Health Canada Santé Canada

State of the Science of Titanium Dioxide (TiO_2) as a Food Additive

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Canada

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Executive Summary

The purpose of this document is to summarize the state of the science concerning the safety of titanium dioxide (TiO_2) used as a food additive. TiO_2 is a white powder that has been approved for use as a food additive in Canada for over five decades and is used to whiten or brighten many foods. TiO_2 is insoluble, poorly bioavailable and has long been regarded as toxicologically inert via the oral route. However, the safety of TiO_2 when used as a food additive has recently come into question, largely as a result of the recognition that food-grade TiO_2, while not intentionally engineered as a nanomaterial, invariably contains a fraction of particles in the nanoscale (<100 nm). Recent analysis of food-grade TiO_2 samples on the European market (where it is referred to as E171 per European labelling requirements for food additives) indicated that the portion of particles with a diameter < 100 nm was as high as 30% on a mass basis and ~70% on a particle number basis (Verleysen *et al.* 2020; 2021). 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, these dispersion methods are intended to identify intrinsic hazards of the constituent particles (both primary particles and reduced agglomerates) and are of uncertain relevance to human health, as TiO_2 used in food preparations is in the form of larger, constituent agglomerates that are not dispersed to the same degree. Moreover, proteins and other macromolecules present in food and biological matrices rapidly and avidly adsorb to TiO_2 particles, forming a "corona" that alters particle size and the surface characteristics that govern their interactions with the gut environment. It is perhaps not surprising therefore, that *in vivo* studies which administer TiO_2 to animals via the diet or *in vitro* studies that employ a simulated food matrix tend not to replicate the findings observed in studies using dispersed TiO_2 in simple matrices.

TiO_2 is photoactive and small particles in particular may undergo photocatalytic reactions in aqueous media to produce hydroxyl radicals upon exposure to light, which may lead to oxidative damage in test systems. TiO_2 is also sonocatalytic and concerns have been raised that the energy applied during sonication to deagglomerate TiO_2 may also generate reactive radicals, potentially producing artifacts in toxicity testing. While a large body of experimental toxicological literature exists for TiO_2, particles used in studies are often poorly described (if at all), making it difficult to establish relationships between particle characteristics (e.g., size, agglomeration state, surface area, particle number, etc.) and toxicity. This also makes it challenging to determine the relevance of these experimental findings to human exposure to the forms of TiO_2 used in food.

TiO_2 is not metabolized to any significant degree and while the vast majority of ingested particles are excreted unchanged in feces, studies in animals and human volunteers indicate a small fraction, likely on the order of 0.001%, may be systemically available via the oral route. In the gastrointestinal tract, TiO_2 particles may gain access to the gut-associated lymphoid tissue (GALT), where they may remain locally in specialized lymphoid follicles known as Peyer's patches or be translocated systemically, dependent on their size. In cadaveric organ donors, TiO_2 has been identified in various organs, notably those rich in macrophages, such as liver and spleen, although there is no established link between organ burden and either age or pathology. The initial concerns with human exposure to TiO_2 particles arose in part from a non-guideline rat study funded by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in which animals were exposed to food-grade TiO_2 dispersed in drinking water at a human relevant dose (~10 milligrams per kilogram of body weight per day; mg/kg bw/d) for 100 days (the Bettini

et al. 2017 study). TiO_2 particles were reported to have accumulated in Peyer's patches and exposed animals developed large aberrant crypt foci (ACF; a colonic lesion which may progress to neoplasia), at higher rates than unexposed controls. However, the findings of ACF in the colon by Bettini *et al.* have not been replicated in subsequent studies, even at doses orders of magnitude higher.

The rodent dietary model successfully mimics human exposure to food-grade TiO_2, as the administration of food-grade TiO_2 incorporated into animal feed resulted in the accumulation of TiO_2 particles in Peyer's patches in rodents, as occurs in humans following exposure to TiO_2 via the diet (Riedle *et al.* 2020). Several studies in which food-grade TiO_2 was administered via the diet are available and these studies were accorded the highest weight in this review. A chronic rodent bioassay conducted 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. This study used a test article that is highly consistent with the forms of TiO_2 currently used in foods, including the fraction of particles in the nanoscale. In addition, a study designed to replicate the findings of Bettini *et al.* (2017) using a dietary model found no evidence of ACF in the rat colon following exposure to food-grade TiO_2 for 100 days up to the highest dose tested (236-300 mg/kg bw/d) in either the presence or absence of a carcinogenic initiator. Similar findings were also observed in a recent extended one-generation reproductive toxicity study performed according to OECD guideline No. 443 (LPT 2020 as cited in EFSA 2021a). This study was conducted with modifications specifically intended to assess the potential for food-grade TiO_2 to produce adverse effects in the colon, and the test article selected was a commercially available food-grade TiO_2 that was determined to have among the highest fraction of constituent nanoparticles of any sample tested on the European market. No adverse effects were observed up to the highest dose tested of 1000 mg/kg bw/d administered via the diet when rats were continuously exposed from pre-conception through to adulthood. Lastly, the available evidence indicates 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.

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 suggests these data gaps are 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.

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1. Background

Titanium dioxide (TiO_2; E171[1]) is permitted for use as a food additive in Canada in accordance with Good Manufacturing Practice (GMP), which means that the amount added must not exceed the amount required to accomplish the purpose for which it has been added.[2] TiO_2 is a colouring agent that is widely used in Canada and internationally in various products such as confectionaries, processed nuts, dairy products, baked goods and condiments.[3] In 1969, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the safety of TiO_2 as a food colour additive and the Committee concluded that this compound was insoluble, poorly bioavailable and did not produce systemic toxicity if absorbed; therefore, an acceptable daily intake (ADI) of "not limited" was assigned (JECFA 1969). Such non-numerical ADIs are assigned to food additives for which no toxicological concerns are identified through their intended food additive uses. TiO_2 was also investigated in a chronic rodent cancer bioassay by the United States National Cancer Institute (NCI) in the late 1970s (NCI 1979). Following two years of exposure to relatively high doses administered via the diet, there were no signs of toxicity that were considered treatment-related and it was determined that TiO_2 was not carcinogenic by the oral route in rats or mice. Food-grade TiO_2 subsequently came to be regarded as a rather innocuous substance that was considered toxicologically inert via ingestion. In recent years, however, additional evidence began to emerge that challenged this view.

In 2016, the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS) re-evaluated E171 as a food additive and concluded the oral bioavailability of TiO_2 was extremely low, and "the use of TiO_2 (E171) as a food additive [did] not raise a genotoxic concern" (EFSA 2016). However, the ANS Panel identified several data gaps that precluded the establishment of a health-based guidance value, specifically the lack of reproductive toxicity data as well as the need for additional data concerning the specifications of E171. The ANS Panel also recommended that the existing European Union (EU) specifications for TiO_2 (E171) be updated to include a characterization of particle size distribution as well as to specify the percentage (in number and by mass) of particles in the nanoscale. At the time, it was considered that E171 mainly consisted of micro-sized TiO_2, with the fraction of particles in the nanoscale being less than 3.2% by mass. Nanoscale particles are known to exhibit properties, attributed to their small size, that are distinct from the constituent individual atoms and molecules, and from the "bulk" form of the material with the same chemical composition (Auffan *et al.* 2009).

[1] E171 is the European designation for titanium dioxide (TiO_2) that meets food additive specifications. European specifications for E171 and other food additives are set out in 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.

[2] GMP is defined in the Marketing Authorization for Food Additives That May Be Used as Colouring Agents, *Food and Drugs Act*. https://laws-lois.justice.gc.ca/eng/regulations/SOR-2012-204/page-1.html#h-784309

[3] TiO_2 used in foods sold in Canada is required to meet food-grade specifications set out in the Food Chemicals Codex (FCC) or the Combined Compendium of Food Additive Specifications. The FCC is a compendium of standards for purity and identity for food ingredients, including food additives, published by the United States Pharmacopeial Convention. The Combined Compendium of Food Additive Specifications, which contains specifications prepared by JECFA, is published by the Food and Agriculture Organization of the United Nations. Both FCC and JECFA specifications for TiO_2 include chemical identity and purity requirements, however, do not include limits on the size of particles in the food-grade material.

In 2017, the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) issued an opinion (2017-SA-0020; ANSES 2017) on a study funded by the French government (Bettini *et al.* 2017) in which rats were exposed to food-grade TiO_2 (E171) via drinking water for 100 days at doses "representing human dietary levels". Bettini *et al.* (2017) reported that E171 treatment produced inflammatory effects in the colon and initiated preneoplastic lesions in the form of aberrant crypt foci (ACF), in addition to promoting the growth of ACF in a chemically-induced carcinogenesis model. The ANSES expert committee concluded that this study provided some new information on the hazards of E171, but did not call into question the conclusions of the 2016 EFSA ANS opinion. The committee also stated the Bettini *et al.* (2017) study could not be used for risk assessment without first confirming these same results are produced when E171 is administered to animals as present in a food matrix.

In 2018, the European Commission requested that the EFSA ANS Panel provide a scientific opinion with respect to four additional studies (Heringa *et al*. 2016; Bettini *et al*. 2017; Guo *et al*. 2017; Proquin *et al*. 2017) that had been published subsequent to their 2016 opinion with a view to evaluating whether these new data merit reconsideration of the existing opinion concerning the safety of E171 as a food additive (EFSA 2018a). The authors of these publications were also invited to a plenary meeting with the EFSA expert ANS Panel in order to present their findings and address comments and questions from panel members. The publication by Heringa *et al.* (2016) was not a primary research study, but rather a risk assessment of oral exposure to TiO_2 nanoparticles (TiO_2-NPs) based on existing data (the pivotal studies of which had already been considered by EFSA). The ANS Panel identified significant limitations in the three remaining studies in which new data were presented, and considered they were useful for hazard identification of TiO_2-NPs in suspension but of limited relevance to the risk assessment of E171 under realistic conditions of exposure via food. Therefore, the ANS Panel concluded these studies did not merit re-opening the existing opinion of the EFSA ANS Panel related to the safety of TiO_2 (E171) as a food additive.

In 2019, ANSES received a formal request from the French government to provide scientific and technical support on the risks associated with ingestion of the food additive E171 (ANSES 2019). Specifically, ANSES was requested to identify oral toxicity studies of TiO_2 that were conducted subsequent to the publication by Bettini *et al.* (2017), and on the basis of these new studies update as necessary the recommendations in the 2017 opinion. The Committee identified 25 additional *in vivo* oral studies using food-grade TiO_2 that were published between 2017 and 2019. The panel pointed out that the majority of the studies were conducted with samples of E171 that had been dispersed in solution by sonication. While the experts considered these studies could help identify "certain hazards" associated with E171, they noted that the sonication step alters agglomerate structure and "is not fully representative of E171 as found in foodstuffs". The Committee concluded that of the 25 new studies examined, none "were able to confirm or refute the potential carcinogenesis promoting effect of E171 reported in the study by Bettini *et al.* (2017)." In their opinion published 12 April 2019, the Committee recommended additional studies be undertaken to better characterize the physicochemical properties and hazard of E171. They also indicated that the process for obtaining marketing authorization for the additive "must be based on clearly established benefits (technological value, substitution impossible, value to the consumer or community)" and pending better characterization of the hazards and risks of E171, restated ANSES' previous conclusion that consumer exposure to nanomaterials in general should be limited.

Further to the publication of the 2019 ANSES opinion, EFSA was requested by the European Commission to provide "urgent scientific and technical assistance regarding the opinion issued by ANSES". Approximately one month following publication of the ANSES opinion, on 10 May 2019, EFSA issued a "statement on the review of the risks related to the exposure to the food additive titanium dioxide (E 171) performed by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES)" (EFSA 2019a). EFSA concluded the recent ANSES opinion reiterated previously identified data gaps and uncertainties, and "[did] not identify any major new findings that would overrule the conclusions made in the previous two scientific opinions on the safety of titanium dioxide (E 171) as a food additive issued by the EFSA ANS Panel in 2016 and 2018."

In June 2019, the EFSA Panel on Food Additives and Flavourings (FAF) published another opinion on TiO_2 that assessed data provided by industry in support of a proposed amendment of the EU specifications for E171 with respect to parameters related to particle size distribution[4]. Interested business operators had responded to EFSA's 2016 call for additional data on the specifications of E171 by submitting data on 5 commercial brands of anatase E171 and 1 form of rutile E171. According to the industry, only three EU-based manufacturers produce food-grade TiO_2 and the samples analysed were representative of all the forms of E171 produced by these manufacturers. The 6 samples were analyzed by 5 different laboratories using various analytical methods including transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM), X-ray disc centrifuge (XDC) and disc centrifuge (DC). For the anatase samples, the average median diameter by SEM ranged from 104 to 166 nm with 11.4 to 45.6% of particles by number having a diameter <100nm; the rutile sample had a median diameter of 151 nm with 5.4% of particles by number having a diameter <100 nm. The FAF Panel concluded that based on the information provided by industry, the specifications for E171 should be revised to stipulate that median particle size should be larger than 100 nm as determined by electron microscopy, which is equivalent to a percentage of constituent particles with a minimum external dimension below 100 nm by number of less than 50%. The FAF Panel also concluded that the previous EFSA ANS Panel opinions (EFSA 2016; 2018a) remained valid and in particular, the new data on particle size distribution did not provide a reason to revisit the conclusion on genotoxicity previously drawn. However, they recommended revisiting the toxicological database in line with the data requirements in the recently published EFSA guidance on nanotechnology (EFSA 2018b). The FAF Panel also noted that industry was planning to perform an extended one-generation reproductive toxicity (EOGRT) study to address the data gap identified by the EFSA ANS Panel in 2016 and that this study would include additional parameters to address the tumour initiation/promotion potential of E171 as raised by Bettini *et al*. (2017).

Also in 2019, the Office for Risk Assessment and Research of the Netherlands Food and Consumer Product Safety Authority (NVWA) published an opinion on possible health effects of the food additive E171 (NVWA 2019). The opinion followed an international workshop in which researchers who study the health effects of food grade TiO_2 and experts in chemical risk assessment were invited to participate. A literature search

[4] Significant technological advances in the methods used to assess particle size distributions emerged following EFSA's 2016 call for greater clarity on the specifications of food-grade TiO_2. In particular, sample preparation steps leading to optimal de-aggregation/agglomeration have been shown to be crucial for the adequate characterization of particle size distributions in both pristine and extracted materials. Sample dispersion effectiveness, and in particular sonication probe size and shape, the acoustic power and the energy delivered can all have a significant impact on the number of particles in the nanoscale detected.

was also conducted to substantiate any conclusions that emerged from the workshop. The conclusion of the workshop participants was consistent with that of EFSA (2018a); specifically that the new studies were not sufficient to call into question the conclusion of EFSA's 2016 assessment. The Authority concluded that the studies conducted in rodents provided an indication that E171 may be associated with tumour promotion in the gastrointestinal tract (GIT), but that none of these studies were conducted in accordance with OECD guidelines and were not sufficient to conduct a well-substantiated risk assessment. They also noted that there were no reliable dose-response data in the animal studies and it is unclear whether the mechanisms that form the basis for the effects in animals also occur in humans to the same extent. More information about potentially sensitive groups such as those with increased intestinal permeability was also identified as a data gap. In addition, the Authority noted that the incidence of colon cancer has doubled in the Netherlands in the last 30 years, and although no direct connection to TiO_2 exposure can be made, there is a need to further investigate realistic intake doses.

In 2021, EFSA's FAF Panel re-assessed the toxicological dataset for TiO_2 in accordance with the principles set out in the Scientific Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (EFSA 2018b, revised 2021b). In contrast to the opinions published by the EFSA ANS Panel in 2016 and 2018, this assessment took into consideration studies of TiO_2-NPs, which were not heretofore considered relevant to the hazard characterization of E171. In May 2021, the EFSA FAF Panel published an opinion that concluded E171 can no longer be considered safe when used as a food additive (EFSA 2021a). Specifically, the FAF Panel raised concerns regarding (i) the potential for bioaccumulation of TiO_2-NPs; (ii) the potential for TiO_2 to produce immunotoxicity, inflammation and neurotoxicity; (iii) the conclusion that no well-designed carcinogenicity studies of TiO_2-NPs were available; and (iv) the genotoxic potential of TiO_2 particles could not be ruled out. The FAF Panel acknowledged that the evidence base was not conclusive, but given the many uncertainties a safe level for the daily intake of TiO_2 could not be established.

