo* genotoxicity studies performed to date (up to 2023) on TiO_2, 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 TiO_2 (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 TiO_2. 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 TiO_2 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 TiO_2 material which is less relevant to the E171 material.

214. Overall, we conclude that there is little evidence in the literature to suggest that there is a health concern related to genotoxicity induction by TiO_2, particularly via the oral route and especially the micro sized TiO_2 fraction (most studies used the nano-sized material).

215. 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 TiO_2.” (COM, 2024b. Not yet published).

COT review and conclusions

216. The COT agree with the COM conclusions. While there is a lack of information specifically on the genotoxicity of food grade E171, there is sufficient information on the different particle types present to reach an overall conclusion.

Inflammation and Immunotoxicity

Studies using E171 or equivalent form of TiO_2

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) TiO_2 at doses of 0, 6.25, 62.5, or 625 mg TiO_2/kg of diet (equivalent exposures of 0 and ≈1, 10, and 100 mg TiO_2/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 TiO_2 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 TiO_2-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 TiO_2 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 TiO_2 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)-1β and tumour necrosis factor (TNF)-α 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 TiO_2 (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 TiO_2 E171 for 3 weeks.

TiO_2 had a low impact on the microbiotal composition in the small intestine/colon. However, TiO_2 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 TiO_2 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 TiO_2 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 TiO_2 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 TiO_2 treatment.

Bettini et al., (2017)

229. Bettini et al. dosed rats with E171 TiO_2, TiO_2 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 TiO_2 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 TiO_2 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.

236. 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 TiO_2 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 TiO_2 in rats as described in paragraph 173. In the 90-day study, TiO_2 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 TiO_2 particles were observed in intestinal lymphoid tissue. However, no treatment-related adverse effects on any endpoints were observed.

Studies using the nanoparticle form of TiO_2

Huang et al., (2017)

239. Huang *et al.*, (2017) treated Raw264.7 and J774a.1 cell lines and primary mouse macrophages with TiO_2 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% TiO_2 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 TiO_2 NPs in wildtype mice, no changes in bodyweight were observed. NP10 and NP50 TiO_2 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 TiO_2 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 TiO_2 NPs was inhibited. These findings indicate that exposure to TiO_2 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 TiO_2 NPs, septic shock following LPS challenge was aggravated with increased levels of inflammatory cytokines in serum

and reduced overall survival. TiO_2 had little or no effect in TLR4-knockout mice, nor did it affect that lack of response to LPS in these animals. TiO_2 did not exacerbate non- TLR4-dependent septic shock induced in wildtype mice.

241. Following exposure *in vitro* to TiO_2 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 TiO_2 NPs in vitro and in vivo, their chemotactic, phagocytic, and bactericidal activities were lower.

242. The authors concluded that "These results demonstrate that TiO_2 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 TiO_2-NPs via the diet. The particles were incorporated into pelleted diet at either 0.2% or 1% TiO_2 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 TiO_2-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 TiO_2-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 TiO_2

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 TiO_2

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 TiO_2 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 TiO_2 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 TiO_2 was administered via the dietary route. Therefore, it is concluded that concerns that food-grade TiO_2 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 TiO_2 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 TiO_2 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 TiO_2 via the oral route. While a few non-guideline studies suggest food-grade TiO_2 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 TiO_2 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 TiO_2 highly comparable to the form of TiO_2 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 TiO_2 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 TiO_2

255. FSANZ identified three studies which fulfilled the criteria for assessing immunotoxicity (two used food-grade TiO_2 administered by the diet and one sonicated food-grade TiO_2 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 TiO_2

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 TiO_2

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 TiO_2. 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 TiO_2 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 TiO_2 dosing. It is suggested therefore that tissue specificity and differential cytokine expression may play a role in the effects of TiO_2.

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 TiO_2-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 TiO_2 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 TiO_2 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 TiO_2 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.

Neurotoxicity

Studies using E171 or equivalent form of TiO_2

EOGRT Study (Leuschner, 2020)

264. Details of the design on the EOGRT study are given in paragraphs 122 – 125. Male and female offspring were tested for auditory startle response between PND 23 and 25, including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64. No differences in the response to an auditory startle stimulus were observed and an increase in hindlimb splay was observed in females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

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

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268. No reliable studies on neurotoxicity using E171 were identified in the published literature.

Studies using the nanoparticle form of TiO_2

Sofranko et al., (2021)

269. Sofranko et al., 2021 investigated the effects of silver and TiO_2 NPs on behaviour and neuropathology in male and female C57BL/6J mice. Mice were fed

pellets containing 10 mg/g TiO_2, 2 mg/g polyvinylpyrrolidone-coated Ag or control pellets for 28 days with and without a 14-day post-exposure recovery period. Inflammation, oxidative stress, and blood-brain barrier integrity were not significantly affected in male or female mice. No consistent significant treatment-related effects on anxiety and cognition were observed for either test material. The authors concluded that there were no substantial neuropathological changes following subacute exposure to foodborne TiO_2 in mice.

Canli et al., (2020)

270. Female albino Wistar rats were given doses (0.5, 5.0 and 50 mg/kg bw per day) of sonicated TiO_2 NPs for 14 days via oral gavage in 200 µL water (n = 6 per group). Control animals (n = 6) received 200 µL water only. Following dosing, total protein, the activities of ATPases and acetylcholinesterase A(ChE), the levels of thiobarbituric acid reactive substances (TBARS) and the different forms of glutathione (GSH) (oxidised, reduced and total) were measured in tissues from the liver, kidney, brain and intestine.

271. The anatase TiO_2 NPs were about 21 nm, >30 m^2/g surface area, >99% purity, and a density of 4.26 g/cm^3. None of the rats died within the 14-day treatment period. TEM images showed that TiO_2 NPs seemed to accumulate in the brain, kidney and liver tissues in a dose-dependent manner. There was no TiO_2 NP deposition in the same tissues from control animals. Brain AChE activity decreased at all TiO_2 NP doses compared to controls, however, brain ATPase activities were generally stimulated. None of these changes showed a dose-response relationship. ATPase activities in the intestine and kidney did not change significantly. Levels of the different forms of GSH did not change significantly. There were no significant changes in TBARS levels, except for a decrease at the highest TiO_2 NP dose.

Grissa et al., (2016)

272. The effects of oral intake of TiO_2 NPs (5–10 nm) at 0, 50, 100, and 200 mg/kg bw for 60 days on the brain of male Wistar rats was investigated. The brain to body weight ratio, AChE activities, level of IL-6, and the expression of glial fibrillary acidic protein (GFAP) were assessed to quantify the brain damage. The results showed

that mid- and high-dose TiO_2 NPs reduced the relative weight of the brain, induced a downregulation of AChE activity and an increase in cerebral IL-6 secretion compared to the control group. Plasma AChE activity decreased and IL-6 levels increased at all dose levels. Elevated levels of immunoreactivity to GFAP were observed in the rat cerebral cortex in mid- and high-dose groups. The authors concluded that oral exposure to TiO_2 NPs can induce neuroinflammation and could be neurotoxic. (Grissa *et al.,* 2016).

EFSA review and conclusions on neurotoxicity of TiO_2

273. With respect to the EORGT study, the EFSA Panel considered that "there was no systematic bias in group testing order and that this was therefore not a plausible explanation for the observed group differences. Grip strength and hindlimb splay belong to the same domain of neurological function, i.e. motor function and/or sensory–motor coordination. However, the effects observed (i.e. increase in hindlimbs play and increase in mean forelimb grip strength) seem to point in opposite directions when it comes to muscle strength. In particular, an increase in hindlimb splay can be interpreted as muscular weakness whereas an increase in mean forelimb grip strength could be indicative of myotonia. The Panel noted that the effects observed were not correlated to any other changes (e.g. alterations in muscle tone, righting reflex, gait, wire manoeuvre, posture). No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in the functional observation battery measurements or locomotor activity were noted. There were no notable histopathological findings in brain or in peripheral sciatic nerve. Based on all the above considerations, the Panel considered that the effects on grip strength and hindlimb splay were not treatment-related. However, the Panel noted that quantitative information on peripheral nerves was not available. Overall, the Panel considered that E171 at these doses had no adverse effects on neurofunctional endpoints in F1 cohort 2A offspring."

274. No neurotoxicity studies performed with E171 were identified from the published literature that were considered sufficiently reliable. Some papers were identified noting effects of TiO_2 NP. EFSA concluded "Overall for neurotoxicity,

adverse effects were seen with TiO_2 NPs < 30 nm. In mice, Zhou et al. (2017; scoring 3 for NSC, nanoscale considerations), reported adverse effects (i.e. inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function) in ex vivo hippocampal neurons of weanling mice after dosing TiO_2 NPs (6–7 nm) during gestation and early lactation at a dose of 1 mg/kg bw per day, the lowest dose tested. In adult female rats (Canli et al., 2020; scoring 3 for NSC), adverse effects (reduced brain cholinesterase, and increased brain Na/K-ATPase activity) were observed with TiO_2 NPs (21 nm) at 0.5 mg/kg bw per day, the lowest of three doses tested, in a 14-day study." EFSA did note that there was an apparent 200-fold difference in potency in the effects of TiO_2 NP on brain cholinesterase activity between Grissa et al. (2016) and Canli et al (2020), which adds to uncertainty.

Health Canada review and conclusions on neurotoxicity of TiO_2

275. Health Canada (2022) summarised the evidence for neurotoxicity of TiO_2 as follows: "The concerns pertaining to potential neurotoxicity associated with TiO_2-NPs in E171 appear to be based predominantly on studies which used test articles that did not correspond to food-grade TiO_2 and/or dosing paradigms that are considered to be of limited relevance to human dietary exposure. In an EOGRT study where developmental neurotoxicity was investigated in rats exposed to E171 at doses up to 1,000 mg/kg bw/d via the diet, no adverse effects on neurodevelopmental or neurofunctional endpoints were observed. Endpoints examined included auditory startle response as well as a functional battery that included grip strength and locomotor activity. No treatment-related changes were observed in any of these endpoints and there were no notable histopathological findings in the brain or peripheral nerves. Similarly, in the only available study in which TiO_2-NPs were administered via the diet (Sofranko *et al.*, 2021), no neurotoxicity was observed."

276. Health Canada noted the study by Sofranko *et al.* (2021), published after the EFSA Opinion, which concluded that sub-acute (28 day) exposure to TiO_2-P25 did not cause neurotoxicity (although P25 is referred to as foodborne, this is not considered as a food additive). Other studies which have been published since the 2021 EFSA Opinion were considered of very limited relevance as they were

conducted *in vitro* using rat cortical cells (Gerber *et al.*, 2022) or involved i.v. injection of sonicated TiO_2 nanoparticles (Ciu *et al.*, 2021; Naima *et al.*, 2021).

FSANZ review and conclusions on neurotoxicity of TiO_2

277. FSANZ identified only one study with neurotoxicological endpoints using food-grade TiO_2 administered via the diet, the EORGT Study (Leuschner, 2020). FSANZ concluded that no adverse neurofunctional effects were observed (in auditory startle response and a functional observation battery).

COT review and conclusions on neurotoxicity of TiO_2

278. Overall, there is no new evidence on neurotoxicity to justify a change to the COT position on this endpoint as stated in its 2021 interim position paper, namely "The findings of the studies on neurotoxicity were considered inconsistent by the COT. It was noted that the EOGRT study did not report any effects and that most of the other studies on this endpoint were of nanomaterials. In the EFSA evaluation, the issue of the test material in the EOGRT not being dispersed was taken into consideration with regards to the conclusions on this endpoint, as they considered that had it been dispersed and stabilised in the nano form some effects could possibly have been observed. The COT, as previously, questioned the relevance of such dispersion to the real-world use of TiO_2. Members noted that the histopathology tests performed for the EOGRT study were standard and were not sensitive enough in comparison to other studies on this endpoint that performed specific neuro-histopathology testing." (COT, 2022.)

279. It should be recognised that this qualified COT opinion on neurotoxicity is more conservative than that of Health Canada, who considered the EOGRT to be sufficiently sensitive and relevant to conclude on the lack of neurotoxicity potential of food grade TiO_2.

Establishment of a Health-Based Guidance Value (HBGV)

280. The Committee concluded that, 1,000 mg/kg bw per day was a robust Point of Departure (POD). This was based on the EOGRT study findings as well as studies by Warheit, Donner and Brown, 2015 and Lee *et al.*, 2019 that reported no effects up to the same dose. There was variability noted in the other studies, but nothing that would undermine the value of 1,000 mg/kg bw per day to be used as the POD. It was also noted by the COT that this is the highest dose of E171 or equivalent TiO_2 tested in a study of this quality.