The United Kingdom's Food Standards Agency (UK FSA) reviewed the most recent opinion by EFSA (2021a) and having identified a number of concerns, referred the publication to their Scientific Advisory Committees, namely the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) and the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) for independent expert review. The preliminary findings of these expert committees were published in the form of an interim position statement in January 2022 (COT 2022). Upon reviewing the EFSA FAF evaluation with respect to genotoxicity, the COM concluded that "the evidence did not allow definitive conclusions to be drawn and therefore they did not agree with the overall EFSA conclusions on the genotoxicity of E171 Titanium dioxide". Due to the heterogeneity in the database and equivocal results, the COM considered that further refinement of the data may be needed before making a definitive conclusion of the genotoxicity and safety of TiO_2 and that the conclusions of the EFSA FAF Panel were not justifiable based on the available evidence. Similarly, the COT questioned the quality and robustness of the dataset and the weight given by the EFSA FAF Panel to studies that were considered to be of low reliability. The COT was informed that the EFSA FAF Panel had indications that when used by industry E171 was dispersed by sonication into nanoparticles (NPs) and therefore studies using particles wholly in the nanoscale were considered relevant by the EFSA FAF Panel. However, the COT questioned this, noting that TiO_2-NPs would not have a technical function in food as they would not provide colour

and would therefore not be of use[5]. The COT concluded that, "on balance, the Committee considered that the weight of evidence did not support the conclusions drawn by EFSA". It should be reiterated that these are interim conclusions and the UK FSA is currently undertaking an independent review of the safety of TiO_2 as a food additive.

The most recent opinion by the FAF Panel (EFSA 2021a) concluded that a safe level for the daily intake of TiO_2 could not be established on account of various uncertainties. In response, a number of additional studies were commissioned by interested business operators in order to address the deficiencies in the evidence base that were identified by the FAF Panel. Perhaps most significantly, samples of the test material used in the 1979 chronic bioassay were obtained from the NCI repository and characterized by modern methods to determine the similarity to current forms of food-grade TiO_2 including the fraction of particles in the nanoscale. Other studies that were conducted included cellular uptake studies, *in vitro* genotoxicity studies and oral bioavailability studies. Additional data on the distribution and homogeneity of E171 in the feed matrix used in the EOGRT study were also provided. Health Canada's Food Directorate put out a call to industry requesting information on the methods used to incorporate TiO_2 into foodstuffs. In response, an industry consortium representing both manufacturers and end users of food-grade TiO_2 informed Health Canada that sonication or other intensive methods intended to break apart agglomerates are not used to incorporate TiO_2 into liquid or solid food preparations in Canada. In light of the aforementioned, Health Canada's Food Directorate undertook the present review with the benefit of several significant new pieces of information that were not available to the FAF Panel at the time of their assessment.

2. Scope

The purpose of this document is to summarize the state of the science concerning the safety of food-grade TiO_2 as well as to illustrate some key uncertainties. Rather than serving as a comprehensive overview of TiO_2 toxicity, it is intended to highlight and provide perspective on recent data, including those studies that have brought the safety of food-grade TiO_2 into question. Recent assessments by competent authorities described above, including those from EFSA (2016; 2018a; 2019a,b; 2021a), ANSES (2017; 2019) and the Netherlands NVWA (2019) were reviewed and served as a pragmatic starting point for Health Canada Food Directorate's review. A search for additional relevant toxicological and toxicokinetic studies in animals or humans was also undertaken by research librarians of the Government of Canada's Health Library in order to identify any critical new data for the assessment of human health risk (details of the search strategy may be found in Appendix A). Following the removal of duplicates, a total of 1134 unique references were identified and subjected to screening. In addition, targeted searches by chemical name and Chemical Abstracts Service registry number were conducted on the PubMed and PubChem websites as well as using the Google Scholar search engine, and citations from relevant review articles were screened. The initial library search was conducted on 29 June 2021 followed by a second library search using the same search strategy on 19 January 2022. For studies obtained by hand searching, the cut-off date for inclusion in this report was 01 March 2022.

[5] Health Canada's Food Directorate has received a statement from industry indicating that sonication is not used to incorporate TiO_2 into liquid or solid food preparations (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022)

Study Screening Criteria

As part of level 1 screening, the titles and abstracts of all studies identified in the literature search were independently screened for potentially relevant data by two senior toxicologist evaluators based on the following criteria:

1) Original research study,
2) Measured titanium (Ti) or TiO_2 exposure or administered TiO_2 as a test article, and
3) Evaluated a toxicokinetic or toxicological outcome *in vivo* (in any mammalian species) or in a relevant *ex vivo* or *in vitro* model.

All studies that met the above criteria were retained and moved forward into 'level 2 screening', which entailed a full text review based on the same level 1 screening criteria. All studies that were confirmed to have met the above criteria based on a full text review were then ranked for relevance and reliability, and a rationale provided for those excluded from further consideration.

Study Ranking Criteria

All studies were independently ranked for relevance and reliability by two senior toxicology evaluators and consensus was achieved in the case of discrepancies; additional details of the study ranking criteria may be found in Appendix B. Each study was evaluated based on five main criteria: 1) representativeness of the test article, 2) mode of exposure, 3) sample preparation method, 4) study reliability/quality, and 5) study type.

1. ***Representativeness of Test Articles:*** All toxicological or toxicokinetic studies that used food-grade TiO_2 or forms of TiO_2 that were highly comparable to food-grade TiO_2 were considered to be relevant to this review. The forms of TiO_2 considered highly comparable to food-grade TiO_2 were determined by comparing the physiochemical properties of a given TiO_2 form to food-grade TiO_2. Forms of TiO_2 that had a similar mean primary particle size, particle size distribution, % of particles <100 nm, isoelectric point, mass-specific surface area, surface composition, elemental composition, crystalline form, *and* purity were considered representative of food-grade TiO_2 (see 'Relevant Reference Materials for Studying Food-Grade TiO_2' section below for more details). Studies that used TiO_2-NPs (engineered NPs that were wholly nano-sized with a mean particle diameter less than ~100 nm) were not consistent with the particle size distribution of food-grade TiO_2 and were deemed to be of limited relevance for the present assessment[6]. Since food-grade TiO_2 can contain a significant portion of primary particles within the nanoscale, studies conducted with food-grade TiO_2 will simultaneously evaluate any TiO_2-NPs that may be present. However, in rare cases, studies that used TiO_2-NPs were considered supporting evidence in the present assessment, if they were considered a key study in an assessment by another competent authority or if the TiO_2-NPs were administered via the diet and the study was deemed to be well conducted.

[6] TiO_2-NPs will be reviewed by Health Canada in a separate assessment specific to nanomaterials.

2. ***Mode of Exposure:*** Studies that used food-grade TiO_2 or forms highly comparable to food-grade TiO_2 in a dietary dosing paradigm were given the highest weight since this paradigm accounts for the contribution of the food matrix and therefore, best reflects the mode of human exposure to TiO_2 from both solid and liquid food preparations[7]. Studies that used food-grade TiO_2 or forms highly comparable to food-grade TiO_2 in non-dietary oral dosing paradigms (oral gavage, drinking water exposure) were considered supporting evidence since interactions with the food matrix may not occur. In the vast majority of dietary studies, the substance in question is typically administered in a solid food matrix (i.e., in feed) and those toxicity findings are applied to a wide variety of food groups, including beverages and other food matrices (e.g., gums, candy coatings, etc.). While this approach is not without some uncertainty, if the substance in question is known to have a significant food matrix effect (as is the case for food-grade TiO_2), this approach is still considered more appropriate than applying findings from toxicity studies that do not account for the contribution of the food matrix. For this reason, Health Canada's Food Directorate considered the findings from dietary studies with food-grade TiO_2 more appropriate to approximate the behaviour and properties of food-grade TiO_2 in beverages and other food matrices compared to studies that administered food-grade TiO_2 in water in non-dietary oral dosing paradigms. See the 'Impact of the Food Matrix and Gastrointestinal Milieu' section for more details.

3. ***Sample Preparation Method:*** Studies in which TiO_2 particles were intentionally dispersed using ultrasonication were considered less representative of TiO_2 when used as a food additive. These dispersion methods are intended to identify intrinsic hazards of the constituent particles – both primary particles and reduced agglomerates. The available evidence suggests TiO_2 particles in food, including beverages and other food matrices, are present in the form of larger, constituent agglomerates, which have not been dispersed to the same degree, and there is also no indication that TiO_2 dissociates or deagglomerates in the GIT (Dudefoi *et al.*, 2021; Marucco *et al.* 2020). In addition, Health Canada's Food Directorate has received confirmation from industry that sonication is not used to incorporate TiO_2 into liquid or solid food preparations (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022). For these reasons, Health Canada's Food Directorate considered studies that used intentionally dispersed TiO_2 particles via sonication not to be fully representative of human dietary exposure to TiO_2 as a constituent of food and therefore, these studies were given less weight in the present assessment compared to studies in which particles were not intensively dispersed and/or stabilised. However, these studies were still considered supporting evidence in the review. See the 'Particle Dispersion' section for more details.

4. ***Study Reliability/Quality:*** Studies conducted in accordance with internationally accepted standards for toxicity testing (i.e., GLP- and OECD guideline-compliant studies) were considered the most reliable and of the highest quality and therefore, were accorded the highest weight in this assessment. Non-guideline studies were still considered for inclusion in this assessment on a case-by-case basis (e.g., non-guideline studies considered key studies by other competent authorities and non-guideline studies considered reliable by Health Canada's Food Directorate).

[7] While the food matrix is often thought of in terms of solid, complex foods, other foods such as beverages also have a food matrix, which consist of various proteins, sugars and polysaccharides, bioactives (i.e., flavonoids, phenolic compounds), lipids, etc.

5. ***Study Type:*** *In vivo, in vitro,* and other studies were considered for inclusion in the present assessment, although *in vivo* studies were given the highest weight.

Ranking of Studies Retained

From the initial library search, a total of 281 test articles/exposures from 188 unique publications were retained following screening by title/abstract and subjected to level 2 screening. Of these, 93 test articles/exposures were screened out while 93 test articles/exposures from *in vivo* studies and 95 test articles/exposures from *in vitro* or other studies were retained and ranked for relevance and reliability. Of the 188 test articles/exposures retained following full text screening, 18 were considered highly relevant, 44 relevant and 126 were determined to be of limited relevance. For the second library search, a total of 15 test articles/exposures from 12 unique publications were retained after screening by title/abstract and subsequently moved forward to full text screening. Three of these test articles/exposures did not meet the criteria for inclusion and were screened out. The remaining 12 test articles/exposures were retained and ranked for relevance and reliability of which, one was considered highly relevant and 11 were determined to be of limited relevance. Details of the ranking results may be found in Table 1 and Appendix C. Additional studies that did not meet the criteria for retention but provided key support for conclusions in other assessments by competent authorities were also considered in this report on a case-by-case basis.

Table 1. Ranking of test articles/exposures from publications retained following level 2 screening for both library searches. The italicized numbers represent the results obtained in the first library search and the numbers in parentheses represent the results obtained in the second library search.

Ranking	Total	*In vivo*	*In vitro*/ other
Highly relevant	*18* (1)	*13* (1)	*5* (0)
Relevant	*44* (0)	*19* (0)	*25* (0)
Limited relevance	*126* (11)	*62*(10)	*64* (1)

Genotoxicity Studies

The current review primarily focused on *in vivo* genotoxicity studies with TiO_2 as these studies were considered more representative of real-world exposure to food-grade TiO_2 (see 'Particle Corona, Food Matrix, and the Gastrointestinal Milieu' section for more details); however, *in vitro* genotoxicity studies with food-grade TiO_2 have also been briefly reviewed. *In vivo* studies that administered TiO_2 particles via the oral route were given the highest weight. To ensure that the genotoxic potential of TiO_2 was assessed under conditions with systemic exposure, *in vivo* studies that administered TiO_2 particles by intravenous or intraperitoneal injection were also considered as supporting evidence in the present assessment. TiO_2 is considered a possible human carcinogen by inhalation through a mode of action not relevant to oral exposure (i.e., particle overload in the lungs) and therefore, *in vivo* studies that administered TiO_2 via inhalation were not considered relevant and were excluded from the assessment.

Relevant Reference Materials for Studying Food-Grade TiO_2

The TiO_2 database consists of studies conducted using a wide variety of TiO_2 materials with distinct physicochemical properties and applications, including food-grade TiO_2, non-food-grade TiO_2 (such as cosmetic-grade TiO_2 and certain forms of pigment-grade TiO_2), and TiO_2-NPs (see Table 4 in Appendix D for a description of the physicochemical properties of TiO_2 forms commonly used as test articles). Food-grade TiO_2, which has been described as pure TiO_2 (> 99%), crystalline-phase anatase or rutile with a specific surface area between ~8 to 10 m^2g^{-1}, with phosphate groups on the surface and not more than 2% aluminum oxide and/or silicon dioxide singly or combined, is the most relevant reference material for studying human exposure to TiO_2 as a constituent of food (Dudefoi *et al.* 2017a; Geiss *et al.* 2020; JECFA 2012). Food-grade TiO_2 can also contain a percentage of TiO_2-NPs as high as ~30% on a particle mass basis and ~70% on a particle number basis (Verleysen *et al.* 2020; 2021), and therefore, studies conducted with food-grade TiO_2 will simultaneously evaluate the toxicity of any TiO_2-NPs that may be present. Verleysen *et al.* (2021) also reported that "hardly any particles smaller than 30 nm were observed" in pristine forms of E171 (i.e., before it is added to food) as well as various food products on the European market that contained TiO_2. Therefore, studies that administer TiO_2-NPs wholly in the nanoscale as test articles, and in particular, TiO_2-NPs < 30 nm (e.g., P25, Aeroxide®) are not considered appropriate reference materials for investigating the toxicokinetics and toxicity of food-grade TiO_2 due to significant differences in the physicochemical properties between food-grade TiO_2 and TiO_2-NPs, including mean particle diameter, particle size distribution, specific surface area, surface composition, and crystalline form. In general, the risk assessment of nanomaterials is a nascent and evolving field with a number of inherent challenges. It has been suggested that given the sheer number of potential variables and their influence on fate and biological interactions, NPs may only be assessable on a case-by-case basis (Carrière *et al.* 2020; Nature Nanotechnology 2020). According to this view, nanotoxicology is not sufficiently advanced to use read-across to conduct hazard or risk assessments, and the results of toxicity tests using non-food-grade TiO_2-NPs should therefore be considered specific to the particular particle being tested and not necessarily relevant nor extrapolatable to the nanoscale fraction of food-grade material. However, occasionally, studies that used TiO_2-NPs were considered supporting evidence in the present assessment, such as when a study was considered a key study in an assessment by a competent authority.

Definitions

A wide variety of terms have been used to describe TiO_2 in the literature[8]. In the present assessment, the term TiO_2 is used when describing TiO_2 in general and encompasses all particle sizes (i.e., both micro- and nano-sizes) as well as all technical uses (i.e., food, cosmetic, pigment, etc.). TiO_2 microparticles (also described as microscale or micro-sized TiO_2 particles) refer to particles with a mean particle size > 100 nm, whereas TiO_2-NPs (also described as nanoscale or nano-sized TiO_2 particles) refer to particles with a mean particle size < 100 nm, of which many are comprised of particles wholly on the nanoscale. The terms food-grade, cosmetic-grade, and pigment-grade TiO_2 are used to describe various forms of TiO_2 approved for use in food, cosmetics, and as pigments in general, respectively. Note that all forms of food-grade TiO_2 are also pigment-grade, but not all pigment-grade forms of TiO_2 are food-grade. In the present review,

[8] A list of depositor-supplied synonyms for TiO_2 (https://pubchem.ncbi.nlm.nih.gov/compound/Titanium-dioxide#section=Depositor-Supplied-Synonyms&fullscreen=true)

the term pristine is used to describe food-grade TiO_2 before it is added to food. There are multiple forms of food-grade TiO_2, including E171, A200, and Unitane® 0-220, which have been described more extensively in the present document. On the European market, food-grade TiO_2 is referred to as E171 as per European labelling requirements for food additives whereas in China it may be referred to as A200. Unitane® 0-220 is the trade name for a form of food-grade TiO_2 previously manufactured in the United States by the now defunct American Cyanamid Company. Note that TiO_2 may be found under other identifiers or trade names in different countries. In the present assessment, the term food-grade TiO_2 was used when discussing the food additive use of TiO_2 in general; however, when a study reported using a particular form of food-grade TiO_2 (i.e., E171, A200, Unitane® 0-220), that particular form was reported instead.

3. Composition, Properties, and Uses

Titanium (Ti) is the ninth most abundant element in the Earth's crust and is widely distributed; Ti may accumulate in soil due to rock weathering and be taken up by plants (Dumon and Ernst 1988). Anthropogenic sources of Ti in the environment include combustion of fossil fuels, incineration of Ti-containing wastes and emissions from industrial processing of Ti-containing metals, minerals and other substances (WHO 1982). Therefore, background levels of Ti may be present in environmental media, food, and drinking water. Although no biological role for Ti in mammals has been identified, humans nevertheless have 10 – 20 mg of Ti in the body, making it more abundant than some essential elements (Buettner and Valentine 2012; Swiatkowska *et al.* 2019).

TiO_2 is a white powder used as a pigment in various industries and is valued for its high refractive index. 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 using only the sulfate (anatase and rutile forms) or chloride processes (only rutile forms) (Ropers *et al.* 2017)[9]. In contrast, TiO_2-NPs may be manufactured by various methods, such as the sol-gel, hydrothermal, or flame-spray pyrolysis processes. For many applications, including cosmetics and some therapeutic products, surface coatings are applied to TiO_2 particles that alter their physicochemical properties. Therefore, toxicity 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). Food-grade TiO_2 also differs in surface composition compared to other TiO_2 materials, such as TiO_2-NPs, that are intended for non-food applications and manufactured using synthetic methods other than the sulfate or chloride processes. The surface of food-grade TiO_2 particles is covered by superficial phosphate groups resulting from the synthetic methods used to manufacture the material, while materials manufactured with alternative methods may have different surface functional groups (e.g., hydroxyl groups). The particular surface properties of TiO_2 materials have a significant impact on the behaviour of the material in various environments, including biological media, thus TiO_2 materials with surface properties that differ from

[9] Potassium Aluminium Silicate-based TiO_2, otherwise known as mica-based pearlescent pigments, are also permitted as food colourants in Canada. However, these substances are chemically and toxicologically distinct from the TiO_2 colour additives that are the subject of this report and are not given further consideration herein. Note that the EFSA FAF Panel (2020) states that potassium aluminum silicate and TiO_2 particles "…are bound to each other by strong physical forces and cannot be separated from each other by standard methods". Therefore, mica-based pearlescent pigments should be considered a separate entity rather than a "carrier" of TiO_2 particles.

food-grade TiO_2 may not be considered relevant models when studying the fate of dietary TiO_2 (Dudefoi *et al.* 2017a).

The ideal particle size distribution of food-grade TiO_2 ranges from 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). TiO_2 particles less than 100 nm are transparent to visible light and are not of functional significance as a pigment. 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. While this has long been recognized, the fraction of primary particles in the nanoscale was thought to be minimal in food-grade TiO_2. Following EFSA's call for data in 2016, new analytical methods with vastly improved size detection limits have been developed and optimized to quantify the particle size distribution of food-grade TiO_2 with greater precision and accuracy. Based on these methodological improvements, recent analysis of pristine E171 from the European market as well as E171 isolated from food matrices using optimized extraction procedures indicated that the mass of primary particles with a diameter of less than 100 nm was as high as 30%, with a mean of ~25% in twelve samples, although in some samples the NP fraction was as low as 2% by mass (Verleysen *et al.* 2020; 2021; Table 2). It is unknown if the manufacturers of those products with only a small fraction of particles in the nanoscale took steps to deliberately reduce their number, although these results suggest it may potentially be feasible to produce food-grade TiO_2 with a minimal fraction of primary particles in the nanoscale. On a particle number basis, the percentage of primary particles with a diameter of less than 100 nm may be 50% or greater, although the percentage by number of particles with a diameter less than 30 nm is on the order of 1% or less (EFSA 2021a).

Table 2. Percentage of particles in the nanoscale (by number and by mass) among 12 pristine samples of food-grade TiO_2 purchased in the European market and analyzed by Verleysen *et al.* (2020; 2021; data extracted from Table W.2 of EFSA 2021a).

Identifier	Structure	Percentage of constituent particles by number<100 nm	Percentage of constituent particles by mass< 100nm
E171-02	Anatase	74	33
E171-03	Anatase	64	29
E171-04	Anatase	67	32
E171-06	Anatase	65	29
E171-07	Anatase	73	31
E171-09	Anatase	71	32
E171-A	Anatase	40	10
E171-B	Anatase	70	30
E171-C	Anatase	56	20
E171-D	Anatase	18	2
E171-E	Anatase	65	27
E171-F	Rutile	20	3

Mass is the most common metric used to express the concentration/doses of TiO_2 particles in *in vitro* and *in vivo* studies and may be the only one practicable in the laboratory. However, whether this exposure metric is toxicologically meaningful given the nature of NPs is questionable. Unless based on a dilution series with the same material, assessing exposure to TiO_2-NPs on a mg/kg basis is equivalent to treating them the same as bulk scale, which essentially ignores the very properties that make nanomaterials distinct. If the toxicity of TiO_2-NPs is in part a function of the size, volume, morphology, density and/or number of particles or their surface area/reactivity, the use of mass as an exposure metric will result in a toxicological bias towards TiO_2 formulations that contain smaller particles, which may not allow for extrapolation to forms of TiO_2 such as food-grade TiO_2 that contain a combination of micro- and nanoparticles. For particles such as TiO_2-NPs that are insoluble or poorly soluble, mass is not directly related to the number of particles and is insufficient to characterize the dose (Delmaar *et al.* 2015). Thus, the use of mass as a dose metric in toxicological studies does not facilitate relevant comparisons with human exposure estimates to characterize risk. In their recent assessment, EFSA's FAF Panel (2021a) states, "the findings in studies with E171 on immunotoxicity and inflammation were considered inconsistent; in studies with TiO_2-NPs > 30 nm effects were seen at a dose of 20 mg/kg bw/d whereas in studies with TiO_2-NPs < 30 nm effects were observed at doses as low as 2.5 mg/kg bw/d". However, if toxicity relates to some other aspect such as particle number or surface area, the "lower dose" expressed on a mass basis may be a mischaracterization. For example, Jones *et al.* (2015) observed that "a particle with a diameter of 500 nm will weigh the same as 125,000 particles with a 10 nm diameter".

TiO_2 particles are highly stable and are considered to be unaffected by food processing (Winkler *et al.* 2018). However, it is well documented that TiO_2 particles, particularly uncoated particles, have a tendency to agglomerate/aggregate, with smaller particles having a greater tendency to agglomerate than larger particles (Parrino and Palmisano 2020). Therefore, to reliably characterize the particle size distribution of food-grade TiO_2, whether in pristine form or isolated from foods, a number of sample preparation steps may be required to deagglomerate particles, such as a combination of optimized pH, sonication, and centrifugation (Verleysen *et al.* 2021). While these dispersion and stabilization methods have been meticulously validated for the purposes of particle characterization, they are also widely used for both *in vivo* and *in vitro* toxicity testing. There is less certainty as to whether the results of such tests are of relevance to human exposure given that TiO_2 as ingested in foodstuffs is not subjected to the same deagglomeration protocols, and therefore the test article when intentionally dispersed was considered less representative of food-grade TiO_2 as encountered in the diet (see 'Particle Dispersion' section below for more details). In their recent opinion, EFSA's FAF Panel (2021a) noted that based on information received from industry, liquid dispersions of E171 or other processes to reduce the formation of agglomerates may be used in some products, although the only example of such applications provided was "incorporation of E171 into a tablet coating or capsule", which does not appear to be typical of use in foods. Health Canada is not privy to the information submitted to the EFSA FAF Panel, although it is possible that reducing the formation of agglomerates in this context refers to separating large, weakly-bound agglomerates that are beyond the ideal particle size range for its technical use; breaking up agglomerates into primary particles such as occurs under certain laboratory protocols would not be of benefit in food preparation as the desired whitening or opacifying effect will be lost or diminished. Health Canada's Food Directorate has received confirmation from industry that sonication is not used to incorporate TiO_2 into liquid or solid food preparations (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.*

13 May 2022). Moreover, the production process of food-grade TiO_2 involves high energy milling that is far more intensive than the methods used to incorporate the material into any known end use application, including food manufacture (TDMA 2019).

Significant advances in the procedures used to characterize the intrinsic properties of food-grade-TiO_2 have been made following EFSA's (2016) call for additional data concerning the specifications of E171 (see for example Verleysen *et al* 2020; 2021; Geiss *et al.* 2020; 2021). It is now feasible to reliably characterize pristine particles on the basis of particle size distribution, shape, composition, surface chemistry, surface charge and zeta potential[10] among other parameters, using conventional methods. However, characterization of TiO_2 particles *in situ* in complex food or biological matrices still presents considerable challenges (Singh *et al.* 2014; Szakal *et al.* 2014). Foods are complex and variable mixtures that often contain particles with dimensions that overlap with the particles of interest (McClements *et al.* 2016). TiO_2 particles are also present in foods in small quantities (typically 1% by weight or less) and it is generally necessary to extract and isolate them to characterize such properties as size distribution or aggregation state. However, the procedures used to isolate the particles from other particulate matter may fundamentally alter the properties of interest and thus not be a true reflection of particle characteristics as used in food. For example, Li *et al.* (2021a) characterized the size distribution of TiO_2 particles in coffee creamer and instant drink powder. The authors state, "To conduct the effective size characterization of TiO_2-NPs, the studied powdered beverages have to be properly dispersed in liquid medium. However, since TiO_2-NPs are well known for their susceptible agglomeration/aggregation, it is important to explore suitable working conditions to minimize these undesirable adverse effects." To this end, to extract and isolate particles from their food matrix and prior to particle size determination, the samples were homogenized, vortexed with a surfactant, defatted with organic solvent (in the case of coffee creamer), centrifuged, further vortexed and sonicated in carrier solutions containing either a pH-adjusted surfactant or multiple non-ionic and ionic detergents. The authors reported that most particles were still present in the form of aggregates and/or agglomerates, although "…many individual NPs can still be found and deemed as primary particles". However, these sample preparation steps are likely to materially alter the agglomeration state of particles, making it difficult to draw inferences from these findings. It has been suggested that complete characterization of nanomaterials present in complex food matrices may require the development of new analytical methods (Bellmann *et al.* 2015; Szakal *et al.* 2014).

Similar to other jurisdictions, food-grade TiO_2 has been approved for use as a food additive in Canada for over fifty years to colour (e.g., whiten or brighten) many foods (see Table 3). Permitted maximum levels of use for TiO_2 are limited by GMP and diet is considered to be the largest source of TiO_2 exposure for the general population (Ramoju *et al.* 2020). In addition to food, other potential sources of human exposure to TiO_2 and TiO_2-NPs from the use of products available to consumers include personal care products such as toothpaste, medicines such as some tablets or capsules, as well as sunscreens and cosmetics that are applied to the skin. TiO_2 is also reported to be the second most commonly used ingredient in tattoo inks, and studies of the fate of tattoo pigments in skin have shown them to accumulate in draining lymph nodes (Schreiver *et al.* 2017; Foerster *et al.* 2020). In addition, many orthopedic prostheses, such as artificial

[10] Zeta potential is a measure of the charge on suspended particles and is used as an indication of the stability of dispersions. A high zeta potential (negative or positive) indicates highly charged particles and physically stable suspensions as agglomeration is less likely due to electrostatic repulsion of individual particles, whereas lower zeta potentials (negative or positive) are associated with agglomeration.

joints, as well as dental implants are made of Ti, which may be a source of exposure to Ti-NPs either locally or systemically due to wear and/or corrosion. Cellular responses to Ti-NPs may contribute to pathological mechanisms underlying adverse local tissue reactions associated with Ti-based orthopedic implants (Yao *et al.* 2017), and blood Ti levels have been proposed as a biomarker of orthopedic implant wear (Swiatkowska *et al.* 2019). Intravenous exposure to TiO_2 particles is unusual but has been documented in intravenous drug users with a history of injecting crushed tablets coated with TiO_2 (Filho *et al.* 1991; Lima *et al.* 2004; Hamilton 2013; Gilbert *et al.* 2021). In the case of intravenous exposure, substantial quantities of TiO_2 particles have been observed primarily in liver and spleen, and to a lesser extent in lungs, lymph nodes and bone marrow.

Table 3. Permitted food additive uses of titanium dioxide in Canada [11] as per the List of Permitted Colouring Agents

Food Additive	Foods Permitted in or Upon	Maximum Level of Use and Other Conditions
Titanium Dioxide	(1) Apple (or rhubarb) and (naming the fruit) jam; Bread; Butter; Concentrated (naming the fruit) juice except frozen concentrated orange juice; Fig marmalade with pectin; Fish roe (caviar); Ice cream mix; Ice milk mix; Icing sugar; Liqueur; Lobster paste; (naming the flavour) Milk; (naming the flavour) Partly skimmed milk; (naming the flavour) Partly skimmed milk with added milk solids; (naming the flavour) Skim milk; (naming the flavour) Skim milk with added milk solids; (naming the fruit) Jam with pectin; (naming the fruit) Jelly with pectin; Pickles; Pineapple marmalade with pectin; Relishes; Sherbet; Smoked fish; Tomato catsup	(1) Good Manufacturing Practice[12]
	(2) Unstandardized foods	(2) Good Manufacturing Practice
	(3) A blend of prepared fish and prepared meat referred to in paragraph B.21.006(n)	(3) Good Manufacturing Practice

NB: "Naming the fruit" and "naming the flavour" refer to standards of identity for certain foods set out in Part B of the *Food and Drug Regulations* (FDR) or the *Safe Food for Canadians Regulations*. Foods with prescribed standards (e.g., B.11.128 [S]. Orange Juice; or B.11.130 [S]. Concentrated (naming the fruit) juice, as set out in the FDR) must comply with the specifications of the standards of identity in every respect. A food that does not meet the requirements of a standard cannot be labelled using the prescribed name for that standardized food.

[11] Item No. 1, *List of Permitted Colouring Agents*. Available online at https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/3-colouring-agents.html

[12] As per the Marketing Authorization for Food Additives That May Be Used as Colouring Agents, "when the words "good manufacturing practice" appear in column 3, the exemption applies if the amount of the agent that is added to the food in manufacturing and processing does not exceed the amount required to accomplish the purpose for which it has been added and if any other condition that is set out in that column is met".

4. Particle Corona, Food Matrix and the Gastrointestinal Milieu

The toxic potential of TiO_2 particles can vary as a function of their size, shape, crystalline structure, surface characteristics and degree of dispersion (Winkler *et al.* 2018). It has also been demonstrated that interactions of particles with complex food matrices and biological substrates may rapidly and substantially alter these attributes (Walczyk *et al.* 2010; McClements *et al.* 2016; McClements and Xiao, 2017; DeLoid *et al.* 2017; Nierenberg *et al.* 2018; Cao *et al.* 2020; Liu *et al.* 2020), such that the toxicokinetics and toxicodynamics of TiO_2-NPs ingested with food may differ substantially from those of pristine TiO_2-NP dispersions. Proteins, lipids and other macromolecules have been shown to adsorb to NPs to form a "corona"[13] that alters surface characteristics and influences biological responses, including interaction with epithelial cells, macrophages and microbiota (DeLoid *et al.* 2017; Winkler *et al.* 2018). The protein corona is a well-recognized limitation in NP-based therapeutics, particularly via the oral route, as it governs their fate and biological identity (Berardi and Baldelli Bombelli 2019; Rampado *et al.* 2020) and can significantly impact therapeutic properties, gastrointestinal transit and oral bioavailability (Zhang *et al.* 2021). The majority of toxicity and genotoxicity studies of TiO_2 particles, however, fail to account for interactions with food and/or biologically relevant matrices to influence the toxicity of these particles via the oral route and thus may not fully represent human exposure to TiO_2 as a constituent of food preparations.

The Particle Corona

The intrinsic properties of pristine particles such as size, shape, crystalline form, zeta potential, etc. are typically characterized prior to their use in *in vivo* or *in vitro* toxicity studies. However, upon entering the complex biological environment of the GIT or interacting with constituents of the food matrix, the surface of particles is immediately coated with proteins and other macromolecules with the resultant corona changing the "synthetic" identity of the particle to its "biological" identity (McClements *et al.* 2016; Breznica *et al.* 2020). It has been emphasized that what a cell, organ or barrier actually "sees" when interacting with particles in a biological medium is more critical than the intrinsic properties of the pristine material, and therefore the biologically relevant unit is not the synthetic particle itself but rather the particle-protein complex (Cedervall *et al.* 2007; Walczyk *et al.* 2010). The interactions with macromolecules and the composition of the resulting corona are a function of the type of matrix, surface chemistry of particles as well as their curvature and diameter (DeLoid *et al.* 2017). Therefore, different-sized TiO_2 particles/agglomerates and same-sized particles/agglomerates in different matrices may be assumed to have distinct corona compositions (Winkler *et al.* 2018) and thus possess fundamentally different toxicological propensities, further complicating attempts to evaluate the human health risks of dietary TiO_2 exposure based on extrapolation from non-food grade test articles and/or particles dispersed in simple matrices such as water. Given that binding of macromolecules alters particle size and surface properties, it is likely that the protein corona also has a significant influence on oral systemic bioavailability. Therefore, studies in which cells or organisms are exposed to dispersions of pristine TiO_2 particles outside of a food or biologically relevant matrix may be useful for identifying intrinsic hazards of constituent particles (both primary particles and reduced agglomerates) but are of questionable

[13] The term "protein corona" was coined by Cedervall *et al.* (2007) to describe the dynamic protein structure that spontaneously forms on NPs when introduced into a biological milieu.

significance for human health risk assessment of dietary exposure to food-grade TiO_2 as the material is encountered in the diet.