281. A standard uncertainty factor of 100 (10 for inter-species differences and 10 for interindividual variability) was agreed by Members and applied to the POD which results in a HBGV of 10 mg/kg bw per day. There is likely to be additional conservatism in the application of this uncertainty factor to the NOAEL of E171 because this was the highest dose of TiO_2 tested and hence the LOAEL (lowest observed adverse level) could actually be appreciably higher and, because there is no metabolism of TiO_2 particles, the inter-/intra-species kinetic differences are likely to be lower than the defaults.

Exposure Assessment

282. The exposures to TiO_2 from medication, personal healthcare products and through dermal, inhalation and intravenous routes are not considered in this assessment. The exposures considered are only those from food.

283. Titanium dioxide can be found in a number of food categories including bakery products, soups, broths, sauces, salads, savoury based sandwich spreads and processed nuts. It is also used in confectionary, chewing gum, food supplements and cake icing (EFSA, 2016).

Occurrence data in food

284. Only food as a source of TiO_2 is considered in this exposure assessment. Food consumption data from the National Diet and Nutrition Survey (NDNS) (Bates, 2014; 2016; Roberts 2018; Bates, 2020) and the Diet and Nutrition Survey of Infants and Young Children (DNSIYC) (Department of Health, 2013) were used to estimate exposure to titanium dioxide. Maximum occurrence levels of titanium dioxide for specific food items, reported by EFSA (2021), were also used in the estimation of exposure (Table 6). Food categories were created by the FSA Exposure Assessment Team (EAT) using data from NDNS and DNSIYC to reflect those created by EFSA for the Food Additive Intake Model and as presented in Annex II of Regulation (EC) No 1333/2008, part D. Foods in NDNS and DNSIYC were matched to food categories associated with the regulation on food additives to enable an assessment of exposure based on maximum levels reported by industry for titanium dioxide and those reported in the scientific literature. Assessments were carried out in Crème which is the software used by the FSA EAT to conduct exposure assessments.

285. The occurrence data used were those reported in EFSA, 2021, which were obtained from industry as reported by the Dutch National Institute for Public Health and the Environment (RIVM), along with levels reported in analytical studies. These levels are presented in Table 6 for sixteen food categories, although titanium dioxide is approved in many other food categories (forty-eight in total) (Table 1, Annex C). For the exposure assessment, only use levels for these sixteen food categories were taken into account, as no data were available for the other categories and it was not possible to use the maximum permitted levels (MPLs) for TiO_2 as they were established at quantum satis, rather than a specific value being ascribed. The assessment was based on maximum use levels reported to provide conservative scenarios of exposure for the population groups considered (Table 7).

Table 6: Occurrence levels of titanium dioxide (E171) used in the exposure assessment scenarios (mg/kg or mg/L as appropriate)

EFSA Food category number	Food category name	Concentration levels used in the exposure assessment (Maximum reported)	MPL (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat-treated products	48	QS
01.8	Dairy analogues, including beverage whiteners	125	QS
03	Edible ices	857	QS
05.2	Other confectionery including breath-refreshening microsweets	4,500	QS
05.3	Chewing gum	16,000	QS
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	20,000	QS
07.2	Fine bakery wares	318	QS
12.5	Soups and broths	193	QS
12.6	Sauces	4,000	QS
12.7	Salads and savoury-based sandwich spreads	3,000	QS
12.9	Protein products, excluding products covered in category 1.8	5,000	QS
14.1.4	Flavoured drinks	70	QS
15.2	Processed nuts	7,000	QS
16	Desserts, excluding products covered in categories 1, 3 and 4	200	QS
17.1	Food supplements supplied in a solid form, excluding food supplements for infants and young children	26,950	QS
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children	26,950	QS

QS – *quantum satis* (no maximum numerical level is specified, and substances will be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled).

Exposure Estimation

286. Exposure assessments were carried out for the following population groups: Infants, toddlers, other children, adolescents, adults and the elderly. There are two toddler groups. One group represents ages 1 - 1.5 years and the data used were from DNSIYC as this survey covers infants and young children aged 4 - 18 months (1.5 years). The other toddler group covers ages 1.5 - 3+ years and data were obtained from the NDNS, as this survey covers all age groups from 1.5 years. The mean and 95th percentile estimates are presented for each population group and food category in mg/kg bw per day in Table 7.

287. The reported data are consumer-based, meaning that only subgroups of the population that consumed these categories of food were considered. The mean and 95th percentile total exposures are derived from the combined exposure for each individual in the respective age group. The mean and 95th percentile total exposures have not been derived by adding up the mean and 95th percentile values respectively, for the food categories, as not all consumers will be exposed in each category. Total exposures were calculated to estimate exposures from the total diet in Table 8. These were calculated from a distribution of individual total exposure of any combination of food categories, rather than by summation of the respective mean/95th percentile consumption values for each of the food categories.

288. The mean calculated total exposures for TiO_2 from foods ranged from 3.3 to 11 mg/kg bw per day. The 95th percentile total exposures for TiO_2 ranged from 9.1 to 26 mg/kg bw per day. The 3 food groups that contribute the most to these exposures are: protein products; Decorations, coatings and fillings, except 4.2.4; and Sauces.

Table 7: Estimated mean and 95th percentile (P95) exposures (mg/kg bw per day)[a] to titanium dioxide E171 from its use as a food additive based on the maximum reported use level. The reported data are consumer-based.

Food group	Infants (4 – 11 months) Mean (P95[b])	Toddlers (1 - 1.5 years) Mean (P95[b])	Toddlers (1.5 - 3 years) Mean (P95[b])	Children (4 - 10 years) Mean (P95[b])	Adolescents (11 - 18 years) Mean (P95[b])	Adults (19 - 64 years) Mean (P95[b])	Elderly (≥65 years) Mean (P95[b])
1.4- Flavoured fermented milk products including heat-treated products	0.24 (0.6)	0.2 (0.49)	0.16 (0.4)	0.097 (0.23)	0.05 (0.12)	0.044 (0.11)	0.049 (0.11)
1.8. Dairy analogues and whitener	0.91 (1.9)	1.6 (5.6)	1.9 (5.7)	0.63 (2.3)	0.21 (0.78)	0.15 (0.47)	0.18 (0.75)
12.5. Soups and broths	0.45 (1.5)	0.53 (1.7)	0.55 (1.3)	0.37 (0.94)	0.23 (0.56)	0.21 (0.54)	0.24 (0.64)
12.6. Sauces	4 (14)	4 (12)	3.5 (11)	3.3 (9.2)	2.2 (6.1)	1.7 (5)	1.4 (3.8)
12.7. Salads and savoury based sandwich spreads	2.2 (9.4)	3 (12)	2.9 (9.2)	1.9 (4.6)	0.92 (2.7)	0.95 (2.7)	0.94 (2.9)
12.9. Protein products	14 (55)	27 (190)	38 (160)	11 (49)	5.3 (18)	5.3 (18)	7.6 (30)
14.1.4. Flavoured drinks	0.15 (0.53)	0.35 (1.1)	0.47 (1.4)	0.44 (1.2)	0.4 (1)	0.24 (0.73)	0.12 (0.32)
15.2. Processed nuts	1.3 (4.6)	2.1 (8.1)	2.8 (9.9)	2 (7.2)	1 (3.6)	1.2 (3.9)	1.2 (3.4)
16. Desserts excluding products covered in categories 1, 3 and 4	1.2 (3.2)	1 (2.6)	0.64 (1.8)	0.33 (0.85)	0.14 (0.38)	0.11 (0.28)	0.15 (0.4)
17.1. Food supplements supplied in a solid form, excluding for infants and young children	1.4 (3.3)	1.7 (2.7)	1.9 (4.2)	1.1 (2.6)	0.82 (1.3)	0.95 (2.1)	0.85 (2.2)

17.2. Food supplements in a liquid form, excluding for infants and young children	3.9 (16)	3.2 (12)	3.3 (11)	1.4 (4)	3.2 (9)	1.7 (5.6)	0.91 (2.3)
3. Edible ices	0.88 (2)	1.1 (3)	1.3 (3.3)	1 (2.5)	0.55 (1.5)	0.32 (0.74)	0.35 (0.84)
5.2. Confectionery and sweets	1.2 (2.9)	2.7 (6.9)	2.6 (7)	2.4 (7.6)	1.5 (4.7)	0.79 (2.9)	0.54 (1.8)
5.3. Chewing gum	0 (0)	1.5 (1.5)	0.73 (1.6)	0.6 (1.7)	0.57 (2.2)	0.41 (1.2)	0.63 (0.82)
5.4. Decorations, coatings and fillings, except 4.2.4	6 (16)	7.2 (19)	6.8 (17)	6.6 (18)	3.8 (10)	2.3 (6)	2.3 (6.3)
7.2. Fine bakery wares	0.3 (0.9)	0.5 (1.4)	0.6 (1.5)	0.55 (1.3)	0.3 (0.76)	0.21 (0.55)	0.23 (0.57)

*Estimates are rounded to 2 significant figures.

**Calculated from a distribution of individual total exposure of any combination of food categories rather than by summation of the respective mean/95th percentile consumption values for each of the food categories.

[a] Exposure per bodyweight was calculated for each individual before calculating the mean and P97.5 exposure. The mean and 95th percentile estimates are presented for each population group and food category in mg/kg bw/day in Table 2. The estimates are based on individual bodyweights and not the average for the population group. However, for context, the average bodyweights of the population groups are 9.11 kg for infants; 10.9 kg for 12-18 months old; 14.6 kg for 1.5 -3 years; 27.1 kg for 4-10 years; 58.9kg fo r 11-18 years; 78.6 kg for 19-64 years; and 70. 6kg for the elderly.

[b] 95th percentile exposures have been reported for the UK to aid the comparison with data reported by EFSA.

Table 8: Estimated mean and 95th percentile (P95) total exposures (mg/kg bw/day)[a] to titanium dioxide E171 from its use as a food additive based on the maximum reported use level.

Age Group	**Infants** (4 – 11 months) **Mean (P95[b])**	**Toddlers** (1 - 1.5 years) **Mean (P95[b])**	**Toddlers** (1.5 - 3 years) **Mean (P95[b])**	**Children** (4 - 10 years) **Mean (P95[b])**	**Adolescents** (11 - 18 years) **Mean (P95[b])**	**Adults** (19 - 64 years) **Mean (P95[b])**	**Elderly** (≥65 years) **Mean (P95[b])**
Total exposure**	3.9 (14)	6.9 (19)	11 (26)	9.5 (24)	5 (13)	3.7 (10)	3.3 (9.1)

[a] Exposure per bodyweight was calculated for each individual before calculating the mean and P97.5 exposure. The mean and 95th percentile estimates are presented for each population group and food category in mg/kg bw per day in Table 2. The estimates are based on individual bodyweights and not the average for the population group. However, for context, the average bodyweights of the population groups are 9.11 kg for infants; 10.9 kg for 12-18 months old; 14.6 kg for 1.5 -3 years; 27.1 kg for 4-10 years; 58.9 kg for 11-18 years; 78.6 kg for 19-64 years; and 70.6 kg for the elderly.

[b] 95th percentile exposures have been reported for the UK to aid the comparison with data reported by EFSA.

** The total exposure includes all of the following food groups:

1.4- Flavoured fermented milk products including heat-treated products

1.8. Dairy analogues and whitener

12.5. Soups and broths

12.6. Sauces

12.7. Salads and savoury based sandwich spreads

12.9. Protein products

14.1.4. Flavoured drinks

15.2. Processed nuts

16. Desserts excluding products covered in categories 1, 3 and 4

17.1. Food supplements supplied in a solid form, excluding for infants and young children

17.2. Food supplements in a liquid form, excluding for infants and young children

3. Edible ices

5.2. Confectionery and sweets

5.3. Chewing gum

5.4. Decorations, coatings and fillings, except 4.2.4

7.2. Fine bakery wares

Assumptions and uncertainties

Evidence base

289. This risk assessment is to assess the safety of TiO_2 E171 as a food additive. There were a few well conducted studies which allowed reasonable conclusions to be drawn. However, during the assessment of the scientific evidence it was noted that the TiO_2 used in the studies was not always well characterised, which can make it difficult to confirm what the effects are for specific forms of TiO_2. Some studies had used food grade TiO_2 or specifically E171. However, for other studies it was less certain what the form of TiO_2 was due to poor characterisation or TiO_2 engineered NPs had been used. These do not have the same characteristics as food grade TiO_2 and would not be used for this purpose. It was therefore uncertain how relevant toxicity data from these studies were to E171 or food grade TiO_2.

290. Another aspect of the dosing regimen was whether the TiO_2 had been sonicated to reduce/remove particle agglomeration. It was uncertain whether the toxicity profile of these would be the same as that of TiO_2 when used as a food additive.

291. A significant number of studies did not use a dietary feeding route for dosing and the relevance of data from those studies, for the assessment of TiO_2 as a food additive, was uncertain. Some studies used drinking water or gavage and potentially the TiO_2 would not have the same physicochemical characteristics or toxicokinetics as when combined with the diet and consumed.

Exposure assessment

292. The exposure assessment takes into account use levels in only sixteen food groups, whereas E171 is approved in more categories (forty-eight). This may introduce underestimations for exposures. However, not all foods within the

categories assessed will contain E171, which means exposure in those categories may be overestimated. In addition, the assessments are based on the assumption that all food in these categories contain E171 at the maximum reported levels. It is very unlikely that all foods in every category assessed will contain E171 and that this will be at the maximum reported levels. This assumption may overestimate exposure.

293. There are differences between the granularity of food groups used by EFSA for the purposes of their exposure assessment, and those used here for the UK population. This could introduce uncertainties about the comparability of the data.

294. The European Union (EU) banned the use of titanium dioxide as a food additive in 2022. This followed EFSA's update on the safety of the food additive and taking into account uncertainties around genotoxicity concerns (Commission Regulation (EU) 2022/63). Titanium dioxide has not been banned in the UK. However, the EU ban on titanium dioxide may lead to a decline in its use by industry, even for foods consumed in the UK. For these reasons, the exposure estimates derived may overestimate current and future exposure to titanium dioxide in the UK population.

295. The exposure estimates do not account for exposures from medicines, toothpaste and other non-food sources. Exposure may be higher if these other sources are taken into account.

Risk Characterisation

296. Exposures for all population groups for the mean total diet are below or very close to the HBGV of 10 mg/kg bw per day. Adverse health effects would not be expected for any of these populations.

Exposures calculated for the 95th percentile total diet range from 9.1 to 26 mg/kg bw per day. The exposures for adults (18 +) and the elderly are below the HBGV and adverse health effects would not be expected. The remaining exposures are 1.3- to 2.6-fold the HBGV. However, for reasons discussed in the assumptions and

uncertainties section, exposures are very likely to be lower than estimated. In addition, the as explained above, the factors used to establish the HBGV are likely to be more conservative than those typically used. Therefore, it is unlikely that there would be a risk to health from exposures of E171 TiO_2 from the diet.

Conclusions

298. Specifically in food, the primary function of TiO_2 is as an opacifier and white pigment. To achieve this function, it is critical that food grade TiO_2 (E171) exists as an aggregate of smaller primary particles with a median particle size of 200 – 300 nm. In engineered nano-TiO_2 all (100%) of the particles are less than 100 nm in diameter and it is colourless and would therefore be unsuitable for use as a pigment in food applications. Hence, there is a limit to the proportion of NP that can be present in food grade TiO_2 while retaining its utility as a food additive.

299. The Committee concluded that there is uncertainty over the toxicological effects of TiO_2 nanoparticles. The Committee therefore considered that if animals and/or humans are exposed to test substances which contain higher levels of NPs than normally found in food-grade TiO_2, that could change the toxicological profile and potentially the risk but it is unclear by how much or in what way.

300. This statement has separated the assessment of the engineered nano form and the food grade TiO_2. The focus and conclusions of the statement are based on food grade TiO_2.

301. The COT concluded that the physical form of TiO_2 will affect the absorption and distribution of TiO_2. The focus of the COT was on food-grade TiO_2 but the wide variance of test materials used (nano, micro and mixtures of nano and micro) was noted. Due to this large variability, as well as the potential impact of the matrix of administration, the Committee could not ascribe a specific percentage for the absorption of TiO_2. However, the Committee considered that absorption of food grade TiO_2 (E171) is very low.

302. The COM reviewed a number of studies to assess the genotoxicity of TiO2.

303. The COM stated that a definitive assessment of the safety of food grade E171 was difficult when there were no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. It was also noted that there is a lack of high-quality data sets on TiO_2 NP that are OECD compliant, and this led to conflicting data and some uncertainty in the risk assessment for TiO_2. (COM, 2024b).

304. The COM opinion is that there is little evidence that TiO_2 micro-sized or nanoparticles are genotoxic in vitro or in vivo based on data from well conducted studies. There is also a lack of replication of study outcomes using the same nanoparticle in different labs. (COM, 2024a).

305. Overall, therefore, the COM concluded that there was little evidence in the literature to suggest that there was a health concern related to genotoxicity induction by TiO_2, particularly via the oral route and especially the micro sized TiO_2 fraction (most studies in the literature used nano-sized material). Hence, the genotoxicity risk from dietary food grade E171 was considered to be low. (COM 2024b).

306. The Committee considered that the data from the relevant studies available indicated that TiO_2 did not induce ACF, nor were there significant effects from studies that assessed inflammation and immunotoxicity, reproductive and developmental toxicity, and neurotoxicity. On balance, the COT considered that the NOAEL of 1,000 mg/kg bw per day was robust.

307. The Committee concluded that 1,000 mg/kg bw per day was a robust Point of Departure (POD). This was based on the EOGRT study findings as well as studies by Warheit, Donner and Brown, (2015) and Lee et al., (2019) that reported no effects up to the same dose. There was variability noted in the other studies, but nothing which would alter the proposed POD for food grade TiO_2 (E171).

308. A standard uncertainty factor of 100 (10 for inter species variability and 10 for individual variability) was agreed by Members and applied to the POD which results

in a HBGV of 10 mg/kg bw per day. There is likely to be conservatism in the application of this uncertainty factor to the NOAEL of E171 because 1,000 mg/kg bw per day was the highest dose of TiO_2 tested and therefore the LOAEL (lowest observed adverse effect level) could actually be appreciably higher and, because there is no metabolism of TiO_2 particles, the inter-/intra-species kinetic differences are likely to be lower than the defaults.

309. Titanium dioxide (E171) can be found in a number of food categories. The exposures calculated and considered in this assessment are only for food and were for infants, toddlers, children, adolescents, adults, and the elderly using food consumption data from UK surveys. Maximum occurrence levels of titanium dioxide for specific food items, reported by EFSA (2021), were also used in the estimation of exposure. All mean total dietary exposures were below or very close to the HBGV and would not be expected to lead to adverse health effects. Ninety-fifth percentile estimates of exposure for infants, toddlers, children and adolescents are 1.3- to 2.6-fold higher than the HBGV. However, the HBGV is more conservative than would typically be the case as the POD was the highest dose tested, so toxicity might not occur until doses appreciably higher than the NOAEL, and the uncertainty factor used allowed for possible variability within and between species in metabolism but TiO_2 is not metabolised, with the result that an uncertainty factor of 100 would be more conservative than the usual default. In addition, exposure was estimated assuming that all food categories assessed contained E171 and that in every case this would be at the maximum reported level. These assumptions would lead to overestimation of exposure. Hence, an apparent exceedance of the HBGV by up to 2.6-fold in some 95th percentile consumers is unlikely to lead to adverse health effects.

310. Based on the uncertainties and assumptions of the exposure assessment as mentioned, the exposure estimates derived probably overestimate the current and future exposure to titanium dioxide in the UK population.

311. The Committee concludes that it is unlikely that there would be a risk to health from exposures of E171 TiO_2 from the diet in any of the age groups or scenarios assessed.

COT October 2024

Statement 2024/05

Abbreviations Table

ABCs	Aberrant crypts
ACF	Aberrant Crypt Foci
A(ChE)	Acetyl Cholinesterase
ADME	Absorption, Distribution, Metabolism and Excretion
ADI	Acceptable Daily Intake
AGD	Anogenital Distance
ANS	Additives and Nutrient Sources
ANSES	Agency for Food, Environmental and Occupational Health and Safety
AOP	Adverse Outcome Pathway
AUC	Area under the curve
BET	Brunauer-Emmett-Teller
BSA	Bovine serum albumin
CA	Chromosome aberration
CAC	Colitis Associated Cancer
CEA	Commissariat à l'énergie atomique et aux énergies alternatives
COM	Committee on the Mutagenicity of chemicals in Food, Consumer Products and the Environment
COT	Committee on the Toxicity of chemicals in Food, Consumer Products and the Environment
COX	Cyclooxygenase
CRAMP	cathelin-related antimicrobial peptide
CY	Cyclophosphamide
DC	Dendritic cells
DLS	Dynamic Light Scattering

DMH	dimethylhydrazine
DNSIYC	Diet and Nutrition Survey of Infants and Young Children
EAT	Exposure Assessment Team
EC	European Commission
ECHA	European Chemicals Agency
EDM-HSI	Enhanced darkfield microscopy with hyperspectral imaging
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EOGRT	Extended One Generation Reproductive Toxicity
ER	Endoplasmic Reticulum
EU	European Union
FACS	Fluorescence-Activated Cell Sorting
FAF	The Panel on Food Additives and Flavourings
FAO	Food and Agriculture Organisation
FBS	Fetal bovine serum
FSA	Food Standards Agency
FSANZ	Food Standards Australia New Zealand
GALT	Gut-associated lymphoid tissue
GD	Gestation Day
GFAP	Glial Fibrillary Acidic Protein
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GLP	Good Laboratory Practice
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor
GSH	Glutathione

HBGV	Health-based Guidance Value
hprt	hypoxanthine phosphorybosyl transferase
IARC	International Agency for Research on Cancer
ICAM	Intercellular Adhesion Molecule
ICP-AES	Inductively coupled plasma – atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IEL	intraepithelial lymphocytes
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
INRS	Institut National de la Recherche et de Securite
i.v.	intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JRC	Joint Research Council
KLH	Keyhole limpet haemocyanin
LOD	Limit of Detection
LOQ	Limit of Quantification
MCHC	mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MN	Micronucleus
MPLs	Maximum Permitted Levels
NCI	National Cancer Institute
NDNS	National Diet and Nutrition Survey
NK	Natural killer
NMs	Engineered Nanomaterials
NOAEL	No Observed Adverse Effect Level
NPs	Nanoparticles

NRCWE	The National Research Centre for the Working Environment
NTP	US National Toxicology Program
OECD	The Organisation for Economic Co-operation and Development
OECD WPMN	OECD Working Party on Manufactured Nanomaterials
pg	pigment grade
PLYz	P-lysozyme
POD	Point of Departure
PSLT	Poorly Soluble Low Toxicity
PND	Postnatal Day
QS	Quantum Satis
RAG	Red, amber, green
RBC	Red blood cell
REG	regenerating islet-derived protein
RIVM	Dutch National Institute for Public Health and the Environment
ROS	Reactive Oxygen Species
SCF	Scientific Committee on Food
SCCS	EU Scientific Committee on Consumer Safety
SD	Sprague Dawley
SDF	Stromal Cell-Derived Factor
SEM	Scanning Electron Microscopy
SOD	Superoxide dismutase
spICP-HRMS	single particle ICP-high resolution MS
STEM	Scanning Transmission Electron Microscopy
TBARS	thiobarbituric acid reactive substances

TDMA	Titanium Dioxide Manufacturers Association
TEM	Transmission Electron Microscopy
TG	Test Guideline
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
uf	ultrafine
WBC	White blood cell
WHO	World Health Organisation
XSDC	X-ray scanning disc centrifuge

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COT 2024/05 Annex A

Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment

Statement on the safety of Titanium Dioxide (E171) as a Food Additive

The search terms

1. A literature search was undertaken. The database Lit-fetch was used to search the following search terms between the dates 2021-01-01 to 2023-04-28. For the first 2 search strings, the numbers in brackets denote (number of hits; number of relevant hits), the 3rd search string just denotes number of hits. An updated search was also carried out for the first 2 search strings for 2023-04-28 to 2024-03-01, but only for in vivo studies.