It is important to note that the protein corona may either enhance or attenuate the targeting, uptake and subsequent toxicity of particles depending on its composition as well as the particular biological environment (Walczyk *et al.* 2010; Jo *et al.* 2016; Liu *et al.* 2020). While a naked particle may have a greater nonspecific binding affinity to cell surfaces than one coated in proteins, the opposite may be true if a particle is coated with proteins for which suitable cellular recognition machinery exists. For example, Runa *et al.* (2017) have shown that bare TiO_2-NPs (21 nm; crystalline form not stated) induce lipid peroxidation of the plasma membrane in HeLa and A549 cells (124 µg/mL, 12 h), as quantified by a malondialdehyde assay, which can be inhibited through passivation of the NP surface by a serum protein corona. Similarly, Proquin *et al.* 2017 showed E171 suspended in Hank's balanced salt solution (HBSS) via sonication (30 minutes at 40 kHz) induced reactive oxygen species (ROS) production under cell-free conditions, while no ROS production was observed in the presence of 0.05% bovine serum albumin (BSA) in the medium, which the authors attributed to the presence of a protein corona on the surface of particles that scavenges ROS. However, deleterious effects of the protein corona have also been reported. Borgognoni and colleagues (2015) investigated the effect of the protein corona of TiO_2-NPs (primary particle size not stated; hydrodynamic modal diameter in culture medium 180 nm, range 80 – 240 nm) on human macrophages. TiO_2 particles (10, 50 or 100 µg/mL) were incubated with macrophages in the presence of 10% fluorescently-labelled BSA. A dose-dependent increase in the induction of pro-inflammatory cytokines IL-6 and IL-1β was observed, although a similar trend was seen for the anti-inflammatory cytokine IL-10. The authors posit that the protein corona interacts with cell surface receptors, inducing cytokine secretion through the activation of signaling cascades, suggesting the presence of the protein corona may facilitate recognition of particles by phagocytic cells such as macrophages and thereby promote inflammation. In addition, just as proteins change the size and reactivity of particles, adsorbed proteins on the surfaces of particles also undergo conformational changes, which may result in new epitopes and altered biological functions or affinities (Parveen *et al.* 2017). The binding of proteins may also be confounded by the surface chemistry of the TiO_2 particles (i.e., the presence of phosphate ions, silica or alumina coating, etc.) which may differ between particles intended for use in food versus other purposes. The protein corona is the subject of extensive research in the field of nanomedicine, where the unique properties of NPs are being explored for their potential application as drug delivery carriers, anti-tumour agents, vaccines and antibacterial products as well as in diagnostics, imaging and as biosensors (for review see Liu *et al.* 2020; Martinez-Negro *et al.* 2021; Akhter *et al.* 2021; Barui *et al.* 2020; Lee 2021; Fasoli 2020). This work has highlighted the complexity of nano-bio interactions under physiologically relevant conditions and the difficulty in predicting the impact of the protein corona on cellular targeting and toxicity.

Lastly, the protein corona should not be regarded as a fixed property of particles but rather as a dynamic entity, the composition of which varies over time and in accordance with the milieu in which it is present. Similar to the "Vroman effect", which describes the competitive binding of blood serum proteins to solids (Vroman 1962), the protein corona initially consists of high abundance proteins which immediately bind to pristine particles upon coming into contact with biological fluids or components of food matrices. Over time, however, the protein composition evolves through competitive binding as abundant but weakly-bound proteins are gradually replaced by less abundant proteins with greater binding affinities until an equilibrium is reached (Vilanova *et al.* 2016; Liu *et al.* 2020). These more stable, high-affinity proteins are

often referred to as the "hard corona", whereas the transient, low-affinity interactions make up the "soft corona". Soft corona proteins may secondarily interact with hard corona proteins and it has been suggested that the soft-hard corona interface is likely critical in establishing the true biological identity of particles (Nierenberg *et al.* 2018). However, due to the complexity of studying these transient interactions *in situ*, the majority of studies of the nano-bio interface have been conducted *in vitro* and thus the toxicological significance of protein-particle transformations within complex food matrices and under physiologically-relevant conditions remains poorly understood.

Impact of the Food Matrix and Gastrointestinal Milieu

Bettencourt *et al.* (2020) assessed the impact of a simulated digestion model on the physicochemical properties of TiO_2 materials (JRC NM-102, NM-103, NM-105) dispersed in water in the presence of BSA using ultrasonication and in accordance with the NANOGENOTOX[14] dispersion protocol. After the dispersion protocol, the TiO_2 suspensions were subjected to three sequential digestion phases consisting of simulated salivary fluid (pH 7), simulated gastric fluid (pH 3) and simulated intestinal fluid (pH 7); the model did not account for the presence of a food matrix. Simulated digestion was not observed to have a significant impact on size distributions of the three TiO_2 materials tested and small particles less than 100 nm in size were still detectable in each TiO_2 suspension after digestion. The hydrodynamic mean size of NM-105 (analogous to Aeroxide® P25 TiO_2-NP[15]) measured by dynamic light scattering (DLS) was observed to decrease slightly after digestion, suggesting that this material may agglomerate less upon digestion. In addition, the size range of TiO_2 agglomerates/aggregates measured using TEM did not change significantly after digestion. The surface charge (zeta potential) measured for each material was also unaffected by the digestion process.

The rather insignificant effect of simulated digestion on the agglomeration status of TiO_2 suspensions generated using ultrasonication in the presence of BSA reported by Bettencourt *et al.* (2020) is in contrast with other studies that demonstrate very significant impacts of digestion on the agglomeration of TiO_2 suspensions. Dudefoi *et al.* (2021) assessed the behaviour of E171 and P25 TiO_2-NPs through a standardized static *in vitro* digestion protocol simulating the oral, gastric and intestinal phases (the test suspensions were not sonicated). The results indicate significant agglomeration of TiO_2 particles as the size distributions of TiO_2 particles were shifted towards larger sizes during the simulated digestion. For all samples, the largest sizes were obtained in the intestinal phase, with size distributions centred at around 90 µm on average and expanding until roughly 500 µm. The authors suggest the extensive agglomeration is mediated by the adsorption of mainly α-amylase and divalent cations onto TiO_2 particles and bridging between particles that are already agglomerated in water. Li *et al.* (2017a) also observed a progressive increase in mean particle diameter of sonicated TiO_2 dispersions as they passed through a simulated GIT model, suggesting that the particles became significantly more aggregated.

[14] Final protocol for producing suitable manufactured nanomaterial exposure media. July, 2011. https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf [Accessed 10 June 2021].

[15] Aeroxide® P25 (manufactured by Evonik Industries, Degussa, Germany) is commonly used as a reference material for TiO_2-NPs in various studies. The material consists of mixed-phase spherical particles (~85% anatase/15% rutile) with a mean particle diameter of 21-24 nm and is considered an optimal photocatalyst.

Marucco *et al.* (2020) studied the molecular processes governing bio-transformation in terms of agglomeration and surface modifications of sonicated food-grade TiO_2 and TiO_2-NP suspensions during the digestion process using an *in vitro* simulated human digestive system. The size distribution measured using DLS for a suspension of aggregated TiO_2-NPs (5-6 nm primary, 100-1000 nm aggregates) shifted slightly to smaller hydrodynamic radii from water to simulated saliva, however, a major shift in distribution to larger sizes was observed in gastric and duodenal fluids. Both food-grade and monodisperse TiO_2-NP suspensions exhibited continuous increases in hydrodynamic radius from water to saliva, duodenal and gastric fluids. The authors also note that agglomeration/aggregation was not only inferred by the shift of the size distribution to higher values, but also by the increase of the variability among the repeat measurements, suggestive of poor stability of the suspensions. This study also demonstrated that agglomeration of TiO_2 materials subjected to duodenal fluid only (pH 6.5) was reversible when the suspensions were centrifuged to remove the supernatant and resuspended in water. However, the agglomeration of these TiO_2 materials, whether subjected to saliva or gastric fluid only, or to all three digestion phases was found to be irreversible, suggesting that irreversible aggregation occurs in the gastric fluid and remains unaltered in the duodenal compartment. Marucco *et al.* (2020) also investigated the separate impacts of pH and ionic strength of the digestive fluids on the agglomeration state, finding that high ionic strength appears to be the main driving force leading to agglomeration. Lastly, the effect of simulated digestion on the cytotoxicity of TiO_2-NPs to human colorectal carcinoma HCT116 cells was examined. A dose-dependent increase in ROS production was observed following exposure to both digested and undigested particles, although simulated digestion attenuated the response. The authors concluded that particles that acquire a protein corona by interacting with simulated digestive fluids are less bioactive and less toxic.

In an example that more closely resembles food matrices in which dietary exposure to food-grade TiO_2 is likely to occur, Cao *et al.* (2019) reported that a series of complex molecular events occurred when TiO_2-NPs (< 30 nm by TEM) were titrated into casein solutions, including dissociation of casein micelles, NP-protein complex formation, and complex aggregation. The authors attribute the high degree of NP aggregation in this system to two effects: first, charge neutralization reduced the electrostatic repulsion between the particles, thereby promoting aggregation; second, bridging flocculation may have occurred where individual casein molecules were adsorbed to the surfaces of numerous NPs, thereby linking them together. The impact of a simulated food matrix on E171 toxicity was further investigated by these authors in an intestinal epithelial model (Cao *et al.* 2020). The food-grade TiO_2 was an anatase crystalline form with a mean particle size of 113.4 ± 37.2 nm by TEM and approximately 40% of particles having at least one dimension < 100 nm. At a concentration of 0.75% or 1.5% (w/w), E171 suspensions produced significant, dose-dependent cytotoxicity and oxidative stress in a fasting food model (FFM) but not in a standardized food model (SFM) based on the "American diet", indicating a significant food matrix effect.

The effect of the food matrix on the corona of food-grade TiO_2 was also examined by Coreas *et al.* (2020). Three food models were processed in a simulated gastrointestinal digestion system in the presence of food-grade TiO_2; a FFM, a SFM, or a high fat food model (HFFM). The test article was the same as used by Cao *et al.* (2020) described above. Following simulated digestion under the three conditions, lipids and proteins were extracted from the corona and profiled. A particle corona formed around the E171 particles under all conditions tested, although the thickest layers were observed in the HFFM model. Corona profiles and particularly lipid profiles varied under the three conditions, although coronas formed under

SFM conditions were more similar to FFM than HFFM. The authors concluded that the particle corona formed on food-grade TiO_2 particles may passivate the surface of these particles, although the extent and composition of the corona will vary as a function of the food matrix and in particular the lipid concentration.

The effect of a simulated digestive environment on the dissolution kinetics, particle size distribution, and morphology of TiO_2 was investigated by Sohal *et al.* (2018). The TiO_2 particles used were an anatase form (identified as TiO_2 A200, from the Shanghai Yuejiang Titanium Chemical Manufacturer Co., Ltd) that was reported to be food-grade with a primary particle size of 100 – 150 nm by TEM and a BET specific surface area of 8.2 m^2/g, consistent with E171. The simulated digestive model had a sequential mouth-stomach-intestinal digestion cascade at appropriate pH and in the presence of relevant enzymes to represent exposure to saliva, gastric, and duodenal and bile fluids. Prior to mixing with digestive fluids, particles were dispersed by sonication in distilled water and "no presence of aggregates" was confirmed. In simulated salivary fluid (pH 7.0), no significant change in the particle size distribution, agglomeration state or ion concentration was observed up to 60 minutes. After dissolution in simulated gastric fluid (pH 2.0) for 8 h, TiO_2 agglomerate size increased from an average of 291.4 nm to 454 ± 49 nm but no significant release of Ti ions was observed. Particles were subsequently exposed to simulated intestinal fluid (pH 7.0) for an additional 8 h during which the agglomerate size continued to increase over time although again no significant ion release was observed. The hydrodynamic diameter of TiO_2 agglomerates as measured by DLS increased from an initial 330 nm to approximately 600 nm by the end of the gastric phase. A sudden decrease in agglomerate size to approximately 500 nm was observed upon transfer to the intestinal phase, which subsequently increased to approximately 900 nm by the end of the digestion cascade. The maximum dissolution for TiO_2 across all phases was estimated to be 0.42% and the authors noted that the pronounced changes in particle size distribution, agglomeration state and dissolution behavior are driven by the absorption of organic components on the particle surface leading to a corona, a conclusion that was supported by TEM observations. Overall, the authors concluded that TiO_2 is biodurable and persistent, with a trend of increasing agglomerate size over time and along the GI tract cascade.

The impact of a SFM on the fate and toxicity of E171 (D_{50}= 113 nm by TEM) in the GIT was also investigated by Zhang and colleagues (2019). The SFM was modelled on the "typical US diet" in terms of protein, sugar, dietary fibre, starch, fat and mineral consumption, based on food studies conducted by the US Department of Agriculture's Food Surveys Research Group. TiO_2 (E171) was dispersed by sonication prior to being added to either 5 mM phosphate buffer (fasting medium) or in the SFM at concentrations of 0.75% w/w or 1.5% w/w. The simulated digestion of the TiO_2 suspension was performed in a 3-step GIT simulator mimicking the conditions of the mouth (2 minute incubation), stomach (2 h incubation) and small intestine (2 h incubation). The cytotoxicity of TiO_2 particles suspended in either fasting medium or SFM was investigated by placing the digestae that had been passed through the simulated GIT in contact with a tri-culture model of intestinal epithelial cells (Caco-2, HT29-MTX, and Raji B cells) and incubated for 24 h. The release of lactate dehydrogenase (a marker of altered cell plasma membranes) was quantified as a marker of cytotoxicity. A dose-dependent increase in cytotoxicity was observed when epithelial cells were exposed to digestae containing TiO_2 particles dispersed in fasting medium. SFM digestae, however, were significantly less cytotoxic than their fasting medium counterparts regardless of concentration, with a 5-fold decrease in toxicity associated with the food matrix effect at the highest concentration of 1.5%

w/w. The authors concluded that these results highlight the importance of the food matrix in studies investigating the toxicity of TiO_2 particles in the GIT.

Similar results have also been observed in simulated gastrointestinal digestion systems using TiO_2-NPs. Li *et al*. (2021b) studied the influence of a standardized food matrix on the physicochemical properties of TiO_2-NPs in a three-stage *in vitro* digestion model (mouth, gastric, intestinal). The particles were engineered TiO_2-NPs with a reported mean particle diameter of 25 nm (crystalline form not stated) and were suspended by brief sonication (10 s) in either 10 mM phosphate buffer (without food matrix) or in SFM at 0.5% w/w, 1% w/w or 1.5% w/w. The hydrodynamic diameter of agglomerates was shown to increase significantly during digestion both with and without a food matrix, with the most significant agglomeration occurring in the gastric and intestinal phases. However, with the exception of the gastric phase, the hydrodynamic size was larger in buffer alone, suggesting the food matrix may also act as a dispersant for pre-dispersed TiO_2-NPs and thus serve to stabilize the suspension under these circumstances. Prior to digestion, the zeta potential was significantly decreased in the presence of the food matrix, and further decreases were observed in all digestive phases, with the largest decreases occurring in the gastric phase. Following simulated digestion, human colon epithelial Caco-2 cells were exposed to digesta mixed with serum free media and incubated for 24 h prior to cell viability analysis. Cell viability upon exposure to TiO_2-NPs digesta was higher in the presence of the food matrix than in its absence. The authors concluded that the physicochemical transformations of TiO_2-NPs in the presence of a food matrix strongly alter their biological effects, and that the toxicity assessment of ingested TiO_2-NPs should take realistic exposure conditions into account.

The methods used to simulate digestion vary considerably in important parameters such as pH, duration, composition of simulated fluids, enzyme and/or microbe type and activity, static versus dynamic systems, and the presence or absence of a simulated food matrix, thereby introducing heterogeneity in the results (for review see Lefebvre *et al.* 2015). Taken together, however, the contrasting aggregation/ agglomeration behaviour of TiO_2 suspensions prepared using ultrasonication in water versus in the presence of common biomolecules or dietary constituents suggest that TiO_2 suspensions in simple matrices may be less relevant as test articles for the study of food-grade TiO_2 as a constituent of the diet. This was also acknowledged by the EFSA ANS Panel (2018) who stated that "the administration of TiO_2 (E 171) by gavage or drinking water is not fully representative of the use of the food additive E 171 in food" and that extrapolating from such studies to the assessment of the food additive introduces uncertainty "because interactions with the food matrix may not occur." Similarly, ANSES (2017) concluded that adverse findings in drinking water studies cannot be used in risk assessment until they have been confirmed by additional studies where the test article is administered in the food matrix.

The results of the various digestive models described above demonstrate that the composition of the particle corona continues to evolve as particle-protein complexes move through different environments, such as salivary fluid through to gastric and intestinal fluid, as the pH and ionic strength of the surrounding fluids change. Foods are likewise highly complex matrices that differ in composition, structure, pH and ionic strength and thus the properties of a given particle may also change depending on the type and amount of food or beverage it is consumed with (McClements *et al.* 2016). An understanding of such changes in the structure and composition of the protein corona that lead to alterations in particle size, agglomeration state, surface properties and ultimately biological activity as a function of the food matrix

and gastrointestinal milieu in which they reside is fundamental to identifying relevant toxicological responses. A major limitation of most *in vitro* studies with respect to their relevance to evaluating the safety of TiO_2 as a food additive is the use of overly simplistic models that fail to account for the influence of these biologically relevant matrices on particle characteristics and toxicity (McClements *et al.* 2016). For example, many studies investigate the cytotoxic or genotoxic potential of pristine TiO_2 particles dispersed in simple matrices such as water, which does not represent the complexity of the GIT environment, including the potential contribution of the gut microbiome or the presence of the food matrix, and may translate to poor if not misleading *in vivo* predictions. The relevance of these studies would be greatly enhanced if particles were first passed through a GIT model to simulate digestion prior to introduction in test systems.

5. Particle Dispersion

Various guidelines, technical reports, and SOPs have been developed to standardize sample preparation and dosimetry for the safety testing of manufactured nanomaterials (e.g. by the OECD, EFSA, the US EPA and within the EU projects NanoDefine, NanoValid, NanOximet, NANoREG or Nanogenotox, etc.). These protocols aim to harmonise sample preparation conditions such that theoretically comparable suspensions in terms of particle size distribution may be produced among different laboratories (ANSES 2019). The necessity of stable dispersions for material characterization as well as accurate and repeatable dosing is well recognized, and the majority of the various methods for sample preparation of nanomaterials published internationally advance the use of sonication for these purposes. As such, in many test systems described in the literature, TiO_2 particles are sonicated in order to create stable, homogeneous dispersions for testing and characterization. Recently EFSA (2021b) has updated their guidance on the risk assessment of nanomaterials in the food and feed chain. This guidance does not refer to sonication specifically, but emphasizes the need to test stable dispersions for identification and characterization of hazards associated with nanomaterials and references several of the protocols mentioned above that do espouse sonication. However, EFSA also cautions that, "In specific cases, and especially when exposure occurs mainly through solid and liquid foods, additional groups with food or drinking water administration *have to be included* to determine whether hazards associated with the nanomaterial are observed *under realistic exposure scenarios*" [emphasis added]. The use of food-grade TiO_2 as a constituent of food is almost certainly one of those specific cases, and the guidance from EFSA is consistent with Health Canada's approach of examining and evaluating the potential health risks of nanomaterials in food products on a case-by-case basis. Both EFSA (2018a) as well as an expert Committee commissioned by ANSES (2019) have also opined that sonicated particles may help in identifying certain hazards associated with food-grade TiO_2 in suspension but due to the disruption of agglomerate structure, sonicated material is not fully representative of the substance as found in foodstuffs.

While it is again acknowledged that sonication is widely used to create stable dispersions of particles for the testing of nanomaterials, in the case of food-grade TiO_2 there is also the potential for this practice to produce artifacts. By increasing collision frequency and decreasing the free energy of particles, sonication may result in deagglomeration of larger particle clusters while simultaneously promoting the re-agglomeration of previously fragmented clusters (Taurozzi *et al.* 2011). TiO_2 is an abrasive material, and high-power sonication may abrade the sonotrode, which is typically constructed of a Ti alloy, leading to a fine fraction of exogenous nano-Ti material in the sample (Marín *et al.* 2017). Ultrasonic disruption of TiO_2

agglomerates may also lead to the formation of highly reactive hydroxyl radicals in a process known as "sonolysis"[16]. This process is exploited in the area of water and wastewater purification, where sonolysis of TiO_2 particles results in the degradation of various water contaminants by hydroxyl radical attack. Although sonication-induced radicals formed in aqueous environments are highly reactive and thus very short-lived, they may still drive oxidative and other chemical transformations, such as changes in particle surface chemistry including oxidation or hydroxylation state, and may subsequently propagate further radical chain reactions or recombine to form more stable (and thus longer-lived) peroxides and peroxyl radicals (Taurozzi *et al.* 2011). Indeed, Taurozzi *et al.* (2011) state that "…it is unquestionable that sonication-specific, radical-mediated changes have been observed as a result of sonication, …and can significantly alter the suspension medium properties or the dispersed material's surface chemistry, and consequently influence its environmental and biological behavior." Similarly, the NANOGENOTOX dispersion protocol, which is widely used in toxicity studies, cautions that unanticipated side effects of the particle dispersion process, such as particle alteration and consumed hydrochemical reactivity/radical formation may occur and "could be mechanisms responsible for toxicological effects" (NANOGENOTOX 2013). The effect of sonication on TiO_2-NP toxicity was shown by Carrière *et al.* (2014). These authors dispersed TiO_2-NPs by either ultrasonication or through ball milling[17], which produced similar particle size distributions. However, the sonicated dispersion produced more deleterious effects on cell membrane integrity and led to intracellular accumulation of ROS in A549 cells. In foods, there are many other molecules in the food matrix to which the energy of excited TiO_2 can be transferred and the matrix can effectively act to insulate the particle surface and prevent binding of O_2, H_2O and OH^-, reducing the formation of highly reactive oxygen-centred radicals. Further, any radicals that may be formed would be much less reactive carbon-centred radicals, with many acting as radical chain terminators, preventing further increase in the concentration of highly reactive, oxidative species.

The dispersion medium may also exacerbate the extent and degree of formation of sonication-induced reactive radicals, which may alter the toxicological properties of the dispersed suspension (Taurozzi *et al.* 2011). For example, BSA is widely used in NP dispersion protocols, and Wang *et al.* (2009) have shown that even low-frequency sonication induces time-dependent, irreversible degradation in BSA structure even in the absence of metal complexes. Prasad *et al.* (2013) compared the genotoxicity of TiO_2-NPs dispersed by sonication in three different media with various BSA concentrations. No differences were observed in the comet assay, although in the micronucleus assay chromosomal damage was observed at the high concentration of BSA only. Fleischer and Payne (2014) investigated the relationship between BSA secondary structure and NP adhesion in a series of cellular binding competition studies using various cell types. These authors observed that when BSA retains its native structure, BSA-NP complexes are recognized by the native albumin receptor and cellular binding of complexes is inhibited by excess BSA. However, in the presence of denatured BSA, such as occurs during sonication, these protein-NP complexes

[16] Ultrasonication refers to the application of sound energy at frequencies largely inaudible to the human ear (higher than ≈ 20 kHz) (Taurozzi *et al.* 2011). Health Canada identified a number of studies that reported sonicating particles at 60 Hz, which would represent an enormous difference relative to the ~20 - 40 kHz used in most studies, as acoustic power is proportional to the square of the frequency. However, it appears that in these instances the authors are erroneously reporting the frequency of the alternating current the device operates on (e.g. 120 V, 60 Hz) rather than the operative frequency of the instrument.

[17] Ball milling is a mechanical technique used to grind materials into fine particles. The material intended to be reduced is placed in a rotating cylinder that is partially filled with balls and high-energy impacts between the balls and the substrate homogenizes and disperses particles (see Bhagyaraj *et al.* 2018 for a more detailed description).

interact with a different set of cell surface receptors (known as scavenger receptors) that have a greater affinity for modified albumins, and excess denatured BSA enhances rather than inhibits cellular binding, thus influencing cellular responses. Recently, Charles and colleagues (2018) undertook a review of *in vitro* genotoxicity studies of TiO_2-NPs with a view to understand why, "even if judged of good quality, the 36 publications selected and analyzed did not lead to a clear picture" with respect to their genotoxic potential. The authors noted that, among other factors, modifications induced by sonication may lead to false *in vitro* genotoxic responses. Whether or not sonication-induced, radical-mediated changes also lead to disparate results in *in vivo* studies of TiO_2 toxicity has yet to be determined. However, given that generation of ROS is often viewed as the initial key event in the adverse outcome pathway of TiO_2 (Brand *et al.* 2020; Braakhuis *et al.* 2021), the fact that sonication of particles may lead to ROS production adds further uncertainty and could possibly be a confounder of the true dose response.

In addition, it is well established that TiO_2 is a photocatalyst capable of producing highly oxidizing ROS upon absorption of a photon with sufficient energy (Carp *et al.* 2004). TiO_2-NPs exposed to light can oxidize hydroxide in water and produce ROS (Haynes *et al.* 2017), such that genotoxicity assays with TiO_2-NPs have been shown to produce conflicting results when performed in the dark versus under laboratory light (Gerloff *et al.* 2009; Woodruff *et al.* 2012; Di Bucchianico *et al* 2017). Ultrasonicated water may also partially dissociate into hydrogen and hydroxyl radicals (Taurozzi *et al* 2011). It is perhaps not surprising, therefore, that a synergistic effect on hydroxyl radical production has been observed between sonolysis and photocatalysis (Selli 2008; Mrowetz *et al* 2003; Verma *et al* 2014) and care should be taken to ensure toxicity tests that are sensitive to ROS synthesis are not conducted under conditions favouring sonophotocatalysis. Photocatalytic activity requires light absorption, and as such the photoactivity of particles varies as a function of their size/agglomeration state (Li *et al.* 2010; Pellegrino *et al* 2017). Larger particles and agglomerates such as those found in food-grade TiO_2 reflect and diffuse light, and therefore have minimal photocatalytic activity relative to smaller particles (Bi and Westerhoff 2019). However, when TiO_2 is deagglomerated by sonication into smaller agglomerates or constituent particles there is a rapid increase in surface area. The effect is that light scattering becomes less dominant and absorption becomes more significant, hence photocatalytic activity increases (Pellegrino *et al* 2017). In addition, food packaging provides a degree of protection from visible and higher energy UV light through light absorption by the polymer packaging itself as well as any added UV blockers to prevent changes to the food product. Furthermore, the food matrix (i.e., the other components of the food product such as proteins, lipids, macromolecules, etc.) will also provide significant protection from visible and UV light by acting as an internal filter and competing for light absorption. The protection from visible and UV light from food packaging as well as the food matrix is generally not taken into consideration in studies that administer food-grade TiO_2 that has been dispersed via sonication in simple liquid matrices, such as pure water, and in open containers or materials intended to be transparent to light-based detection methods (e.g., DLS).

Health Canada's Food Directorate is aware that sonication is used by the food industry for certain applications, such as the preparation of botanical extracts, inactivation of pathogenic organisms, the removal of food allergens or pesticides, the modification of functional characteristics of lipids and proteins as well as emulsification or homogenization, typically at smaller scales (Arvanitoyannis *et al.* 2017; Mumtaz *et al.* 2019; Ranjha *et al.* 2021; Arruda *et al.* 2021). Food manufacturers also disperse and stabilize TiO_2 in food matrices although Health Canada's Food Directorate is not aware of the use of high-energy ultrasound for these purposes. Indeed, aside from not being readily scalable, such methods would

presumably be counter-productive if sufficient energy is applied to break apart agglomerates, unless they are very large (i.e., outside the ideal particle size range for its technical use). TiO_2-NPs in food-grade-TiO_2 will contribute to the efficacy of the product as a whitening agent if they are agglomerated but not if they are dispersed into primary particles or agglomerates that are too small to scatter light and impart opacity. A variety of mechanical methods such as high-shear mixing, colloid and disk mills, and high-pressure homogenizers may be used to disperse aggregates of TiO_2 by food manufacturers. However, the shear forces generated by these devices are orders of magnitude lower than those produced by sonication and insufficient to separate agglomerates into their constituent particles. Hielscher (2011) states that conventional devices such as high-shear mixers "do not deliver sufficient power to separate nanomaterial into its individual particles. Particularly for substances in the range of a few nanometers to a few micrometers, the use of high power ultrasound has been proven as a highly efficient and effective means to destroy the agglomerates, aggregates and even primary particles." Similarly, Akpan *et al.* (2019) report that the mechanical forces generated by high-shear mixing are "far less powerful compared with the high-energy ultrasonication". Dispersion of agglomerates requires the application of shear stress that is greater than the binding energy of the particles. Huang and Tarentjev (2012) state that mechanical high shear mixing may produce a strain rate as high as 4,000 s^{-1} and shear stress of no more than 20 kPa in viscous liquids such as epoxy resins; in low-viscosity liquids such as water, shear stress delivered "will drop to below 50 Pa, offering very little hope of achieving dispersion". In the case of sonication, however, extremely high strain rates are produced during cavitation, up to 10^9 s^{-1}, leading to shear stress approaching 100 MPa, or roughly 10^5 – 10^6 fold greater than the maximum stresses produced by high shear mixing.

Health Canada's Food Directorate requested additional information from stakeholders regarding food industry practices in dispersing TiO_2 and received notice that sonication or other intensive methods intended to break down agglomerates into their constituent particles are not used to disperse TiO_2 in food or beverage manufacture (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022). This is consistent with information previously provided to Health Canada's Food Directorate indicating that the production process of food-grade TiO_2 involves much greater forces than the methods used to incorporate the material into any known end use application, including food manufacture (TDMA 2019).

6. Toxicokinetics

Oral intake via food, personal care products and medicines or natural health products is the primary route of TiO_2 exposure for those who are not occupationally exposed. A number of studies have investigated the oral bioavailability, systemic distribution and elimination of TiO_2 in both rodents and humans. In general, all TiO_2 particles are insoluble in aqueous solutions and only minimally degraded under conditions representative of the gastrointestinal milieu (Winkler *et al.* 2018), with the vast majority of ingested TiO_2 particles being eliminated unchanged in feces. However, the gastric epithelium is protected by the gut-associated lymphoid tissue (GALT), which immunologically surveils contents of the gut. In the small intestine, this mucosal lymphatic tissue forms aggregates that are known as Peyer's patches. The epithelial layer of Peyer's patches contains specialized cells called microfold (M) cells, with the unique ability to violate the mucosal barrier and capture and transport particles from the intestinal lumen (Dillon and Lo 2019). While these cells typically traffic calcium phosphate particles, TiO_2 particles are also within the

optimal size range of M cell uptake and thus may "hijack" this physiological pathway and gain entry into the gut lymphatics (Barreto da Silva *et al.* 2020). Upon accessing the lymphatic system, small particles may be translocated systemically whereas those greater than ~1 µm are effectively trapped in the Peyer's patches and persist undegraded (EFSA 2016). Particles may accumulate to the point of resembling tattoo-like structures at the bases of Peyer's patches, with the density of pigmentation observed to increase with age in children/youth aged 1.6 to 18.1 years (mean 12.2 years) undergoing intestinal biopsies for suspected inflammatory bowel disease, although particles were detected in less than half (42%) of the biopsies conducted and were not confirmed to be TiO_2 specifically (Hummel *et al.* 2014). Particles taken up by M cells may also be transcytosed to adjacent dendritic cells located within Peyer's patches, and dendritic cells themselves may directly sample particles in the intestinal lumen via tight-junction like structures with epithelial cells (Rescigno *et al.* 2001).

Studies in Rodents

Riedle *et al.* (2020) investigated whether a murine dietary model could be used to mimic human exposure to TiO_2. The authors posited that in order to be considered successful, it must be demonstrated that administration of food-grade TiO_2 incorporated into diet results in the accumulation of particles in Peyer's patches, as occurs in humans. Male and female C57BL/6 mice were exposed to 1, 10, or 100 mg/kg bw/d food-grade TiO_2 in feed for 18 weeks (anatase D_{50} 119 nm, range 50 – 350 nm), at which time they were euthanized and the GITs harvested. Peyer's patches were excised and examined via confocal microscopy and SEM equipped with energy-dispersive X-ray spectroscopy (SEM-EDX). As observed in humans, dose-dependent uptake of TiO_2 particles in the base of Peyer's patches was observed, leading the authors to conclude that the dietary model is a physiologically relevant oral dosing approach for human health risk assessment of food-grade TiO_2. The authors did not examine whether particles could be detected in tissues other than those of the GIT. Additional endpoints examined in this study are described below in the section on 'Immunotoxicity, Inflammation and Effects on the GIT'.