- Search "In Vitro Techniques"[Mesh] OR "Caco-2 Cells"[Mesh] OR "Lysosomes"[Mesh] OR "Hypoxanthine Phosphoribosyltransferase"[Mesh] OR "Autopsy"[Mesh] OR "Micronucleus Tests"[Mesh] OR "Pathology"[Mesh] OR Autops*[tiab] OR histopath*[tiab] OR "in vivo"[tiab] OR "in vitro"[tiab] OR GALT[tiab] OR Caco 2 cell*[tiab] OR Colonic Epithelial Cell*[tiab] OR Colorectal epithelial cell*[tiab] OR Comet assay*[tiab] OR HPRT[tiab] OR "Hypoxanthine Phosphoribosyltransferase"[tiab] OR "hypoxanthin phosphoribosyl transferase"[tiab] OR Lysosom*[tiab] OR "M cell"[tiab] OR "M cells"[tiab] OR "Microfold cell"[tiab] OR "Microfold cells"[tiab] OR Micronucleus assay*[tiab] OR Micronucleus test*[tiab] OR "read across"[tiab] OR SCGE assay*[tiab] OR single-cell gel electrophoresis assay*[tiab] OR "xanthine guanine phosphoribosyl transferase"[tiab] OR "xanthine guanine phosphoribosyltransferase"[tiab] OR xprt[tiab] AND titanium dioxide (322; 19)

- Search "Aneugens"[Mesh] OR "Aneuploidy"[Mesh] OR"apoptosis"[Mesh] OR "Chromatids"[Mesh] OR "Chromosomes"[Mesh] OR "DNA Damage"[Mesh]

OR "DNA Repair"[Mesh] OR "Mutagens" [Pharmacological Action] OR "Mutagens"[Mesh] OR "Mutagenesis"[Mesh] OR "Oxidative Stress"[Mesh] OR Aneugen*[tiab] OR Aneuploid*[tiab] OR Chromatid*[tiab] OR Chromosome*[tiab] OR Clastogen*[tiab] OR "DNA binding"[tiab] OR DNA fragmentation*[tiab] OR DNA damage*[tiab] OR "DNA lesion"[tiab] OR "DNA lesions"[tiab] OR "DNA repair" [tiab] OR genotox*[tiab] OR mutagen*[tiab] OR Mutat*[tiab] OR oxidative stress*[tiab] AND titanium dioxide (209; 16)

- (Titanium dioxide AND Nanoparticles AND E171 AND toxicology) (1); (Titanium dioxide AND Nanoparticles AND E171 AND genotoxicity) (8); (Titanium dioxide AND Nanoparticles AND E171 AND human health) (13); (Titanium dioxide AND Nanoparticles AND E171 AND adverse effects) (7); (Titanium dioxide AND Nanoparticles AND E171 AND reproductive toxicity) (5); (Titanium dioxide AND Nanoparticles AND E171 AND developmental toxicity) (2); (Titanium dioxide AND Nanoparticles AND E171 AND Distribution) (8); (Titanium dioxide AND Nanoparticles AND E171 AND Metabolism) (4); (Titanium dioxide AND Nanoparticles AND E171 AND Excretion) (1); (Titanium dioxide AND Nanoparticles AND E171 AND ADME) (1); (Titanium dioxide AND Nanoparticles AND E171 AND immunotoxicology) (0); (Titanium dioxide AND Nanoparticles AND E171 AND neurotoxicology) (0).

COT 2024/05 Annex B

Summary table of studies

Absorption, Distribution, Metabolism and Excretion (ADME) – E171 animal studies

Reference	TiO_2 characterisation	Quality of study e.g., OECD/GLP	Method and duration of dosing	Study methodology to include species, numbers, controls	Results
Bettini et al., 2017	1) E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles < 100 nm; 2) TiO_2 NPs (NM-105), anatase/rutile, 15–24 nm.	OECD	Series One Dosage: 200 µ L with TiO_2 NM-105, E171 (10 mg/kg of BW/day) or water for 7 days by gavage. Series Two Dosage: E-171 at 200 µ g or 10 mg/kg of	Series One: rats (n = 10 rats/group) dosed daily by intragastric gavage (200 µ L) with TiO_2 NM-105, E171 (10 mg/kg of BW/day) or water for 7 days. Tissue imaging, flow cytometry and cytokine assays, tissue inflammation	Titanium was detected in the immune cells of Peyer's patches. Dendritic cell percentage were increased, observed days after exposure but no effect at 100 days. No effects in the spleen. Regulatory T cells and T-helper cells were significantly decreased days after exposure and at 100 days for rats exposed to E 171. Stimulation in vitro of immune cells isolated from Peyer's patches had

			BW/day via drinking water for 100 days (with or without DMH treatment). Series Three Dosage: No treatment followed by a single dose of 10 mg/kg E-171.	and gut permeability measurements were conducted. Series Two: rats (n = 11 to 12 per group) were treated or not with 1,2-dimethylhydrazine (DMH) to induce colon carcinogenesis and exposed to E-171 at 200 µ g or 10 mg/kg of BW/day via drinking water for 100 days. Control animals (n = 12) received water only. Flow cytometry and cytokine assays were assessed for gut inflammation and ACF.	a decrease in T-helper (Th)-1 IFN-γ secretion and splenic Th1/Th17 inflammatory responses increased. With exposure to TiO_2 NP there was an observed increase in the percentage of dendritic cells in Peyer's patches with no decrease in the percentage of Tregs. E 171 exposure (1 week) did not initiate intestinal inflammation, but E 171 treatment (100-days) showed colon microinflammation - significantly increased IL-1β, IL-8 and TNF-α expression in the colon and increased IL-10.

				Series Three: untreated rats (n = 4) were evaluated for ex-vivo cytotoxicity and proliferative assays on isolated immune cells. E-171 particle agglomeration was assessed in the luminal contents of the jejunum and colon collected from 4 rats 4 h after a single dose of 10 mg/kg was delivered.	
Talamini et al., 2019	E171 (35% nano determined by TEM), 99.3% pure anatase, 201.2 ± 8.5 nm in suspension (NTA). No sonification or deagglomeration to simulate	This work was reviewed by the Institute for Pharmacological Research Mario Negri IRCCS Animal Care and Used Committee (IACUC) and then approved by the Italian	Treatments were given 3 days per week for 3 weeks for a total of 9 treatments in 21 days. Average daily dose of ~2 mg/kg bw.	NFR male mice (22/group) were administered either water (control) or 5 mg/kg bw E171 suspended in water. Ti concentrations in tissues were determined by	Ti concentrations in the liver (0.94 ± 0.57 µg/g tissue) and large intestine (1.07 ± 0.38 µg/g tissue) were significantly higher in treated mice compared to controls. Ti concentrations in the brain, kidney, and testes were below the quantification limit (<0.03 µg/g). Ti concentrations in lungs, spleen, stomach, and small intestine were

	realistic conditions.	National Institute of Health (code:42/2016-PR).	Treatments were dripped slowly into the mice's mouths, allowing each drop to be swallowed.	single particle ICP-MS analysis.	not statistically significant between treated and control mice.
Riedle et al., 2020	E171, anatase, 119 nm.	N/A	Mice were exposed to 0, 1, 10, or 100 mg/kg bw/d E171 via the diet for 6, 12 and 18 weeks.	Mice were divided into 4 groups of 18 and given 0, 6.25, 62.5, or 625 mg/kg diet (equivalent to approximately 0, 1, 10, or 100 mg/kg bw). Then 6 mice per group were euthanized at 6, 12 and 18 weeks.	No evidence of gross alteration of immune-cell physiology or inflammation at doses up to 100 mg/kg bw/d via the diet. Authors demonstrate E171 uptake by Peyer's patches, validating the delivery model. Presence of E171 particles detected by reflectance confocal microscopy (no quantification of particles completed). Weak signals observed at the base of Peyer's patches at low and mid-doses. Higher signals observed at highest dose, indicating evidence of dose-response.
Comera et al. 2020	Food grade TiO2 (E171) 95% anatase.	European legislation (Council Directive	Mice (4 per group).	Adult C57BL/6 mice (12–18 weeks).	Small intestine: TiO2 absorption peaked at 4 h in jejunal and ileal villi and returned to basal levels at 8 h and

		2010/63/UE) and French Decree 2013–118-compliant.	Dosage: single dose (200 µl) of either E171 at 40 mg/kg of body weight (BW) or 200 µl of vehicle (water) by intragastric gavage. In addition, In some experiments, the gavage solution from sonicated E171 particles was equilibrated in 30% corm oil and vortexed before oral delivery.	Animals were terminated at 2, 4, 8, and 24 hours to recover the intestine.	undetectable at 4 h but was present at 8 h in the jejunal Peyer's patches. Colon: Low absorption. Blood: TiO2 particles were detected at 4- and 8-hours post-treatment. 30 minutes post-exposure to TiO2 in the presence and absence of pharmacological inhibitors of paracellular tight junction (TJ) permeability, TiO2 absorption by the jejunal villi was decreased by 66% ($p < 0.001$ vs. control) in the presence of triaminopyrimidine. Other inhibitors had no significant effect. Absorption via a goblet cell (GC)-associated pathway was also detected.
Dudefoi 2017b	Food-grade TiO2 (E171-1, 17% NPs and 100% anatase and E171-6a).	N/A	Dosage: 100–250 ppm.	Method: A defined model intestinal bacterial community.	At these low concentrations no impact on gas production and only a minor effect on fatty acids profiles was observed with limited effect on bacterial communities.

Proquin et al. 2018	E171 in combination with azoxymethane (AOM)/dextran sodium sulphate (DSS) vs E171 only.	N/A	Dosage: 5 mg/kg bw per day of E171 by gavage for 2, 7, 14, and 21 days.	BALB/c mice.	E171 induced a downregulation of genes involved in the immune system which were indicative of impairment. Additionally signalling genes involved in a variety of types of cancer including colorectal cancer were modulated and effects were observed which indicated a potential association with oxidative stress.
Jensen et al. 2019	Vegetable carbon (E153) and food-grade titanium dioxide (E171), mean TiO2 particle size of 270 nm.	N/A	Dosage: 10 weeks by oral gavage once a week.	Rats.	TiO2-only Results: Decreased gene expression of protein TJP1 was observed in the rats only exposed to E171 (500 mg/kg/week) and shorter lung telomeres. This study found no oxidative DNA damage in the liver or lung and no changes in the DNA repair activity of oxidative DNA damage in the lung.
Farrell TP and Magnuson B. (2017).	4 different food grade TiO2 test items containing a range of particle sizes and morphologies.	GLP-compliant, OECD TG 41.	Dosage: Four grades of TiO2 (200 ppm) or control (0 ppm) via the diet for 7 days followed by a control	Male and female Sprague-Dawley rats were given TiO2 by the diet equivalent to 30 mg/kg bw/day for 7 days.	Ti in kidney, liver and muscle were below LOD (<0.1-<0.2 mg/kg ww) Ti in tissues, where above LOD, was 0.1-0.3 mg/kg w. Ti in blood was <0.04 mg/L for all samples.

			diet for 1, 24, or 72 hours.	Animals were then terminated post-feeding at 1, 24 and 72 hours.	Ti in urine was equal to <2% daily dose/L and below LOQ. Ti in faeces was found to be the main route of excretion. No differences in absorption were found between the different grades of TiO2.

Absorption, Distribution, Metabolism and Excretion (ADME) – non-E171/Nanoparticle animal studies

Reference	TiO_2 characterisation	Quality of study e.g., OECD/GLP	Method and duration of dosing	Study methodology to include species, numbers, controls,	Results
Warheit, Boatman and Brown, 2015	1) anatase/rutile (89/11%) (uf-1), d50=43 nm d50=23 nm Methods: XSDC and TEM respectively Shape: Irregular. 2) anatase (100% nano) (uf-2) d50= 42 nm d50=19 nm Methods: XSDC and TEM respectively Shape: Irregular.	OECD Guideline 414.	Sterile water-based TiO_2 sample formulations were administered by oral gavage to time-mated rats from the time of approximate implantation until the day prior to expected parturition. Dose levels: 0, 100, 300 or 1,000 mg/kg bw per day. Dosage volume: 5	Three studies (Group size n=22): Time-mated pregnant Sprague–Dawley rats, (Crl:CD(SD)) exposed to TiO_2 (uf-1, uf-3 and pg-1) by gavage on Gestational Days 6–20. Three additional studies (Group size n=22-23) pregnant Wistar rats exposed to TiO_2 (uf-2 and pg-2) by gavage from Gestational Days 5 to 19. Necropsy:	At 1,000 mg uf-1/kg per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. Mean male fetuses: 7.2 Mean male fetuses control group: 5.5 Test facility historical control group data range: 5.2 to 7.4. Mean female fetuses: 4.8 Mean female fetuses control group: 6.7. Test facility historical control group data range: 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mguf-1/kg bw per day group: 60% (males/females)

	3) rutile (100% nano) (uf-3), d50=47 nm d50=22 nm Methods: XSDC and TEM respectively Shape: rod-like. 4) anatase (27% nano) (pg-1), d50=153 nm d50=120 nm Methods: XSDC and TEM respectively Shape: irregular. 5) rutile (11% nano) (pg-2), d50=195 nm d50=165 nm Methods: XSDC and TEM respectively. Shape: irregular.		mL/kg bw per day.	• Gross examination of the dam, • Counting of corpora lutea, • Implantation sites, • Resorptions, • Live and dead fetuses, • Fetal sex, • Fetal weight, • Fetal pathological external, visceral and skeletal examinations for abnormalities.	Mean control group fetal sex ratio: 46% Test facility historical control group data range: 43% to 53%. A few incidental changes in body weight and feed intake were noted, no other changes were observed in the dams or the fetuses.

Tassinari et al., 2014	TiO_2 nanoparticles (anatase, primary size <25 nm, BET surface area 45-55 m^2/g, purity 99%).	All experiments on animals were performed according to the European Community Council Directive 86/609/EEC (EEC 1986).	TiO_2 nanoparticles were administered by oral gavage over 5 consecutive days at a dose of 0, 1, 2 mg/kg body weight per day.	Sprague-Dawley rats were divided into 3 treatment groups (7 rats/sex/group). Treatment groups were high dose (2 mg/kg bw), low dose, (1 mg/kg bw), and controls (CTRL) (vehicle only (distilled water)).	In the high dose treatment group, significant increases in total Ti tissue levels were found in spleen (0.036 ± 0.009 vs. 0.046 ± 0.008 µg/g fresh weight; $p \leq 0.05$) and ovaries (0.28 ± 0.07 vs. 0.12 ± 0.04 µg/g fresh weight; $p \leq 0.01$). Sex-related histological alterations were observed at both dose levels in thyroid, adrenal medulla, adrenal cortex (females) and ovarian granulosa, without general toxicity. Altered thyroid function was indicated by reduced T3 (males). Testosterone levels increased in high-dose males and decreased in females. In the spleen of treated animals TiO_2 aggregates and increased white pulp (high-dose females) were detected, even though Ti tissue levels remained low reflecting the low doses and the short exposure time.
Ammendolia et al. 2017	Nano-sized titanium dioxide (anatase, primary size <25 nm, BET surface area 45–	N/A	TiO2 NPs at 2 mg/kg bw per day for five days in male and female rats.	N/A	Nanoparticle deposition the intestinal tissue and increased serum testosterone levels. There was no evidence of oxidative stress or cellular alteration at low concentrations

	55 m^2/g, purity 99%).				of TiO2 NPs however NP treatments associated with testosterone or Insulin-like Growth Factor-1 showed increased cell proliferation.
Geraets et al. 2014	TiO2 NPs (sizes NM-100, NM-101, NM-102, NM-103, and NM-104) with differing particle sizes and structure.	N/A	Dosage: Oral and intravenous administration of a single or five repeated doses.	TiO2 nanoparticle kinetics were investigated using intravenous injection and oral dosing in rats. For orally dosed rats, liver, spleen and lymph nodes were targeted for analysis.	Following oral exposure, Ti levels in the liver and spleen were only occasionally above the LOD and was detected in lymph nodes at low levels. Following intravenous exposure, Ti distribution was observed across all tissues (liver, kidney, lung, spleen, heart, brain, thymus and reproductive organs with the liver identified as the main target. Recovery 24 hours post-exposure was 64-95% or 59-108% respectively for male and female rats. The maximum relative decrease of TiO2 up to 90 days post-exposure was 26%.
Hendrickson et al. 2016	2 test items TiO2 NPs (5-10 nm and 20-25 nm respectively).	N/A	Dosage: Intragastric administration of TiO2 NPs (1	Male rats.	GIT and secondary organ translocation were size-dependent.

			of 2 test items) for 28 days at a dose of 250 mg/kg of body weight per day.		Larger nanoparticle exposure showed deposition in liver, kidneys, spleen, and small intestine (0.01– 0.29 µg/g of organ). Smaller nanoparticle exposure resulted in Ti deposits in brain, lungs, heart, liver, kidneys, spleen, small intestine, testicles, and blood (0.004–0.227 µg/g of organ).
Hendrickson et al. 2020	TiO2 NPs	N/A	Dosage: A single dose suspension of TiO2 NPs (250 mg/kg of body weight).	Model: A Physiological model designed to mimic the intestinal lumen of an experimental animal.	TiO2 NPs were found in the small intestine mucosa, liver, and spleen. TiO2 NPs resulted in different changes in the cellular ultrastructures in the endoplasmic reticulum, mitochondria, extensions into the perinuclear spaces and caused myelin-like structures to appear. The most sensitive organ was noted to be the spleen.
Kreyling et al. 2017a	TiO2 anatase NPs. Median agglomerate size: 70 nm.	N/A	Dosage: 40–400 µg/kg bw single intravenous dose in	Female Wistar rats. Clearance and biokinetics were observed from 1-	Highest TiO2 NP accumulation occurred in the liver after one day (95.5%) then then spleen (2.5%), carcass (1%), skeleton (0.7%) and blood (0.4%). TiO2

			aqueous suspension.	hour post-dosage to 4 weeks.	NPs were detected in all other organs at levels lower than these. TiO2 NPs in the blood decreased quickly 24 h after exposure. Organs and tissue NP levels were stable until day-28.
Kreyling et al. 2017b	TiO2 NPs.	N/A	Dosage: Oral dosage of a single dose of an aqueous TiO2 NP suspension at 30–80 µg/kg bw.	Female Wistar-Kyoto rats. Assessed 1 h, 4 h, 24 h and 7 days post-oral exposure.	0.6% of the administered dose passed the gastro-intestinal-barrier after 1 hour. 0.05% of the dose were still distributed in the body after 7 days distributed across the following organs: liver (0.09 ng/g), lungs (0.10 ng/g), kidneys (0.29 ng/g), brain (0.36 ng/g), spleen (0.45 ng/g), uterus (0.55 ng/g) and bone deposits (0.98 ng/g). Faecal excretion was complete after 4-5 days.
Sadiq et al. 2012	TiO2 NPs.	N/A	Dosage: 1) Intravenous 0.5, 5.0, and	6–7-week-old male B6C3F1 mice.	Blood results: No increase in Pig-a mutant frequency or %MN-RETs,

			50 mg/kg TiO2 NPs. 2) Intravenous three daily doses of 50 mg/kg TiO2 NPs Ti levels in bone marrow measured after 4, 24, and 48 hours of the last treatment.	In vivo micronucleus and Pig-a (phosphatidylinositol glycan, class A gene) mutation assays using TiO2 NPs to evaluate genotoxicity. Blood: Samples taken one day before the treatment and on Day 4, and Weeks 1, 2, 4, and 6 after the beginning of the treatment. Pig-a mutant frequencies were determined at Day −1 and Weeks 1, 2, 4 and 6, percent micronucleated-reticulocyte frequencies were measured only on Day 4.	Tissue results: Ti NPs peaked at 4 hours post-exposure. %RETs was reduced in treated animals on Day 4 (dose-dependent) which suggests cytotoxicity of TiO2-NPs in the bone marrow. No evidence of genotoxicity.

Absorption, Distribution, Metabolism and Excretion (ADME) – E171 and non-E171/Nanoparticle Human Studies

Reference	TiO_2 characterisation	Quality of study e.g., OECD/GLP	Method and duration of dosing	Study methodology to include species, numbers, controls,	Results
Pele et al. 2015	Pharmaceutical/food grade TiO2, anatase, 50-250nm.	This study was conducted based on ethical approval under EC01/037.	Dosage: A single dose of 100 mg TiO2.	Test subjects: Humans with normal intestinal permeability. Blood samples were collected at between 0.5 to 10 h post-oral exposure. Blood samples were analysed for visual TiO2 reflective particles using dark field microscopy.	Early absorption occurred by 2 hours with a peak maximum at 6 hours. Following oral dosing. This mirrors the results of a previous study by Bockmann et al (2000) which the authors were attempting to replicate.
Guillard et al. 2020	Basal Ti level in human placenta study. TiO2 with 55% NPs, 20 to 440 nm.	N/A	Samples were taken of placenta and meconium from human babies (n=22) and tested for TiO2 and other metals and	TiO2 in human placentas was analysed by ICP-MS and STEM coupled to EDX spectroscopy.	All placenta contained TiO2 at 0.01 to 0.48 mg/kg of tissue with the majority below 100nm in size (over 50%). Meconium samples also contained TiO2 between 0.02-1.5 mg/kg.

			trace elements.		
Heringa et al. 2018	Post-mortem analysis of human liver and spleen TiO2 analysis.	N/A	N/A	High resolution ICP-MS was used to detect TiO2 in the liver and spleen in 15 deceased humans (9 female and 6 male).	7 of the 15 livers sampled contained TiO2 and 13 of 15 of spleen samples contained the same. Particle sizes respectively for liver and spleen: 85–550nm and 85–720nm with 24% <100 nm in size. Particle mass concentration for liver and spleen respectively: to 0.3 mg titanium/kg tissue and 0.01 to 0.4 mg titanium/kg tissue. Average concentration in liver and spleen samples: 40 ng/g and 80 ng/g.
Peters et al. 2020	Postmortem tissue analysis of deceased persons for the presence of TiO2. Detected particle sizes were in the range of 50–500 nm, with a			15 humans sampled, 8 female and 7 male aged 64-98 years. Postmortem liver, spleen, kidney, jejunum and ileum were sampled.	Findings of between <0.01 to 2.0 mg Total Ti/kg with median values (mg Ti/kg): Liver = 0.02, Spleen = 0.04, Kidney = 0.05, Jejunum = 0.13, Ileum = 0.26.

	mode of 100–160 nm.				Particulate TiO2 were observed from 0.01 to 1.8 mg Ti/kg with median values (mg Ti/kg): Liver = 0.02, Spleen = 0.02, Kidney = 0.03, Jejunum = 0.08, Ileum = 0.25. Particulate TiO2 accounted for 80% of the total-Ti concentration.

Aberrant Crypt Foci (ACF) as a marker for carcinogenicity

Reference	**TiO_2 characterisation**	**Quality of study e.g., OECD/GLP**	**Method and duration of dosing**	**Study methodology to include species, numbers, controls,**	**Results**
Blevins et al., 2019	E 171, anatase, 110–115 nm (SEM), 36% particles < 100 nm.	N/A	E-171 concentrations: 0 mg/kg* dose (22.3 ± 1.2, 23.7±1.8 ppm) 40 ppm dose (59.6 ±1.1, 61.0±2.6 ppm) 400 ppm dose (384 ± 8, 387±13 ppm) 5000 ppm dose (4310 ± 132, 4610±160 ppm).	Six-week-old male Wistar Han IGS (Crl:WI (Han)) rats. Test material: Food grade sample E-171. DIfferent grades	E-171 consumption did not alter T-cell-mediated mechanisms of immune control. Dietary E-171 did not induce inflammation peripherally or in the GI tract.

				of commercially-available E-171 were averaged to produce the test material supplied. Test material was added to feed. Two feed batches: batch one was fed throughout the 7-day study and through week 10 of the 100-day study. Batch two was fed post-week 10 of the 100-day study. 7-day study: 4 groups of 5 animals (randomised based on weight). Total food and water consumption were calculated at the end of the study. Body weights measured at the start and end of	An increase was observed in the relative spleen weight in 22.4 mg E-171/kg bw per day + DMH compared to not initiated animals and an increase in IL-17A in colon (22.4 mg E 171/kg bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects. No changes were observed in spleen cellularity. No changes were observed in the percentage of CD103+ DC, CD4+ T helper cells or total or activated Treg in peripheral blood, spleen or Peyer's patches in animals exposed to E-171 + DMH compared to animals treated with only DMH. No treatment related histopathological changes were observed in the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E-171.

				the 7-day exposure period. 100-day study: 8 groups of 16 animals. Groups 1-4 were dosed with 180 mg/kg BW dimethylhydrazine dihydrochloride (DMH · 2HCl) in 1.5% EDTA-0.9% NaCl, pH 6.5 by intraperitoneal injection (the highest dose that showed no signs of toxicity in pilot studies). Animals in groups 5-8 were given a dose of 1.5% EDTA-0.9% NaCl, pH 6.5. by intraperitoneal injection. Seven days post-injection, feeding	Rats treated with DMH only and those which received E-171 in the diet after the initiation displayed several histopathological abnormalities. A significant surface area of the epithelial surface of the sampled colon (proximal, middle and distal) was obscured when observed by light microscopy therefore the entire surface of the colon samples for ACF and ABC could not be examined. No change in the number of ACF and ABC were observed due to E-171 exposure alone.

				of the test material was commenced at 0, 40, 400, or 5000 ppm E-171 in groups 1-4 and 5-8 for 100 days.	
Akagi et al., 2023 – 28 Day Study	6 nm TiO_2 nanoparticles.	N/A	5 female and 5 male F344/DuCrlCrlj rats.	TiO_2 NPs with a crystallite size of 6 nm were examined in male and female F344/DuCrlCrlj rats by repeated oral administration of 10, 100, and 1000 mg/kg bw/day (5/sex/group) for 28 days.	No mortality was observed in any group, and no treatment-related adverse effects were observed in body weight, urinalysis, haematology, serum biochemistry, or organ weight. Histopathological examination revealed TiO_2 particles as depositions of yellowish-brown material. The particles observed in the gastrointestinal lumen were also found in the nasal cavity, epithelium, and stromal tissue in the 28-day study. Overall, no effects were observed after repeated oral administration of TiO_2 with a crystallite size of 6 nm at up to 1000 mg/kg bw/day regarding general toxicity,

					accumulation of titanium in the liver, kidneys, and spleen, abnormality of colonic crypts, and induction of DNA strand breaks and chromosomal aberrations.
Donner et al. 2016	One of three pigment-grade or one of three ultrafine /nanoscale anatase and/or rutile TiO2 test materials.	OECD 474 Guidelines.	Dosage: Single oral gavage doses of 500, 1000 or 2000 mg/kg body weight with negative (water) and positive controls (cyclophosphamide).	Male and female rats. Blood samples were collected 48 and 72 h post-exposure.	There were no relevant increases in the micronucleated RET frequency in any TiO2-exposed group at either time point at any dose level. All tests showed negative results for in vivo genotoxicity effects, no significant blood or liver TiO2 increases following exposure to the highest dose.