The absorption, distribution and excretion of food-grade TiO_2 incorporated into diet were assessed in a GLP-compliant study in rats carried out in accordance with the OECD 417 (Toxicokinetics) test guidelines (Farrell and Magnuson 2017). Food-grade TiO_2 (anatase, D_{50} = 133 – 146 nm) was incorporated into feed at a nominal concentration of 200 ppm and male and female Sprague Dawley rats (*n* = 10 per sex per group; dose equivalent to 30 mg TiO_2/kg bw/d assuming daily consumption of 30 g diet/d by a 200 g rat) received either TiO_2 or a control diet (no TiO_2 added, background concentration 7 – 9 ppm) for seven days. After seven days, the TiO_2 diet was withdrawn and three rats per sex were sacrificed at 1 h, 24 h and 72 h following replacement of the TiO_2 containing diet with the control diet. Animals were housed individually in metabolic cages after withdrawal of the test diet and all excreta including cage washings were retained. The Ti content in excreta, whole blood, liver, kidneys and muscle was determined by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). The majority of Ti was excreted in feces with concentrations in urine below the limit of quantification (LOQ; <0.04 mg/L; equivalent to <2% daily dose/L) for all samples except the 0 to 24 h urine sample for one rat (0.05 mg/L); collection of urine and feces began after withdrawal of the test diet and therefore no mass balance recovery was estimated. Whole blood Ti concentrations were also below the limit of detection (LOD) in both treatment and control groups. In tissues, Ti levels were below the 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 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. The authors conclude 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 d.

A second GLP-compliant, multi-site toxicokinetics study of 5 different grades of TiO_2 was carried out in accordance with OECD 417 test guidelines and a summary report of this unpublished study was submitted to Health Canada by industry (EBRC 2022). In this study, male and female CD rats (*n* not stated, although the test guideline stipulates a minimum of four animals per sex per dose group) 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 96 h post-dosing. The relative oral bioavailability of the various grades of TiO_2 was compared to a soluble Ti reference substance (Titanium(IV) bis(ammonium lactato) dihydroxide solution – 50 wt. % in H_2O) that was administered orally (100 mg/kg bw) or intravenously (10 mg/kg bw). The test articles included a food-grade form of TiO_2 identified as E171-E which had a median particle diameter (SD) of 99.9 ± 2.0 nm and contained approximately 50-51% of constituent particles in the nanoscale (LNE 2020). The other four particles were identified as G6-3 (a rutile TiO2-NP coated with alumina and hydrophobic organic, D_{50} = 9.2 ± 2.0 nm), G2-5 (uncoated anatase TiO2-NP, D_{50} = 5.5 ± 2.0 nm), G3-1 (uncoated pigmentary rutile TiO_2, D_{50} = 146.9 ± 5.9 nm) and G4-19 (pigmentary rutile TiO_2 coated with alumina and polyol, D_{50} = 177.5 ± 3.9 nm). Details of the vehicle and the dispersion protocol were not provided. Blood was collected at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h post-dosing and Ti concentrations measured by ICP-MS/MS following microwave-assisted acid digestion of whole-blood samples using H_2SO_4. This method accounts for the bioavailability of particulate TiO_2 as well as any dissolved Ti. Blood Ti concentrations of vehicle-control treated rats were highly variable with several males considered statistical outliers and excluded from data processing. The mean blood Ti concentrations of male and female rats were below 0.2 µg Ti/g blood following oral administration of all test articles. Administration of the soluble Ti reference resulted in blood Ti concentrations up to 90 µg/g blood and 0.9 µg/g blood following i.v. and oral dosing, respectively. The highest blood Ti concentrations following oral dosing were observed in the group that received the food-grade TiO_2 test item E171-E. The resulting areas under the curve (AUC) were plotted for rats exposed to the reference substance orally or by i.v. and compared to the AUC of rats that received E171-E by oral administration. The maximum relative oral bioavailability of E171-E was determined to be 0.0013% (rounded to one significant figure for the purpose of this report). The measured blood Ti levels of the other four forms of TiO_2 were below the LOD after background correction (LOD not stated). The authors concluded that oral bioavailability of all TiO_2 grades tested was close to the LOD of the analytical system. They also stated that most reagents used in the process contain low but measurable background concentrations of Ti, which makes analysis of low levels challenging. In addition, the authors reported that the background level of blood Ti in controls rats was highly variable, especially in males, which is consistent with time zero levels measured during dietary studies.

As part of a series of experiments, Bettini *et al.* (2017) examined the fate of TiO_2 in rats that were administered ultrasonicated particles in water (NANOGENOTOX dispersion protocol). In the first experiment, adult male Wistar rats (*n* = 10 per group) received a vehicle control (water) or 10 mg/kg bw/d of food-grade TiO_2 (E171) by intragastric gavage for 7 days. Confocal microscopy imaging using laser

diffraction to detect light-diffracting TiO_2 particles and cell autofluorescence to detect cellular structures were used to examine the fate of TiO_2 along the gut-liver axis. In an additional experiment, the agglomeration state of particles recovered from the luminal contents of the jejunum and colon of rats (*n* = 4 males) 4 h after receiving a single gavage dose of 10 mg/kg bw E171 were examined. The authors report that light-diffracting TiO_2 particles were found in the Peyer's patches of the small intestine, the colonic mucosa and the liver of rats receiving E171 for 7 days, but not in the controls. They also state that in comparison with the initial bolus, the dispersed particles did not re-agglomerate in the gut or during intestinal transit. In images reported from this experiment, a large number of laser-reflective particles are observed in the TiO_2 test suspension prior to gavage, as well as along the intestine in the luminal content of the jejunum. A much lower number of laser-reflective particles are observed in the colon. Based on other evidence reported in this study, it is likely that the observed laser-reflective particles are accurately identified as TiO_2. However, it is noteworthy that the spatial resolution of the methodology used is insufficient to demonstrate the agglomeration state of the imaged particles. TEM images of the colonic mucosa show the presence of E171 aggregates (>200 nm in size), but do not clearly indicate the presence of deagglomerated NPs. It is also worth noting the dispersed particles were stabilized with BSA, which is known to induce changes in binding affinity both among particles and with other ligands, and thus may not fully reflect particle disposition under typical conditions of dietary exposure.

Talamini and colleagues (2019) also investigated whether E171 administered to mice via the oral route would lead to accumulation in tissues. Male mice were treated with either vehicle or E171 in water (without deagglomeration to better simulate human exposure) at 5 mg/kg bw/d, three days per week for three weeks. Following terminal sacrifice three days after the last dose was administered, total Ti concentrations were measured in tissues of four mice from each group by triple quadrupole inductively coupled plasma mass spectrometry (ICP-MS) and E171 and TiO2 particles from the large intestine were also measured by single particle ICP-MS (sp-ICP-MS). In mice administered E171, significant increases in Ti content in the liver and large intestine were observed (1.8 and 3.6 fold increases versus control, respectively). In the large intestine, particles were determined to be primarily micro-scale although a detectable proportion of TiO_2-NPs was also observed (single particle analysis was undertaken in large intestine samples only). Additional details of this study are reported in the section on 'Immunotoxicity, Inflammation and Effects on the GIT' below.

Coméra *et al.* (2020) also examined absorption kinetics after a single dose of 40 mg E171/kg bw (dispersed in water following the NANOGENOTOX dispersion protocol) in fasted C57BL/6 mice (sex not specified). The presence of TiO_2 particles in segments of the gut (jejunum, ileum and colon) and in the blood were measured by confocal microscopy or TEM following administration of the test article or vehicle control. The authors estimate that approximately 0.007% of the administered dose was absorbed in jujenal and ileal villi at the 4 h time point, although levels returned to baseline at 8 h and relatively little absorption occurred in the colon. In Peyer's patches, levels were undetectable at 4 h but significantly increased at 8 h. Laser-reflecting confocal microscopy was used to detect reflective particles in blood that were presumed to be indicative of TiO_2 particles; reflective particles were observed at baseline but were significantly increased by 3.5- and 4.1-fold at 4 and 8 h, respectively. However, blood Ti as quantified by ICP-MS remained below the LOD (< 0.02 mg Ti/kg) at both time points. In a second experiment by the same authors, *ex vivo* jujenal loops of anaesthetized mice were pre-treated with a phosphate-buffered saline (PBS) control or one of the following pharmacological inhibitors: 100 mM (2,4,6-

triaminopyrimidine) (TAP, an inhibitor of paracellular tight junction permeability); 100 µM of 5-(N-ethyl-N-isopropyl) amiloride (EIPA, to inhibit micropinocytosis); 30 µM Pitstop 2 (to block clathrin-mediated endocytosis) or 17 µM methyl-β-cyclodextrin (MβCD, to inhibit raft-dependent endocytosis). After 30 minutes of incubation, the lumen contents were rinsed and replaced with PBS with or without 300 µg/ml of sonicated E171 and further incubated for 30 minutes. Inhibition of the paracellular pathway by TAP reduced absorption of E171 particles by 66% relative to controls, whereas inhibition of endocytosis showed only slight but non-significant decreases, suggesting only a minor contribution of transcellular transport. There are, however, some design issues with intestinal loop studies that complicate interpretation of results. These include disruption of the gastrointestinal mucous barrier by multiple rinsings and the lack of a food matrix.

Several studies have also investigated the fate of orally ingested TiO_2-NPs. Cho and colleagues (2013) investigated the absorption, distribution, and excretion of TiO_2-NPs in male and female Sprague Dawley rats (*n* = 11/group). The particles (80% anatase/20% rutile phase TiO_2 with a mean diameter of 21 nm) used for testing appear to be similar to P25 (NM-105) and were suspended in distilled water (details of the suspension protocol are not reported). Rats were treated by oral gavage with either a distilled water control or TiO_2-NPs at doses of 260.4, 520.8, or 1041.5 mg/kg bw/d for 90 days. Five animals per group were randomly selected and placed in metabolic cages immediately after gavage and urine and feces were collected for 24 h. Upon terminal sacrifice, blood as well as tissue samples from the liver, spleen, kidney, and brain were collected and analyzed for Ti content by ICP-MS (method detection limit = 0.1-1 ng/L). Following 90 days of exposure there was no significant increase in the Ti content in sampled organs, even at the highest dose tested, and no evidence of a dose response. Very high concentrations of TiO_2 were detected in feces, with no significant increase in urine (levels in feces were approximately 4 orders of magnitude higher than urinary levels). The authors concluded that TiO_2-NPs were minimally absorbed in the rat, even after repeated dosing at levels up to several orders of magnitude higher than human exposures, with no significant systemic translocation through the GIT.

Geraets *et al.* (2014) exposed male Wistar rats (*n* = 3/group) by oral gavage to one of 4 different TiO_2-NPs for either 1 or 5 consecutive days at a dose of 2.3 mg/rat/d (corresponding to doses of 6.8-8.6 mg/kg bw/d). The test articles were NM-101 (anatase; 6 nm primary particle size), NM-102 (anatase; 20 nm primary particle size); NM-103 (rutile, 20 nm primary particle size, coated with Al_2O_3 and a polysiloxane polymer layer) and NM-104 (rutile, 20 nm primary particle size, coated with Al_2O_3). The particles were dispersed in ethanol and rat serum albumin by probe sonication and diluted to their final concentration in 10% PBS; control males and females received vehicle only. The rats were sacrificed 24 h after the last exposure (day 2 or day 6) and liver, spleen and mesenteric lymph nodes (MLN) were collected. Tissue samples were analyzed for the presence of Ti by high-resolution ICP-MS. Of the 30 liver/spleen samples of exposed animals, one liver sample (NM-102 group) was at the LOD and 1 liver sample (NM-103 group) was above the LOD but below the LOQ; the LOD and LOQ were 0.03 µg Ti/g tissue and 0.09 µg Ti/g tissue, respectively. No Ti levels above the LOD were detected in spleen of any exposed animals, although of the three untreated controls, one had a Ti concentration at the LOD in both the spleen and liver. All MLN samples contained Ti concentrations above the LOD although only in the NM-104 group were levels in exposed animals greater than unexposed controls. In this group, the total MLN Ti content was estimated to represent approximately 0.003% of the administered dose, which was considered a worst case scenario, although it should be noted that this is a fine (~24 nm) coated cosmetic-grade rutile particle and

thus not representative of TiO_2 as used in food. Based on all nanomaterials and all organs tested, the authors estimated approximately 0.02% of the administered dose was absorbed and therefore concluded that TiO_2-NPs have very limited oral bioavailability in the rat. Similar results were reported by Janer *et al.* (2014) and MacNicoll *et al.* (2015); the results of both these studies indicated that Ti content of blood, tissues and internal organs were not significantly different from controls following oral administration of TiO_2-NPs (additional details may be found in Appendix E).

Given their insolubility in biological matrices and lack of known excretory mechanisms for systemically absorbed particles, concern has been raised that the absorbed fraction, while small, may persist and accumulate in tissues following chronic exposure. However, high variability in TiO_2 intake and background (basal) tissue levels of Ti in both humans and laboratory animals complicates accurate determination of kinetic parameters such as the elimination half-life (EFSA 2021a). In their recent opinion, the EFSA FAF Panel (2021a) identified the potential for TiO_2-NPs to accumulate in liver, lung and spleen with half-lives ranging from 83 days in liver and lung, 350 days for spleen and 450 days for whole body. These estimates, however, are based on intravenous injection of TiO_2-NPs in rats and are extrapolated from the last sampling time point at 28 d. There is inherent uncertainty given the length of the sampling period relative to the estimated half-lives and the fact that no experimental study could appropriately measure a 450-day half-life in rodents. It has been recommended that the sampling scheme duration should be at least 4 times the average measured half-life in order to have confidence in the reported half-life values (Colucci *et al.* 2011). In this case, however, the estimated half-life of 450 days is 16-fold greater than the sampling duration of 28 days.

The biokinetics of orally and intravenously administered TiO_2-NPs in female Wistar-Kyoto rats were investigated by Kreyling and colleagues (2017a,b) in two vanadium (V) radiotracer studies. Following dispersion, anatase NPs formed approximately spherical aggregated/agglomerated entities of roughly 50 nm in diameter that consisted of smaller primary particles with a diameter of 7 – 10 nm. Suspensions of [^{48}V]TiO_2-NPs were administered to non-fasted rats in a single bolus by oral gavage in the range of 30–80 µg/kg bw or in a single intravenous injection in the range of 40 to 400 µg/kg bw. Rats were housed individually in metabolism cages for collection of urine and feces; at 1 h, 4 h, 24 h, and 7 days post-dose, four rats per time point for each route of exposure were sacrificed and ^{48}V radioactivity was measured in blood, organs, tissues and all excreta. An additional four rats were sacrificed at 28 days in the intravenous experiment only. In the oral kinetics study (Kreyling *et al.* 2017a), almost all retained [^{48}V]TiO_2-NP beyond the gut distributed to the organ free carcass, with only small and heterogeneous fractions observed in organs such as liver and spleen, whereas the liver collected virtually all [^{48}V]TiO_2-NPs following intravenous administration. The authors noted this is likely due to the observation that orally ingested TiO_2 is taken up and translocated via the lymphatics, where it may be retained in lymph nodes and only slowly released into the blood (Kreying *et al.* 2017a). Assuming ^{48}V is a reliable surrogate marker for TiO_2-NPs, approximately 0.6% of the administered dose was absorbed in the first hour after gavage, and the concentration declined to 0.05% after 7 days. The rapid decline of the retained fraction from 0.6% after 1 h to 0.2% after 4 h indicates the existence of active early clearance mechanisms. However, low but detectable levels persisted in organs such as the brain and uterus seven days after dosing, suggesting the potential for bioaccumulation of TiOs-NPs under conditions of repeated exposure, assuming these measures are not confounded by the leaching of soluble ^{48}V from ^{48}V-labelled TiO_2 particles. The authors note that studies on long-term biokinetics following repeated oral doses are still lacking. When comparing

the oral and intravenous kinetics of TiO_2-NPs in the rat, strikingly different biokinetics were apparent, leading the authors to conclude that "intravenous injection appears not to be an adequate surrogate for assessing the biodistribution and potential health effects occurring after oral exposure to TiO_2 nanoparticles" (Kreyling *et al.* 2017a).

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 how humans are exposed 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.

Studies in Humans

The JECFA (1969) evaluation of TiO_2 references an unpublished report from the chemical industry in which five male volunteers ingested 5 g of food-grade TiO_2 suspended in milk for three consecutive days (West and Wyzan 1963, as cited in JECFA 1969). Urine was collected for five days and no increases in urinary Ti levels were detected, from which the Panel concluded that no significant absorption of Ti occurred. However, significant technological advances have improved analytical capacity to detect Ti in biological matrices since that time and urine does not appear to be a significant route of Ti excretion (Cho *et al.* 2013). Pele *et al.* (2015) more recently investigated the oral bioavailability of food-grade TiO_2. Healthy volunteers with normal intestinal permeability (n = 7) ingested capsules containing 100 mg of food-grade TiO_2 (anatase, D_{50} of 260 nm) in a fasted stated (participants were offered breakfast and lunch 1 h and 4 h post-dose, respectively, and although the Ti content of the meals was not quantified, they were reported to be "free from added TiO_2"). Blood samples were collected at baseline as well as at intervals up to 10 h post-ingestion and analyzed for TiO_2 particles semi-quantitatively using dark field microscopy as well as for total Ti by ICP-MS. Findings from dark-field microscopy roughly mirrored Ti measurements by ICP-MS in the same samples. Significant increases in blood Ti were observed from 2 h onwards, peaking at 6 h. The highest blood Ti level observed at the 6 h time point was approximately 11 ng/ml, compared to an average baseline level of roughly 2 ng/ml, although the size of particles absorbed was not quantified.

Jones *et al.* (2015) conducted a study in humans to compare the absorption of TiO_2-NPs with that of larger particles. Human volunteers (four adult males and five adult females) received single oral doses of 5 mg/kg bw TiO_2 (equivalent to 300 mg for a 60-kg individual) consisting of various particle sizes dispersed in water: 15 nm (anatase; ~ 100% by number < 50 nm), 100 nm (rutile; 95% by number between 48 and 154 nm) and < 5000 nm (rutile; 100% by number > 100 nm). Doses were administered at least 4 weeks apart and serial blood (baseline, 2, 4, 24 and 48 h post-dose) and urine samples (24 h baseline up to 72 h post-dose) were collected. Titanium content in blood and urine was measured by ICP-MS. No significant differences between pre- and post-dose biomarker levels were observed for any particle size, and neither blood nor urine measurements yielded curves indicative of absorption and/or elimination. Interestingly, the average baseline blood Ti level in the Jones *et al.* study was 13.7 ng/ml, which is greater than the peak level

observed post-ingestion in Pele *et al.* (2015); whether or not TiO_2 was administered in a fasted state in the Jones *et al.* study is not stated. However, the baseline Ti levels in this study appear unusually high, as recent investigations using high resolution ICP-MS consistently indicate basal Ti levels in serum and whole blood are less than 1 ng/ml (Balcaen *et al.* 2014; Swiatkowska *et al.* 2019), suggesting the possibility of contamination or interference(s). Swiatkowska *et al.* (2019) also cautioned that standard ICP-MS, which was used by both Pele *et al.* (2015) and Jones *et al.* (2015) to quantify blood Ti, is not an appropriate method to measure Ti in biological samples, due to a range of polyatomic and isobaric interferences.

The maternal-fetal transfer of TiO_2 particles during pregnancy was investigated by Guillard *et al.* (2020) by analyzing the Ti content of the placenta and meconium as well as through an *ex vivo* perfusion model to study transplacental passage of food-grade TiO_2. In the first study, full-term placentae were collected from 22 uncomplicated pregnancies in Toulouse, France. Meconium samples (n = 18) were also collected in the first few days after birth, although these samples were not from the same infants associated with placenta collection. Ti was detected in all placenta samples with concentrations ranging from 0.01 to 0.48 mg/kg tissue. The size distribution of Ti particles in the placenta (n = 2) was characterized using TEM with Energy Dispersive X-ray analysis (TEM-EDX) and the majority of particles (17 of 26) were determined to be below 100 nm. In meconium, Ti was detected by ICP-MS in nine of 18 samples, with concentrations ranging from 0.02 to 1.50 mg/kg tissue. Size analysis by TEM-EDX of 33 particles from two meconium samples determined 82% were in the nano-range (<100 nm), although this included particles of Si, Al, Fe and Zn in addition to Ti. The EFSA FAF Panel (2021a) also noted that the authors did not control for the possibility that Ti particles in meconium may have come from the diapers, diaper cream or from mother's milk as infants had nursed prior to collection. In the second experiment, the authors used an *ex vivo* placental profusion model to characterize transplacental transfer of E171. Placentae were perfused with (n = 13) or without (n = 2) E171 at a concentration of 15 µg/L for 1 h with samples collected from the fetal flow every 5 minutes. Following perfusion, Ti was detected by ICP-MS in most fetal exudates (0.41 to 3.46 ng Ti/mL), although 92% were in the range of the blank level (0.33 to 1.92 ng Ti/mL). The method did not allow for quantification of the total number of particles in fetal side exudate in which TiO_2 particles increased from 10 minutes after E171 perfusion, reaching a plateau at 20-30 minutes. Pooled liquid fractions of placental exudate 20 – 30 minutes after perfusion with E171 were analyzed by SEM-EDX. A total of 300 TiO_2 particles were sampled and measured by taking the smallest dimension of each particle within agglomerates. With the exception of three particles all had a diameter less than 200 nm and 70 and 100% were in the nanoscale in two perfusion experiments, respectively.

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). Heringa and colleagues (2018) investigated the human organ burden of nano- and micro-scale TiO_2 particles in 15 post-mortem human livers and spleens (six men and nine women, aged 56 to 104 years). In the liver and spleen, total-Ti content ranged from 0.02 to 0.09 mg Ti/kg tissue (mean 0.04 ± 0.02 mg/kg) and 0.02 to 0.4 mg Ti/kg tissue (mean 0.08 ± 0.1 mg Ti/kg), respectively. Ti content was below the blank-corrected LOD (0.01 mg/kg tissue) in eight of 15 liver samples and one of 15 spleen samples. Particle size ranged from 85-550 nm in the liver and 85-720 nm in the spleen, with 24% of TiO_2 particles <100 nm, although this is likely an

underestimate as the spICP-HRMS method used for particle size characterization had an LOD of 85 nm. Using SEM-EDX, the authors determined that the larger particles consisted of agglomerates of smaller primary particles in the 75-150 nm range. Based on total Ti concentrations and the maximum Ti concentrations in particles, and taking into account the LOD_{size} of 85 nm, it was assumed that all Ti was present as particles. Within subjects, no correlation was observed between the Ti particle burden in liver and spleen. Two subjects had Ti implants (type not stated) although these individuals did not have higher Ti or TiO_2 particle levels in tissues.

Systemic uptake of TiO_2 was also evaluated in post-mortem tissue samples by Peters and colleagues (2020). Samples of liver, spleen and kidney were obtained from seven men and eight women who died at the age of 64–98 years; parts of the jejunum and ileum were also examined in a subset of 12 individuals. Ti was below the LOD (0.01 mg/kg tissue) in four liver samples, two spleen samples, one kidney sample and one jejunum sample. For tissues where Ti was present above the LOD, median values were: 0.02 (liver), 0.04 (spleen), 0.05 (kidney), 0.13 (jejunum), and 0.26 mg/kg tissue (ileum) of which particulate TiO_2 accounted for about 80% of the total Ti concentration. Primary particles, aggregates and agglomerates were identified that ranged in diameter from 50 – 500 nm, with a mode of approximately 100 – 160 nm; approximately 17% of the detected TiO_2 particles had a diameter <50 - 100 nm. The smallest detectable TiO_2 particle size was 50 nm in this spICP-HRMS method. Given the age of the subjects, it is reasonable to assume that steady state levels had been achieved in organs, and no association of particle burden with age was observed.

Additional details on studies that inform the toxicokinetics of TiO_2 in humans and experimental animals may be found in Appendix E.

7. Toxicity

Acute Toxicity

TiO_2 particles are of low acute oral toxicity in animal studies. Two OECD guideline-compliant studies were identified in the published literature, one in mice (Wang *et al.* 2007; crystalline structure not specified, 25, 80 and 155 nm particles) and one in rats (Warheit *et al.* 2007; 79% rutile and 21% anatase, median particle size 140 nm). Both studies estimated an LD_{50} effect level of greater than 5000 mg/kg bw, which was the highest dose tested. An additional study that investigated the acute oral toxicity of TiO_2-NPs (average size 28.3 ± 3.1 nm) according to the OECD Guideline 420 (Fixed Dose Method) observed no mortality at the highest dose tested of 2000 mg/kg bw (studies summarized in Appendix F). Four additional, unpublished study reports are also summarized in the European Chemicals Agency (ECHA) dossier for TiO_2, although basic information on the test article (crystalline form, size, purity, surface treatments, etc.) and dispersion methods is generally lacking. These reports consistently demonstrated that the acute oral LD_{50} of TiO_2 particles was greater than the highest dose tested in all cases, which included a 1971 study in which male ChR-CD rats were administered TiO_2 by oral gavage at doses up to 25,000 mg/kg bw. Under many classification schemes for acute toxicity (such as the GHS[18]), substances

[18] The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) is a system developed by the United Nations to standardize the classification and labelling of chemicals on a global basis.

with an acute oral LD_{50} of greater than 5000 mg/kg bw are regarded as being practically non-toxic or of low acute toxicity via the oral route.

Carcinogenicity and Chronic Toxicity

Most of the available carcinogenicity research on TiO_2 pertains to exposure via the inhalation route in occupational settings. The International Agency for Research on Cancer (IARC) classified TiO_2 as possibly carcinogenic to humans (Group 2B) based on sufficient evidence of carcinogenicity in experimental animals (i.e., increased incidence of lung tumours in rodents following long-term inhalation exposure). At the same time, IARC noted there was inadequate evidence in humans to assess the carcinogenicity of TiO_2 (IARC 2010). While many other international reviews/regulations related to the carcinogenic potential of TiO_2 via the inhalation route have been published (e.g., most recently by the EU[19]), inhalation exposure to food-grade TiO_2 outside of occupational settings is not expected in the general population nor is it relevant to dietary exposure and therefore, it will not be considered further in this document.

No oral carcinogenicity or chronic toxicity studies with food-grade TiO_2 were identified in the published literature. However, one oral cancer bioassay with Unitane® 0-220 (a test article consistent with the current forms of food-grade TiO_2) was identified (NCI 1979). Three different lots of Unitane® 0-220 were used for this bioassay. According to the identity/purity reports obtained from the National Toxicology Program (NTP), all three lots of Unitane® 0-220 were described by the manufacturer (American Cyanamid Company, Wayne, New Jersey) as anatase with 98% minimum TiO_2 and met the United States Food and Drug Administration (FDA) food-grade specifications[20] (MRI 1974; 1975a,b). The elemental analysis conducted by the Midwest Research Institute (Kansas City, Missouri) confirmed all lots contained low levels of impurities of the trace elements aluminum, phosphorous, and silicon in the 0.1-1.0% range (MRI 1974; 1975a,b), which is consistent with the elemental composition of food-grade TiO_2 (Dudefoi *et al.* 2017a). Based on a draft report obtained from the chemical industry, Unitane 0-220 had a D_{50} by number ranging from 113 to 135 nm when analyzed by SEM and 109 to 124 nm when analyzed TEM, a mass-specific surface area ranging from 8.4 to 9 m^2/g, and was found to contain a significant portion of particles on the nanoscale (20-40% and 26-44% of particles < 100 nm by number as determined by SEM and TEM, respectively; TDMA 2022a), which is consistent with the D_{50} by number, specific surface area, and portion of NPs in the current forms of food-grade TiO_2 (Dudefoi *et al.* 2017a; EFSA 2019b; 2021a; Verleysen *et al.* 2020; 2021; Geiss *et al.* 2020; 2021). The EFSA ANS Panel (2016) reported a slightly higher arithmetic mean diameter for Unitane® 0-220, which ranged from 180 to 320 nm, although no analytical method was specified and this may be an inference based on its use as a white pigment. Similarly, Winkler *et al.* (2018) inferred from the optical properties of Unitane® 0-220 that a significant portion of particles had a mean diameter in the 200-300 nm range conferring a white colour. Overall, given that manufacturing methods for food-grade TiO_2 have not changed significantly over time, the information presented above supports that Unitane® 0-220 is comparable to the current form of TiO_2 added in food and therefore, it is considered an appropriate test article for characterizing the potential health hazards associated with oral exposure to food-grade TiO_2.

[19] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2020:044:FULL&from=FR
[20] FDA Regulation 8.316.

Unitane® 0-220 was evaluated in a two-year cancer bioassay with male and female B6C3F1 mice and Fischer 344 rats (NCI 1979), which was conducted according to the 1976 NCI guidelines for carcinogenicity testing in small rodents (Sontag *et al.* 1976)[21]. The dietary concentrations selected for the cancer bioassay were based on a 13-week range-finding study in which male and female B6C3F1 mice and Fischer 344 rats (*n* = 10 per sex per group) were exposed to Unitane® 0-220 at levels up to 100,000 ppm in the diet without evidence of adverse effects[22]. Based on these findings, the NCI set the highest dietary concentration at 50,000 ppm (5% w/w), which was the "maximum amount allowed for use in chronic bioassays in the Carcinogenesis Testing Program", and the lowest dietary concentration was set at 25,000 ppm (2.5% w/w). Animals (*n* = 50 per sex per group) were exposed to Unitane® 0-220 incorporated in the diet for 103 weeks at concentrations of 0, 25,000 or 50,000 ppm (equivalent to doses of 0, 3250 or 6500 mg/kg bw/d and 0, 4175 or 8350 mg TiO_2/kg bw/d for male and female mice, respectively, and doses of 0, 1125 or 2250 mg/kg bw/d and 0, 1450 or 2900 mg/kg bw/d for male and female rats, respectively)[23]. Surviving animals were euthanized on week 104 and a full histopathological evaluation of major tissues/organs as well as all gross lesions was completed. In female mice, a statistically significant and dose-related trend in mortality was observed, with 45/50 (90%) surviving to 104 weeks in the control group versus 39/50 (78%) and 33/50 (66%) in the low- and high-dose groups, respectively. In treated animals, increased incidences of several non-neoplastic lesions also occurred in both sexes of both species, but were not considered treatment-related by the study directors, who concluded, "there was essentially no evidence of toxicity of titanium dioxide in the dosed rats or dosed mice".

An increased incidence of neoplastic lesions was observed in treated male (pheochromocytomas of the adrenal medulla and fibromas of the subcutaneous tissue) and female rats (endometrial stromal polyps) relative to concurrent controls, but was considered by the study directors to be within the range of historical controls of the same strain and age (NCI 1979). A statistically significant Cochran-Armitage test for a positive dose-related trend in the incidence of keratoacanthomas in male rats was also reported (0/49, 0/50, 3/50); however, in a separate statistical analysis, the study directors noted that the increase in the high-dose group did not achieve statistical significance and therefore, was not considered treatment-related. A statistically significant Cochran-Armitage test for a positive dose-related trend in the incidence of C-cell adenomas or carcinomas of the thyroid was also observed in female rats (1/48, 0/47, 6/44), but the incidence of these tumours was not statistically significant from concurrent controls and the study directors did not consider the effect to be treatment-related. TiO_2 is also poorly absorbed via the oral route with very low levels of TiO_2 particles detected systemically in experimental animals and human cadavers (see 'Toxicokinetics' section above for details), and therefore, the biological plausibility of neoplasia outside of the GIT in rats is not clear at this time. A slight increase in the incidence of hepatocellular carcinomas was also observed in male mice in the high-dose group relative to concurrent controls, but was considered by the study directors to be within the range of normal background lesions

[21] Although the 1976 NCI guidelines for carcinogenicity predate the modern (OECD) and International Council for Harmonisation (ICH) test guidelines, many elements are similar with the biggest difference being the recommendation that studies are conducted with three dose groups instead of two dose groups (Hayes *et al.* 2011; Hentz 2010).

[22] 13-week range-finding study described in NCI (1979).

[23] Calculated by the EFSA ANS Panel (2016) according to guidance on converting chemical substance concentrations in feed to daily doses in experimental animals (see EFSA 2012 for more details).

recorded in mice of the same strain and age. The study directors reported no other treatment-related differences in the incidence of neoplasia between treated mice of either sex and concurrent controls and no dose-response relationship/trend was observed for any lesion, including neoplastic lesions in the intestinal tract. Based on these findings, the study directors concluded that dietary exposure to Unitane® 0-220 was not carcinogenic to male or female B6C3F1 mice or Fischer 344 rats at concentrations up to 50,000 ppm diet (5% w/w), which was the highest dietary concentration tested[24]. Since Unitane® 0-220 is highly comparable to the current form of TiO_2 found in food, the above findings also provide strong support that chronic exposure to food-grade TiO_2 is not carcinogenic in male and female mice and rats when administered at very high concentrations in the diet (5% w/w), equivalent to doses of 2250 to 8359 mg/kg bw/d depending on sex and species.