Reproductive toxicity

Reference	**TiO_2 characterisation**	**Quality of study e.g., OECD/GLP**	**Method and duration of dosing**	**Study methodology to include species, numbers, controls,**	**Results**
Leuschner, 2020 –	Test substance: Anatase E-171, median particle	OECD Test Guideline 443.	Dosages (by oral diet, dependent on endpoint for each cohort):	96 male and 96 female CD® (Sprague Dawley)	Results:

Main Study	diameter 99.9 (± 2.0 nm), 51% of particles < 100 nm. Dietary particle size: 109.2 (± 1.4) to 113.7 (± 4.9 nm), 31-43% of particles < 100 nm.		F0, F1-1A and 1B - 0, 100, 300, and 1000 mg/kg bw/day over 10 weeks (prior to mating and up to the end of weaning periods). F1-2A and 2B: indirectly exposed to test item via milk of feed-dosed rats. Additional background levels of TiO2 in feed were estimated to be approximately 1.4 mg/kg bw/day. Diet mixtures were prepared weekly. Food intake was monitored and volumes of dosed feed was adjusted according to previous consumption weekly. Administration of keyhole limpet hemocyanin (KLH) and cyclophosphamide KLH via intravenous	IGS Rat (Crl:CD(SD)). Test Grouping Sizes: F0 – 4 groups, 20 male, 20 female per group. F1-1A and 1B – 20 male, 20 female. F1-2A, 2B and 3– 4 groups, 10 male, 10 female. Endpoint Groupings: F1-1A and 1B - reproductive & developmental toxicity F1-2A and 2B - developmental neurotoxicity. F3 - developmental immunotoxicity.	F0 - Dose-dependent marginal increase in TiO_2 blood and urine concentration in rats dosed with 1000 mg/kg bw/day. No test item-related effects on sexual function or fertility in males or females. No test item-related pre- or postnatal loss observed. No test item-related thyroid hormone or haematological effects. No test item-related differences in splenic lymphocyte subpopulation distribution. No test item-related changes related to histopathology examinations including the testis and epididymides and intestinal examinations for ACF. **Reproductive & developmental toxicity F1-1A and 1B**

			bolus injection in F3 cohort.	Test Item Exposure: F0 - Exposure to test item was from 10 wks prior to mating until F1 generation weaning. F1 – Exposure to test item was from weaning to PND 4/PND 8 of F2 generation. F2 – Exposure from birth until PND 4 or PND 8 (via milk).	No test item-related effects on sexual function or fertility in males (F1-1A – sexual hormones and maturation and sperm) or females (F1-!B - sexual maturation and hormones, number and length of estrous cycles, follicle number or corporea lutea). No treatment-related pre- or postnatal loss. No external or internal abnormalities detected in pups. No test item-related effects on growth or sexual development. No test item-related differences in body weight or food consumption. No test item-related thyroid hormone or haematological effects reported at any dose for F1-1A cohort. No test item-related differences in weight or histopathology (spleen, thymus, lymph nodes, bone marrow, white blood cell

					count, and splenic lymphocyte subpopulation distribution in F1-1A. No test item-related effects on pathology or histopathology. There was a statistically significant decrease in the antigen specific IgM level was measured at the highest dose tested (1,000 mg/kg bw/d) in males only (–9%) and without an apparent dose-response. No dose-dependent increase in TiO_2 blood concentration in rats up to 1000 mg/kg bw/day Observation of pale faeces not of toxicological relevance. **Developmental neurotoxicity** **F1-2A and 2B** No test item-related pre- or postnatal loss observed. No external or internal

					abnormalities detected in pups. No test item-related effects on growth or sexual development. No test item-related differences in body weight or food consumption. No test item-related effects on neurofunctional endpoints (F1-2A). Dose-dependent elevations of blood TiO_2 levels were found exceeding 300 mg/kg No test item-related effects on pathology or histopathology. Observation of pale faeces not of toxicological relevance. **Developmental immunotoxicity** **F3** No test item-related differences in body weight or food consumption.

					No mortality and no test item-related effects reported at any dose for any generation. No ACF were found in the colons of animals in any dose group. Observation of pale faeces not of toxicological relevance.
Leuschner, 2020 – Satellite study	Test substance: Anatase E-171, 51% of particles < 100 nm. Dietary particle size: 31-43% of particles < 100 nm.	OECD Test Guideline 443.	F0 satellite group: 0, 100, 300, and 1000 mg/kg bw/day over 10 weeks (prior to mating and up to the end of weaning periods).	CD® (Sprague Dawley) IGS Rat (Crl:CD(SD)). F0 satellite group – 30 male, 30 female per group + additional 40 (20 male, 20 female) for use as an F1 generation of satellite animals to be used as the positive control group in the KLH-assay (?) Endpoint: ACF.	No test item-related effects in behaviour or external appearance. No test item-related thyroid hormone effects. No test item-related effects on body weight, food consumption and water consumption. No test item-related effects on haematology and biochemical parameters or urinalysis.

					No test item-related effects on thyroid and sexual hormones or sperm. No test item-related changes in bone marrow or organ weights. No test item-related histopathological effects in the high dose group. No test item-related induction of aberrant crypt foci (ACF) in rat colons. Satellite animals of F1 used as the positive control for the KLH assay (at a different time and ages from F1-3 cohort). No additional controls were tested. No test animals died prior to termination. Observation of pale faeces not of toxicological relevance.
Lee et al., 2019	TiO_2 NPs P25 (15–24 nm).	OECD Guideline 414 (Pre-natal Toxicity Study).	Test item: Nanoparticles in deionised water. 80/20 anatase/rutile.	Sprague–Dawley rats (12 females per group).	No statistically significant differences in general clinical signs, body weight, organ weights (absolute and relative to body weight),

			Mean diameter of approximately 21 nm (minimum of 100 particle sizes averaged) administered daily by oral gavage. Dosage: Test item was administered from Gestational Days 6 to 19 at dose levels of 0, 100, 300 and 1000 mg/kg with a dose volume of 10 mL/kg.	Quantitative analysis in blood/tissues. Four groups of twelve females per group in the toxicology group (total test animals: 48) and four groups of four females in the tissue distribution group (total test animals: 16).	macroscopic findings except a statistically significant decrease in food intake but no correlated decreased body weight or body weight gain during the study period of the females of the high-dose group. No statistically significant differences in caesarean section parameters and fetal external and visceral examination. A small but statistically significant increase (4%) was observed in the number of ossification centres in the metatarsals of both hindlimbs of the fetuses of 100 mg/kg bw per day group which may have been incidental.

Immunotoxicity

Reference	TiO_2 characterisation	Quality of study e.g., OECD/GLP	Method and duration of dosing	Study methodology to include species, numbers, controls	Results
Han et al., 2020	E171, anatase, 150 nm, 99.5% purity.	Study conducted according to OECD TG 408.	E171 suspended in distilled water, sonicated for at least 10 minutes. E171 administered by oral gavage at doses of 0, 10, 100 or 1,000 mg/kg bw/d for 90 days. Quantitative analysis in Sprague-Dawley rat's tissues.	Sprague–Dawley rats (10/sex/group) were administered E171 by oral gavage at doses of 0, 10, 100 or 1,000 mg/kg bw/d for 90 days. Ti concentrations were measured in the colons, kidneys, and spleens harvested from all rats at necropsy.	Statistically significant decreases in GM-CSF plasma levels (~30% in females) and plasma IgM (~12% in females and 9% in males) were observed at the highest dose compared to controls. E171 accumulation in the stomach wall of several rats administered 1,000 mg/kg E171 for 90 days. Ti concentration increased in the colons of both sexes administered 1,000 mg/kg E171 compared with the control, while colonic, superoxide dismutases (SOD)-1 (male and female) and SOD-2 (female) protein levels were down-regulated.

					When exposed to AGS cells (human stomach epithelial cell line) for 24 h, E171 (40 µg/mL) was located in the perinuclear region. The E171 treatment affected expression of ER stress-related proteins but did not induce cell death up to the used maximum concentration (40 µg/mL). A gene profile analysis also showed that immune response-related microRNAs were most strongly affected by E171 exposure.
NCI, 1979	TR-097: Titanium Dioxide (CASRN 13463-67-7) (nih.gov) Titanium dioxide anatase Purity: 98%.	N/A	Groups of 50 rats of each sex and 50 mice of each sex were administered titanium dioxide in the diet at one of two doses, either 25,000 or 50,000 ppm, for 103 weeks and then observed for 1 additional week. Matched controls consisted of 50 untreated rats of each	Administration of the titanium dioxide had no appreciable effect on the mean body weights of rats or mice of either sex. With the exception of white feces, there was no other clinical sign that was judged to be related to the	In the male and female mice, no tumours occurred in dosed groups at incidences that were significantly higher than those for corresponding control groups. It is concluded that under the conditions of this bioassay, titanium dioxide was not carcinogenic by the oral route for Fischer 344 rats or B6C3F1 mice.

			sex and 50 untreated mice of each sex. All surviving rats and mice were killed at 104 weeks.	administration of titanium dioxide. Survival of the rats and the male mice at the end of the bioassay was not affected by the test chemical; mortality in female mice was dose related. Sufficient numbers of dosed and control rats and mice of each sex were at risk for development of late-appearing tumors.	
Akagi et al., 2023 – 28 Day Study	6 nm TiO_2 nanoparticles.	N/A	5 female and 5 male F344/DuCrlCrlj rats.	TiO_2 NPs with a crystallite size of 6 nm were examined in male and female F344/DuCrlCrlj rats by repeated oral administration of 10, 100, and 1000 mg/kg bw/day (5/sex/group) for 28 days.	No mortality was observed in any group, and no treatment-related adverse effects were observed in body weight, urinalysis, haematology, serum biochemistry, or organ weight. Histopathological examination revealed TiO_2 particles as depositions of yellowish-brown material. The particles observed in the gastrointestinal lumen

					were also found in the nasal cavity, epithelium, and stromal tissue in the 28-day study. Overall, no effects were observed after repeated oral administration of TiO_2 with a crystallite size of 6 nm at up to 1000 mg/kg bw/day regarding general toxicity, accumulation of titanium in the liver, kidneys, and spleen, abnormality of colonic crypts, and induction of DNA strand breaks and chromosomal aberrations.
Akagi et al., 2023 – 90 Day Study	6 nm TiO_2 nanoparticles.	N/A	10 female and 10 male F344/DuCrlCrlj rats.	TiO_2 NPs with a crystallite size of 6 nm were examined in male and female F344/DuCrlCrlj rats by repeated oral administration of 100, 300, and 1000 mg/kg bw/day (10/sex/group) for 90 days.	No mortality was observed in any group, and no treatment-related adverse effects were observed in body weight, urinalysis, haematology, serum biochemistry, or organ weight. In addition, they were observed in Peyer's patches in the ileum, cervical lymph nodes, mediastinal lymph nodes, bronchus-associated

					lymphoid tissue, and trachea in the 90-day study. Overall, no effects were observed after repeated oral administration of TiO_2 with a crystallite size of 6 nm at up to 1000 mg/kg bw/day regarding general toxicity, accumulation of titanium in the liver, kidneys, and spleen, abnormality of colonic crypts, and induction of DNA strand breaks and chromosomal aberrations.
Pinget et al., 2019	E171, anatase, 30-300 nm. E171 was dispersed in drinking water using sonication.	N/A	Mice were exposure to E171 via drinking water for 4 weeks at doses of 0, 2, 10, 50 mg/kg bw/d. Dose is calculated based on water intake measured per cage. Microbiota populations in fecal samples or the small intestine were examined through 16S rRNA sequencing. Canonical correspondence	Male C67BL/6JAusb mice were exposed to E171 via drinking water at doses of either 0, 2, 10, or 50 mg TiO2/kg BW/day for 3 weeks to determine impact on colonic microbiota composition and on gut bacterial metabolites (10 mice/group).	At the highest dose tested, TiO2 had minimal impact on the composition of the gut microbiota. Alterations in bacterial metabolites were observed from 10 mg/kg bw/d. Doses of 10 and 50 µg/ml TiO2 significantly promoted biofilm formation by commensal bacteria. There was reduced expression of the colonic mucin 2 gene, a key component of the intestinal

			analysis (CCA) constrained to the 4 distinct TiO_2 concentrations used.	Incubated commensal bacteria derived from mouse colons anaerobically for 5 days with dose of 0, 2, 10, 50 µg/ml of TiO_2 biofilm formation (6 mice/group). Impact of TiO_2 on colonic epithelial function was determined by comparison of gene expression of key markers Muc2, Tjp1, Defb3, and Gzmb in colonic tissue of mice administered 0, 2, 10, or 50 mg TiO2/kg BW/day in drinking water (n = 5–8 mice per group). To investigate impact of TiO2 on colonic	mucus layer, and increased expression of the beta defensin gene, indicating that TiO_2 significantly impacts gut homeostasis. These changes were associated with colonic inflammation, as shown by decreased crypt length, infiltration of CD8+ T cells, increased macrophages as well as increased expression of inflammatory cytokines.

				inflammation, flow cytometric analysis, gene expression determined by qPCR and tissue staining were used on mice administered 0, 2, 10, or 50 mg TiO2/kg BW/day in drinking water (n = 8–10 mice per group). TiO2 impact on adaptive immune cell infiltration into the colon was investigated using gene expression via qPCR and flow cytometric analysis on mice treated with 0, 2, 10, or 50 mg TiO2/kg BW/day in drinking water (5-8 mice/group).	

Neurotoxicity

Reference	TiO_2 characterisation	Quality of study e.g., OECD/GLP	Method and duration of dosing	Study methodology to include species, numbers, controls,	Results
Sofranko et al., 2021	10 mg/g TiO_2, 2 mg/g polyvinylpyrrolidone-coated Ag.	OECD 424 Neurotoxicity study in the rodents.	N/A	10 female and 10 male C57BL/6J mice. The mice were fed ad libitum with food pellets dosed with 10 mg/g TiO_2, 2 mg/g polyvinylpyrrolidone-coated Ag or control pellets for 28 days.	The neurotoxicity of TiO_2 and Ag NMs, applied in food pellets, in male and female C57BL/6 J mice in a 28-day oral exposure study with or without a 14-day post-exposure recovery period. No major neuropathological changes regarding neuroinflammation in biochemical and immunohistochemical analyses could be observed and behavioural changes in anxiety and cognition were absent. However, in the Ag NM exposed mice motor performance effects were observed by the rotarod test that differed between sexes. The female mice that were exposed to Ag NM for 28 days, showed a consistent diminished motor coordination and increased cortical activity of specific tyrosine kinases. Female mice that were exclusively investigated in a

					subsequent toxicokinetic study also revealed whole brain levels of Ag that steadily increased during the 28 days of exposure and persisted up to 4 weeks post-exposure. Our study demonstrates that subacute exposure to foodborne TiO_2 and Ag NMs does not cause marked neurotoxicity in mice. However, our toxicokinetic and specific toxicodynamic findings with Ag NMs indicate that long-term oral exposures to this nanomaterial may cause adverse effects on the central nervous system in a sex dependent manner.
Grissa et al. (2016)	TiO_2 NPs, anatase, 5–12 nm (TEM, XRD).	N/A	Internal exposure: quantitative in male Wistar rat tissues; methodology with important flaws.	N/A	There was a statistically significant dose-related increase in the level of NO in 100 and 200 mg/kg bw per day TiO_2 NPs groups observed and a statistically significant dose-related increase in brain TNF-α in 200 mg/kg bw per day TiO_2 NPs group.
Gerber et al., 2022	TiO_2 NPs, average primary particle size of 26.2 ± 10.7 nm.	N/A	N/A	The aim of the study was to investigate the effects of two common types of NP, titanium dioxide NP (TiO_2 NP) and silver	Acute and sub-chronic exposure to TiO_2 NP is without effects, whereas chronic exposure only modestly reduces neuronal

				NP (AgNP), on neuronal function following acute (0.5 h), sub-chronic (24 h and 48 h) and chronic (14 days) exposure in vitro rat cortical cells.	function without affecting morphology.
Ciu et al., 2021	TiO2 NPs.	N/A	36 male Sprague Dawley rats aged postnatal day 21 (PND 21) were injected intraperitoneally with TiO2 NPs (20 mg/kg) and/or BEO (200 mg/kg).	N/A	TiO_2 NPs exposure during the adolescent period induced anxiety-like behaviour, cognitive impairment, neuroinflammation and oxidative damage in hippocampus, and BEO treatment could significantly ameliorate the neurotoxicity induced by TiO_2 NPs exposure.
Naima et al., 2021	TiO2 NPs.	N/A	Rats were injected intravenously with a single dose of TiO2-NPs (20 mg/kg body weight) and were subjected to cognitive and emotional tests using Morris water maze and elevated plus maze.	N/A	Acute intravenous injection of TiO_2-NPs impaired behaviour performances through brain biochemical and structural changes and precautions should be taken to their usage in food additive and medical applications.
Canli et al., 2020	TiO2 NPs	N/A	Oral administration of TiO2 for 14 days (0, 0.5, 5, and 50 mg/kg bw/day).	Female rats.	Results showed that brain AChE activity decreased at all treatment levels, and ATPase activities increased.

					Intestine and kidney ATPase activities showed no significant change. Levels of GSH showed no significant change but the TBARS level at the highest NP dose showed a significant decrease. TiO2 NPs accumulated (dosage-dependent) in tissues. The brain was found to be the most sensitive organ against the effects of TiO2 NPs.

COT 2024/05 Annex C

Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment

Statement on the safety of Titanium Dioxide (E171) as a Food Additive

1. The occurrence levels of food grade E171 TiO_2 used in the exposure assessment scenarios are shown in Table 1.

Table 1: Occurrence levels of titanium dioxide (E171) used in the exposure assessment scenarios (mg/kg or mg/L as appropriate)

EFSA Food category number	Food category name	Concentration levels used in the exposure assessment (Maximum reported)	MPL (mg/L or mg/kg as appropriate)
01.4	**Flavoured fermented milk products including heat-treated products***	**48**	**QS**
01.5	Dehydrated milk as defined by Directive 2001/114/EC	N/A	QS
01.6.3	Other creams	N/A	QS
01.7.1	Unripened cheese, excluding products falling in category 16.	N/A	QS
01.7.3	Edible cheese rind	N/A	QS
01.7.4	Whey cheese	N/A	QS
01.7.5	Processed cheese	N/A	QS
01.7.6	Cheese products, excluding products falling in category 16	N/A	QS
01.8	**Dairy analogues, including beverage whiteners***	**125**	**QS**
03	**Edible ices***	**857**	**QS**
04.2.4.1	Fruit and vegetable preparations, excluding compote	N/A	QS

04.2.4.1	Fruit and vegetable preparations, excluding compote	N/A	QS
04.2.5.3	Other similar fruit or vegetable spreads	N/A	QS
05.2	**Other confectionery including breath-refreshening microsweets***	**4,500**	**QS**
05.3	**Chewing gum***	**16,000**	**QS**
05.4	**Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4***	**20,000**	**QS**
06.3	Breakfast cereals	N/A	QS
06.5	Noodles	N/A	QS
06.6	Batters	N/A	QS
06.7	Pre-cooked or processed cereals	N/A	QS
07.2	**Fine bakery wares***	**318**	**QS**
08.3.3	Casings and coatings and decorations for meat	N/A	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	N/A	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	N/A	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	N/A	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	N/A	QS
09.3	Fish roe	N/A	QS
12.2.2	Seasonings and condiments	N/A	QS
12.4	Mustard	N/A	QS
12.5	**Soups and broths***	**193**	**QS**
12.6	**Sauces***	**4,000**	**QS**
12.7	**Salads and savoury-based sandwich spreads***	**3,000**	**QS**
12.9	**Protein products, excluding products covered in category 1.8***	**5,000**	**QS**
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)	N/A	QS

13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	N/A	QS
13.4	Foods suitable for people intolerant to gluten as defined by Regulation (EC) No 41/2009	N/A	QS
14.1.4	**Flavoured drinks***	**70**	**QS**
14.2.3	Cider and perry	N/A	QS
14.2.4	Fruit wine and made wine	N/A	QS
14.2.5	Mead	N/A	QS
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	N/A	QS
14.2.7.3	Aromatised wine-product cocktails	N/A	QS
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	N/A	QS
15.1	Potato-, cereal-, flour- or starch-based snacks	N/A	QS
15.2	**Processed nuts***	**7,000**	**QS**
16	**Desserts, excluding products covered in categories 1, 3 and 4***	**200**	**QS**
17.1	**Food supplements supplied in a solid form, excluding food supplements for infants and young children***	**26950**	**QS**
17.2	**Food supplements supplied in a liquid form, excluding food supplements for infants and young children***	**26950**	**QS**

QS – *quantum satis* (no maximum numerical level is specified, and substances will be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled).

* the 16 food categories used in the exposure assessment.

COT 2024/05 Annex D

Studies used to review the toxicokinetics and absorption of the nanoparticle form of TiO_2

Studies in rats and mice

Disdier et al., (2015)

1. The test material used was 75% anatase, 25% rutile titanium dioxide nanoparticles 21.5 ± 5 nm.

2. Adult Fischer rats were treated with 1 mg/kg titanium dioxide or saline buffer (control); no dispersion protocol was applied for the in vivo experiment. The dose was administered intravenously, and samples were taken at 30 minutes, 1, 2, 6 and 24 hours and 7-, 28-, 90- and 365-days post treatment from blood, liver, brain, spleen, kidney and lungs. Blood and brain samples were additionally collected at 5 and15 minutes post injection. Titanium concentrations were determined by ICP-MS.

3. The authors reported that titanium burdens in the liver, spleen and lungs of the treated group were significantly higher for all time points post injection, however the levels declined over time. Levels in the liver were higher than the spleen and lungs. Titanium burden after a year remained high, suggesting biopersistence (approximately 33% of the titanium burden of the early time points). The titanium burden in the kidneys increased significantly from 30 minutes to 24 hours but decreased significantly 7 days after i.v. administration. No statistically significant results were reported for the blood samples. For the time points before 24 hours, there was a statistically significant increase if titanium concentrations in the brain. After 24 hours, titanium content did not differ from controls. No further details were given in the text, but graph 2 provides more details on the levels of titanium in the different organs. The authors estimated that they recovered approximately 44 % of

the administered dose in the liver, 10 % in lungs and 2 % in spleen 6 hours post administration.

Kreyling et al., (2017a)

4. The test item used was titanium dioxide anatase nanoparticles, 7-10 nm.

5. Female Wistar- Kyoto rats were dosed with 10-20 µg of radiolabelled titanium dioxide given as a single i.v. injection of nanoparticles suspended in water.

6. The animals were terminated at 1, 4, 24 hours and 7and 28 days post administration (n=4 per time point). All organs as well as blood and all excretions of the animals were collected. The results of a separate intravenous study performed to investigate the absorption and biodistribution of soluble ionic48V were used to correct48V release from [48V] titanium dioxide nanoparticle.

7. The highest [48V] titanium dioxide accumulations were found in liver (95.5%ID on day 1), followed by spleen (2.5%), carcass (1%), skeleton (0.7%) and blood (0.4%). Detectable nanoparticle levels were found in all other organs. The [48V] Titanium dioxide NP content in blood decreased rapidly after 24h while the distribution in other organs and tissues remained rather constant until day-28. Particularly, 4 hours post, administration, 99.5% of the radioactive dose was found in the liver and at 28 days 88.9% of the dose was detected in the liver. The spleen and the kidneys contained: spleen between 2.5% and 4%, and kidneys between 0.05% and 0.2%. All other tissues had lower contents. The bones (including the marrow) and the remaining tissues contained 1% and 0.7%. The radiolabelled compound was excreted in urine and within 28 days the excretion amounted to roughly 1%. Highest excretion occurred on day 1. Excretion by the faeces, indicative of biliary excretion, amounted to 3% over 28 days.

Kreyling et al., (2017b)

8. A similar experiment to the one described above was also carried out, with the animals exposed via the oral route (a single dose of an aqueous, radiolabelled-nanoparticle suspension by intra-esophageal instillation) in female Wistar-Kyoto rats.

9. Titanium concentrations were determined at five retention time points 1 h, 4 h, 24 h, 7 d and 28 d after gavage in four rats for each time point. However, after observing in the seven-day experiment that fecal excretion of the test item was complete after 4–5 days, no further animals were terminated for a 28-day biodistribution.

10. Blood, all organs, tissues and excreta were collected, and the concentration of radioactive titanium dioxide was measured. Most of the radioactivity was excreted in the faeces. Absorption was calculated as the fraction of the dose that could not be accounted for by the radioactive content of the intestinal tract plus faeces. Approximately 0.6% of the dose was absorbed within an hour post-treatment. Seven days post treatment, roughly 0.05% of the dose administered was still present.

11. The authors noted that the distribution patterns between animals were variable and that several data were below the LOD during the first 4 hours. The spleen, kidneys, heart and uterus contained detectable levels even after 4 hours post treatment. Maximum retention was reached in the spleen, kidneys and heart at 24 hours post-treatment. In the liver, lung and blood, nanoparticle retention declined from 4 hours to 7 days. In the brain, uterus and kidneys, the highest concentrations were observed at day 7. The peak concentration in liver and spleen was 12.5% (4 hours) and 2.6% (24 hours) of the absorbed dose, respectively. According to the authors, due the slow excretion kinetics, accumulation of systemically circulating particles in specific cells and organs is likely to occur in subjects chronically exposed to titanium dioxide nanoparticles. When comparing the biodistribution of the radioactive titanium dioxide nanoparticles retained after oral administration with the results obtained after intravenous injection (Kreyling et al., 2017a), the authors concluded that the kinetics patterns are very different and intravenous injection does not appear an adequate surrogate for assessing the biodistribution occurring after oral exposure to titanium dioxide nanoparticles.

Geraets et al., (2014)

12. In the EFSA 2021 Opinion, only the i.v. experiment was discussed; however, the paper also contains the result of an oral administration experiment.

13. The test items used were NM-100, NM101, NM-102, NM-103 and NM-104. More information is provided on these in paragraph 46.

14. In the oral study, the single dose groups received a gavage dose of 2.3 mg/rat corresponding to 6.8-8.6 mg/kg bw. The repeat dose groups received five consecutive daily administrations of 2.3 mg titanium dioxide in one mL per rat resulting in a cumulative dose range of 34.1- 42.4 mg/kg bw for male rats and 54.5-59.9 mg/kg bw for female rats.

15. In the i.v. study, the suspensions prepared contained 2.3 mg titanium dioxide/mL. The single i.v. dose treated rats received a dose of 8.4-9.8 mg/kg bw and 12.4- 14.1 mg/kg bw for male and female rats respectively, via the tail vein. The repeated dose treated rats received a cumulative dose that ranged from 42.3-49.4 mg/kg bw and 61.2-71.9 mg/kg bw for male and female rats, respectively. Thus, the actual dose in mg/kg bw depended on the weight of the rats.

16. After repeated oral exposure (overall dose of 11.5 mg Titanium dioxide) titanium levels were near or below the detection limit in liver and spleen, indicating a very low absorption. In two out of 30 liver/spleen samples of exposed animals (for NM-102 and NM-103) titanium levels were above the LOD, whereas all mesenteric lymph nodes (MLN) samples (including controls) contained titanium amounts above LOD. Only a small increase in titanium content was observed, because the background levels in MLN were 2–3 times the LOD. MLN from control rats contained 0.14 µg titanium whereas the highest titanium average was 0.36 µg and was located in MLN from NM-104 exposed rats. This gives an increase of 0.226 µg titanium in MLN or 0.003% of the 6895 µg Titanium exposure in the dose. The total recovery of dosed titanium in all tested organs (expressed as % of the total dose) was estimated to be approximately 0.02%.

17. In the i.v. study, the highest levels were observed in the liver, but redistribution to the spleen was observed over the 90-day post-exposure period Day 2/Day 6 and Day 90). Redistribution to remaining tissues was not identifiable. The authors hypothesised that release of particles from liver and possibly other organs may be responsible for the increase in spleen levels. Titanium was detected in all

investigated tissues in the present study, i.e., blood, liver, spleen, kidney, lung, heart, brain, thymus and reproductive organs.

18. Both after single and repeated i.v. exposure, blood titanium levels in blood decreased rapidly during the first minutes after which the titanium levels slowly decreased and approached the limit of detection at 24 hours post exposure.

19. Based on the available data, the authors concluded that elimination of total Titanium dioxide has a long half-life. For the liver, which was considered the main target organ, the estimated half-life was 28–248 days.

20. The authors considered that the data showed that at the long run Titanium dioxide particles will accumulate in the spleen. Finally, they noted that the expected accumulation with daily exposure as a consequence of the negligible elimination might indicate a potential concern for human health risk.

Hendrickson et al., (2016)

21. The test materials used were NM-101 (5-10 nm) and NP-25 (20-25 nm).

22. Male Sprague Dawley rats were treated with 250 mg/kg bw/d of either one of the test materials, dispersed in an aqueous starch solution containing 0.1% Tween-80 and sonicated via intragastric administration for 28 days.

23. Within a day of the last exposure, the animals were terminated and blood samples as well as samples from the lungs, liver, spleen, testes, small intestine heart, stomach and kidneys were harvested.

24. For animals treated with NM-101, titanium dioxide nanoparticles were detected in all organs and tissues. The organs with the highest concentrations were the spleen (0.227 µg/g) and liver (0.147 µg/g). In the kidneys, small intestine and testicles similar amounts of nanoparticles were detected (0.092, 0.098 and 0.089 µg/g respectively). Titanium dioxide nanoparticles were detected at 0.028 µg/g in the heart, 0.04 µg/g in the lungs and 0.049 µg/g in the brain.

25. In NP-25 treated animals' accumulation of titanium dioxide nanoparticles were detected in the small intestines and liver (0.29 µg/g in the liver) and at low levels

(0.01 µg/g) in the kidneys. In the spleen, it was detected at levels of 0.11 µg/g of the organ. No titanium dioxide nanoparticles were detected in the lungs, brain, testicles, heart or blood.

26. The authors concluded that biodistribution differs between smaller (NM-101) and larger (NP-25) titanium dioxide nanoparticles, with the smaller particles showing a greater distribution spread and accumulation in all organs. The larger particles exhibited reduced but similar tendency. The main difference was that the larger nanoparticles could not overcome the blood brain barrier and penetrate the brain.

27. Due to the fact that the detected levels accounted for less than 1% of the administered dose, the authors concluded that the data was evidence of the limited bioavailability and efficient excretion of titanium dioxide.

Ammendolia et al. (2017)

28. The test material used was titanium dioxide nanoparticles (anatase, primary size < 25nm BET) surface area 45-55 m^2/g, purity 99%, suspended; the suspensions were sonicated.

29. Sprague-Dawley rats (10/sex/group) were treated with 1 or 2 mg/kg bw/d titanium dioxide nanoparticles or vehicle only (ultrapure water) via gavage for 5 consecutive days.

30. Twenty-four hours after the last dose, the animals were terminated, and the small intestine was excised. A piece of jejunum was used for histological analysis and the remaining part of small intestine was sampled for studying either tissue accumulation of titanium dioxide nanoparticles, determined as titanium by ICP-MS.

31. Titanium was detected in small intestine tissue at 0.08 ± 0.02 lg/g in the control, 0.09 ± 0.02l g/g in the low dose group and at 0.13 ± 0.03 lg/g at the high dose group.

Hendrickson et al., 2020

32. The test material used was titanium dioxide nanoparticles, rutile, rod/ needle like shape, 5 x 30 nm.

33. Wistar rats were treated with 50 mg/kg bw titanium dioxide using an isolated intestinal loop technique.

34. Three hours post treatment, the isolated loop was cut out. The liver and spleen were collected.

35. The presence of particles in tissues was studied by TEM and diffraction analysis. Loose agglomerates (100 nm and larger) were detected. Diffraction analysis was used to confirm that the particles were titanium dioxide. Titanium dioxide nanoparticles were detected on the surface and between the microvilli of the mucosal cells of the small intestine and also in the mucosal tissue. Nanoparticles were detected in the Peyer's patches, both as single nanoparticles and agglomerates of sizes ranging between 20 and 60 nm. In the liver, parenchymal tissue aggregates of titanium dioxide nanoparticles (150–200 nm) and up to 300 nm were seen. In the spleen red pulp, single nanoparticles (20–30 nm), agglomerates (up to 100 nm) and conglomerates (up to 800 nm) were observed.

Chen et al., 2020a

36. The test material used was titanium dioxide nanoparticles anatase, 29 nm (SEM).

37. Sprague- Dawley rats were dosed with 0, 2, 10, 50 mg/kg bw by oral gavage for 90 days. The test material was sonicated prior to treatment.

38. The tissue distribution of the titanium dioxide nanoparticles was evaluated by determining the titanium content in blood and tissues including liver, stomach, small intestine, colon, spleen, heart, lung, kidneys and testicles by high resolution ICP-MS.

39. Significantly increased titanium dioxide nanoparticle levels were only detected in the colon of rats exposed to 50 mg/kg test material, compared with the control group. There was no dose-response relationship, however. The authors hypothesised that the significant increase of titanium dioxide in colon tissue was due to the titanium dioxide nanoparticles attaching on the surface of the colonic mucosa tissue and not in mucosa cells. As most of orally ingested test material was excreted

through feces, it resulted to long-term retention in large intestine. The titanium dioxide nanoparticles did not enter the colon epithelial cells and were mainly deposited in the intestinal cavity or between villi. The content of Ti in all tissues was very low, which was approximately 0.0001%-0.00001% or 100-1000 ng/g tissue, except for the colon of the high dose group. All spleen and heart tissue samples from rats contained very low titanium levels, which were below the LOD of 0.032 µg/g. Finally, they concluded that the results indicate that the absorption and distribution of titanium dioxide nanoparticles was very low after low-dose and long-term oral administration.

Warheit, Boatman and Brown, 2015

40. The COT noted that TiO_2 is able to move into the body through the GI tract. Warheit, Boatman and Brown (2015) evaluated the potential maternal or developmental toxicity of six different forms of predominantly NP form of TiO_2 in pregnant rats (22 or 23 rats per group). The dose levels for each form of TiO_2 were 0, 100, 300, and 1000 mg/kg bw per day. The TiO_2 formulations were in sterile water and delivered via oral gavage. This study noted that in a set of companion analyses, one was of the pigment-grade (pg) TiO_2 and the other was of the ultrafine (uf) TiO_2 test material (BET (Brunauer-Emmett-Teller) and the surface areas of pg and uf samples ranged from 7-17 m^2/g and 50-82 m^2/g respectively). Each material was evaluated for potential systemic exposure/uptake from the GI tract to the blood for circulation.

41. In three studies, Warheit, Boatman and Brown (2015) dosed time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22 per group), daily with TiO_2 by gavage on Gestational Days 6 to 20. Three additional studies included pregnant Wistar rats (n = 22 – 23 per group) exposed daily to TiO_2 by gavage from Gestational Days 5 to 19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day with the TiO_2 specifications detailed below:

vii. anatase/rutile (89/11%)(uf-1), d50 = 43 nm (XSDC), d50 = 23 nm (TEM), irregular,

viii. anatase (100% nano) (uf-2), d50=42 nm (XSDC), d50=19 nm (TEM), irregular,

ix. rutile (100% nano) (uf-3), d50=47 nm (XSDC),d50=22 nm (TEM), rod-like,
x. 4)anatase (27% nano) (pg-1), d50=153 nm (XSDC), d50=120 nm (TEM), irregular,
xi. rutile (11% nano) (pg-2), d50=195 nm (XSDC), d50=165 nm (TEM), irregular.

42. No dose-dependent increase in TiO_2 was observed in the rat blood at 48 or 72 hours, or in the liver at 72 hours following a single exposure (2000 mg/kg bw TiO_2). These results suggested that there was little to no absorption of particles from the GI tract into the blood, which conflicted results from a previous study by Tassinari *et al*., 2014.

Tassinari et al., 2014

43. In the Tassinari *et al*. (2014) study, rats were dosed with distilled water (controls) or TiO_2 NPs (anatase, primary size < 25nm, BET surface area 45–55 m2 /g, purity 99%) by gavage for 5 consecutive days at 0, 1, or 2 mg/kg bw/day (7 rats/sex/group). Twenty-four hours after the last treatment (day 6), the animals were terminated, and the uterus, ovary, testes, thyroid and adrenals were excised and weighed. The spleen was sampled both for histopathological examination and for studying tissue deposition of Titanium dioxide nanoparticles.

44. The highest total titanium concentration was detected in the thyroid. However, the difference in levels was not of statistical significance between the treated animals and the controls (0.24 ± 0.09 mg/g and 0.22 ± 0.04 mg/g of fresh thyroid weight in animals dosed with 2 mg/kg bw/day and controls, respectively). A significant increase was observed in the ovaries of animals dosed with 2 mg/kg bw/day TiO_2 NPs compared with control animals (0.28 ± 0.07 vs. 0.12 ± 0.04 mg/g fresh weight, respectively). Levels in uteri were low (0.051 ± 0.006 vs. 0.49 ± 0.04 mg/g fresh weight). A significant increase in the concentration of titanium in the spleen was observed in animals dosed with 2 mg/kg bw per day when compared to controls. (Tassinari *et al*. 2014). Overall, the authors considered that these results indicated the potential for titanium dioxide bioaccumulation. The COT considered that the strength of evidence for the absorption of TiO_2 from the GI tract into the blood was not clear.

Bettini et al., 2017

45. In addition to the rats dosed with E171 as described in paragraphs xx-xx, Bettini et al also assessed TiO_2-NPs using NM-105. As described previously, rats (10 per group) were dosed daily by gavage with NM-105 (10 mg/kg bs/day) for 7 days (the control group was given water). As described previously the NanoSIMS analyses of subcellular TiO_2 NM-105 showed a similar distribution in the immune cells of Peyer's patches, with the greatest concentration of particles in the central zones of the Peyer's patches which have a high concentration of immune cells. Ti was also located in the cytoplasm and nuclei of the Peyer's Patches cells.