Preneoplastic Lesions in the Colon

Six studies were identified in the published literature that investigated the potential for food-grade TiO_2 to induce the formation of preneoplastic lesions in the GIT. Three of these studies were published by a research group from the Universidad Nacional Autonoma de México and Maastricht University (Urrutia-Ortega *et al.* 2016; Medina-Reyes *et al.* 2020; Proquin *et al.* 2018a). These studies were not guideline-compliant and evaluated the potential for subchronic exposure to E171 dispersed by sonication in drinking water or by oral gavage to induce the formation of intestinal tumours and other markers of tumour progression. However, due to limited reporting of study details as well as some identified limitations in the study methodology and/or design, none were considered appropriate for inclusion in the present assessment. The main concerns with Urrutia-Ortega *et al.* (2016) pertained to the lack of a vehicle control for the gavage administration of E171, the administration of a single E171 dose level, the lack of individual incidence data reported for the neoplastic findings, and uncertainty as to whether some of the histopathological and molecular evaluations were standardized due to inadequate descriptions of study methodology. For the Medina-Reyes *et al.* (2020) study, the evaluation of intestinal adenomas appeared to have been conducted via gross observation, volume was used to measure adenoma size making it is difficult to interpret these findings without subsequent histological or morphometric evaluations, the incidence of intestinal adenomas in control mice fed a regular diet after 16 weeks of treatment was significantly higher than those previously reported in mice of the same sex and strain at 24 months (38% versus 4%; Brayton *et al.* 2012), and the observation of increased goblet cells in E171 treated animals (which is evidence of an anti-tumorigenic effect) is inconsistent with the observation of intestinal tumours. The results of the Proquin *et al.* (2018a) study were also difficult to interpret given the limited description of alterations in crypt architecture at 7 days as well as the low number of mice in each experimental condition (n = 2 per sex per group per time point).

Of the remaining three studies identified in the literature, two were non-guideline subchronic studies that administered E171 to rodents either in drinking water (Bettini *et al.* 2017) or via the diet (Blevins *et al.*

[24] A reviewer of the cancer bioassay noted the potential significance of the increased incidence of C-cell adenomas or carcinomas of the thyroid in female rats and recommended revising the conclusion to reflect the "equivocal findings in female rats". A recommendation to revise the conclusion to state, "no firm conclusion can be reached about the possible carcinogenicity of [Unitane® 0-220] to Fischer 344 rats" received no objection from the external review panel, although it does not appear that the study directors modified their conclusion. It was noted by an NCI staff member that the "incidence of C-cell tumors of the thyroid was not an unexpected finding in the Fischer rat".

2019) whereas the third was performed in accordance with OECD guideline 408 (repeated dose 90-day oral toxicity study) in which E171 was administered to rats by oral gavage (Han *et al.* 2020). A seventh, unpublished GLP- and OECD 443 guideline-compliant EOGRT study, which was commissioned by industry to address endpoints of concern raised by the EFSA ANS Panel (2016; 2018a) and NVWA (2019), investigated the potential initiating effects of undispersed E171 in the colon using a dietary model (LPT 2020 as cited in EFSA 2021a).

Bettini *et al.* (2017) investigated the potential for subchronic exposure to E171 when dispersed in drinking water by ultrasonication to initiate and promote the formation of preneoplastic lesions in the colon of adult male Wistar rats. Additional experiments were performed by Bettini *et al.* (2017) and are described elsewhere in this report (see the 'Toxicokinetics', 'Genotoxicity' and 'Immunotoxicity, Inflammation and Effects on the GIT' sections). To examine the potential promoting effects of E171, rats (*n* = 12 per group) were pre-treated with 1,2-dimethylhydrazine (DMH; 180 mg/kg i.p. in 9 g/L NaCl) to initiate colon carcinogenesis seven days prior to E171 exposure at nominal doses of 0, 200 µg or 10 mg/kg bw/d in drinking water for 100 days. To evaluate the potential initiating effects of E171, a second group of rats were exposed to nominal doses of 0 (control, *n* = 12) or 10 mg/kg bw/d E171 (*n* = 11) in drinking water for 100 days without pre-treatment with DMH. A French commercial supplier of food colouring provided the samples of E171 used in the above experiments. The E171 sample was primarily composed of anatase, had a median particle diameter of 118±53 nm (range 20 to 340 nm) with 44.7% of particles < 100 nm as determined by TEM, and was "close to purity" based on TEM-EDX analysis. The E171 particles were dispersed in drinking water by ultrasonication using the NANOGENOTOX dispersion protocol (i.e., sonication for 16 minutes at 20 kHz in a 0.05% w/v BSA). The E171 drinking water dispersions were prepared two to three times per week and the water bottles were shaken by inversion three times per day to avoid the sedimentation of E171 in the bottles (Dr. Houdeau, *pers. comm.* 15 February 2022). The stability of the E171 drinking water dispersions and stock solutions were analyzed in triplicate by DLS over an 8-day period and the hydrodynamic diameter of E171 was reported to be stable, ranging from 297.4 to 407.9 nm and 322.3 to 383.4 nm across drinking water and stock solution samples, respectively (Dr. Houdeau, *pers. comm.* 15 February 2022). Control rats were exposed to drinking water mixed with ~5 ng/mL BSA to account for the addition of BSA in the E171 dispersions (Dr. Houdeau, *pers. comm.* 15 February 2022). For both experiments, rats from the same dose group were housed together in groups of three and the E171 dose administered was approximated based on the mean body weights of the rats per cage and the average water consumption over the 100-day period (~22-23 mL/day/rat; Dr. Houdeau, *pers. comm.* 15 February 2022). The nominal concentration of E171 in drinking water for both E171 dose levels was adjusted every 15 days to account for changes in the mean body weight per cage, ranging from 0.0036 to 0.0057 mg/mL (mean 0.0049 mg/mL) and 0.179 to 0.286 mg/mL (mean 0.247 mg/mL) for the 200 µg and 10 mg/kg bw/d doses, respectively (Dr. Houdeau, *pers. comm.* 15 February 2022). The E171 doses varied slightly over the course of the 100-day experiment with mean exposure doses of 225 µg/kg bw/d (range 200 to 250 µg/kg bw/d) and 11.97 mg/kg bw/d (range 10.65 to 13.84 mg/kg bw/d) for the low- and high-dose E171 groups, respectively (Dr. Houdeau, *pers. comm.* 15 February 2022). The low and high doses used in both experiments were selected to approximate dietary exposure to E171 in adults and children, respectively.

For both experiments, the colons were removed at the end of the 100-day treatment period, stained with methylene blue (0.1%) for 6 minutes and the entire epithelial surface was examined for aberrant crypts

(ABCs) and ACF at 40x magnification using a light microscope by two readers blinded to dose group (Dr. Houdeau, *pers. comm.* 15 February 2022) based on the procedure outlined by Bird (1987)[25]. ABCs/ACF were first identified in mice by Bird (1987) as the earliest morphologic precursor lesion in the colon following treatment with a colon carcinogen and they have since been identified in humans (e.g., Pretlow *et al.* 1991); although the predictive value of ACF as a biomarker of colorectal cancer in rodents and humans is controversial (see Appendix G for more information of ACF as a potential biomarker of colorectal cancer). In rats pre-treated with the genotoxic carcinogen DMH and subsequently exposed to dispersed E171, a statistically significant increase in the mean number of ABCs and large ACF per colon (defined as >3 ABCs per ACF) were observed in the high-dose E171 group relative to DMH-only controls (ABCs: ~540 versus ~460, respectively and large ACF: ~45 versus ~35, respectively). No statistically significant differences in the mean number of ABCs or large ACF per colon were observed between the low-dose E171 group and the DMH-only controls. The mean number of ACF per colon did not achieve statistical significance, with all three treatments generating ~200 ACF per colon. Based on the above results, Bettini *et al.* (2017) concluded E171 when dispersed in water by ultrasonication promoted the development of preneoplastic lesions in the form of ABCs and ACF in the colon of carcinogen-treated rats. However, the mean number of ACF per colon in the DMH-only controls was not different from the low- or high-dose E171 groups and while a statistically significant difference was observed between the mean numbers of large ACF per colon in DMH-only controls compared to the high-dose E171 group (~35 versus ~45), this difference was small in magnitude. Since very few of these lesions progress to neoplasia, with most lesions spontaneously regressing over time in rodents (Choi *et al.* 2015; Shpitz *et al.* 1996) and humans (Schoen *et al.* 2008) and considering DMH pre-treatment in rodents can result in inter-individual variability of approximately 20-40% in ACF formation (see Blevins *et al.* 2019; Rodrigues *et al.* 2002; Won *et al.* 2012 for examples of inter-individual variability within DMH treatment groups), the slight increase in large ACF per colon in the high-dose E171 group was not considered toxicologically significant.

In the absence of a colon carcinogenesis initiator, the spontaneous development of ACF was observed in four of the 11 rats exposed to dispersed E171 in drinking water (Bettini *et al.* 2017). In these rats, one to three ACF were observed per colon, of which no more than three ABCs were observed per ACF, with the exception of one ACF with 12 ABCs. No ACF were observed in the remaining seven rats treated with dispersed E171 or in controls. Based on these findings, Bettini *et al.* (2017) concluded that E171 when dispersed in drinking water by ultrasonication initiated preneoplastic lesions in the colon. However, the spontaneous formation of one to three ACF were only observed in the colon of approximately a third of the E171-treated rats (4/11; ~36%) of which only one ACF had greater than three ABCs per focus and therefore, met the authors' definition of a large ACF. The spontaneous formation of ACF is not an uncommon observation in control mice and rats in the absence of a colon carcinogenesis initiator despite colon cancer in these species being exceedingly rare. For example, Furukawa *et al.* (2002) reported that ~42.5% of untreated, male and female F344 rats spontaneously developed ACF in the colon over a 16-week period (~112 days), of which ~77% of ACF had 1-3 ABCs per focus and ~23% of ACF had ≥4 ABCs per focus, but the spontaneous development of colorectal tumours was found to be ≤ 0.34%. In another study, approximately 35 ACF spontaneously developed per colon in Wistar rats receiving saline by gavage for 90 days, with the majority of ACF comprised of ≤3 ABCs and only one or two ACF with ≥4 ABCs per focus; no tumours were reported (Singh *et al.* 2022). However, neither of these studies were likely long enough to

[25] ABCs were classified as larger and having a thicker epithelium compared to healthy colonic crypts, whereas ACF are defined as an aberrant focus with two or more ABCs (Bird 1987).

fully assess whether any of the ACF would progress to neoplasia nor were they directly comparable to the findings reported in Bettini *et al.* (2017) due to the difference in rat strains and dosing paradigms. In the Bettini *et al.* (2017) study, given that so few ACF were observed per colon (including only one large ACF with >4 ABCs) and considering most ACF do not progress to neoplasia, particularly ACF with three or fewer ABCs per focus (e.g., Alrawi *et al.* 2006a; Magnuson *et al.* 1993; Pretlow *et al.* 1992; Kristiansen 1996), the finding of ACF in the colon of E171-treated rats in the absence of a colon carcinogenesis initiator was not considered conclusive evidence of a potential toxicological hazard related to exposure to dispersed E171 in drinking water.

On the other hand, not all studies report finding ACF in control rodents in the absence of a colon carcinogenesis initiator (e.g., Rodrigues *et al.* 2002) and in Bettini *et al.* (2017), more convincingly, no ACF were observed in the concurrent control rats, which is considered the best comparison group. There is also evidence that at least some ACF may progress to adenomas and cancer over time (e.g. Shpitz *et al.* 1996), with some arguing that the clinical significance of the potential for a single ACF to progress to colorectal cancer must not be overlooked (Clapper *et al.* 2020); although, as mentioned above, the utility of ACF as a biomarker of colorectal cancer risk or progression in rodents and humans is controversial. Therefore, it is recognized that the Bettini *et al.* (2017) study provides evidence that subchronic oral exposure to E171 particles when intentionally dispersed via ultrasonication to release primary particles and reduce agglomerate size may initiate the formation of ACF in the rat colon; however, these experimental conditions are not fully representative of human dietary exposure to TiO_2 as a constituent of food. TiO_2 particles in solid and liquid food preparations are present in the form of larger agglomerates that have not been dispersed to the same degree and the available evidence does not indicate that TiO_2 in foodstuff deagglomerate or disassociate in the GIT (see the 'Particle Dispersion' and 'Particle Corona, Food Matrix and the Gastrointestinal Milieu' sections for more details). Health Canada's Food Directorate has also received confirmation from industry that sonication is not used to disperse TiO_2 in food or beverage manufacture (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022). Moreover, in Bettini *et al.* (2017), E171 was administered to the rats in drinking water, which does not account for the contribution of the food matrix. Proteins, sugars, and other macromolecules are present in both solid and liquid food preparations, which can interact with food-grade TiO_2 particles altering their physicochemical properties and influencing their interactions in the GIT (see the 'Particle Corona, Food Matrix and the Gastrointestinal Milieu' section for more details). As a result, the applicability of the sample preparation method and mode of exposure in Bettini *et al.* (2017) to human exposure to TiO_2 from foodstuff is uncertain and therefore, this study was considered to have unclear relevance to the hazard characterization of TiO_2 as a food additive.

The above interpretation of the Bettini *et al.* (2017) study is consistent with previously published opinions by other competent authorities, including ANSES (2017) and the EFSA ANS Panel (2018). The ANSES expert committee considered the increase in large ACF in rats pre-treated with DMH and subsequently exposed to E171 to be moderate, concluding that E171 may have a potential promoting effect in the colon (ANSES 2017). However, due to uncertainty in the predictive nature of ACF for colonic tumours and considering it was the only biomarker evaluated and the presence of ACF does not guarantee these lesions would progress to neoplasia, the expert committee recommended that the potential promoting effects of E171 be confirmed in experiments that evaluated additional biomarkers and/or had longer exposures to assess

whether E171 induced tumours in the colon. Concerning the potential initiating effects of E171, the expert committee considered the number of rats evaluated to be too low to determine the potential significance of this finding. Overall the expert committee acknowledged that the Bettini *et al.* (2017) study could identify potential hazards associated with E171, but questioned whether the experimental conditions tested in the study (i.e., the administration of E171 particles dispersed in drinking water by ultrasonication) were representative of E171 when used as a food additive and recommended additional studies with E171 be conducted to confirm these findings in a dietary matrix before the study could be used in risk assessment (ANSES 2017). Similarly, the EFSA ANS Panel (2018) noted that the use of a single biomarker (ACF) by Bettini *et al.* (2017) to evaluate the potential for E171 to induce preneoplastic lesions in the colon was a limitation and questioned the predictive nature of ACF for later tumour development. The ANS Panel also considered the appearance of ACF in the colon of a few E171-treated rats in the absence of a colon carcinogenesis initiator to be "unusual"; although, neither this finding nor the observation of ACF in carcinogen-treated rats also exposed to E171 were considered sufficient to raise concerns about the potential initiating or promoting effects of E171 on account of the negative two-year cancer bioassay in mice and rats conducted by the NCI (1979). The ANS Panel also concluded "the administration of TiO_2 (E171) by gavage or drinking water is not fully representative of the use of the food additive E171 in food" (EFSA 2018). Therefore, the relevance of the results of the Bettini *et al.* (2017) study to the assessment of E171 as a food additive is uncertain, because interactions with the food matrix may not have occurred.

The subchronic oral toxicity of E171 dispersed in an aqueous solution by ultrasonication was also investigated in an OECD 408 guideline-compliant repeated dose 90-day study (Han *et al.* 2020). Five-week-old male and female Sprague Dawley rats (n = 10 per sex per group) were exposed to E171 via oral gavage at doses of 0, 10, 100, or 1000 mg E171/kg bw/d in distilled water. The E171 particles (HOMBITAN® FG, purity 99.5%) had a single anatase phase with a diameter of approximately 150 nm as measured by TEM. E171 suspensions were prepared at least once per week by dispersing the particles in distilled water for at least 10 minutes by sonication (energy not specified), which were reported to be stable for 7 days when stored in a refrigerator (Dr. Yoon, *pers. comm.* 22 September 2021). Tissues and organs were collected during necropsy, stained with hematoxylin and eosin, and examined for macroscopic and microscopic abnormalities. No treatment-related abnormalities in organ weight, gross anatomy, or histopathology (including the gastrointestinal tract) were observed in male or female rats exposed to 1000 mg E171/kg bw/d compared to controls; although the authors reported E171 accumulated in the stomach walls of rats at the highest dose. Based on the observation of E171 in the stomach walls, the authors concluded the no-observed-adverse-effect level (NOAEL) for oral exposure to E171 was between 100 to 1000 mg/kg bw/d. However, based on a review of the photomicrographs, E171 appeared to be present only on the surface of the stomach and in the mucosa; therefore, the NOAEL for oral exposure to E171 was considered to be 1000 mg/kg bw/d. The EFSA FAF Panel (2021a) also considered the highest dose administered in this study (1000 mg/kg bw/d) to be the NOAEL. Nevertheless, due to the use of ultrasonication to disperse the E171 particles in distilled water, which Health Canada has confirmed with industry does not reflect its use in food manufacture (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022), as well as the gavage administration of E171 in distilled water, which does not take into account the impact of the food matrix, this study was considered to have unclear relevance to the hazard characterization of TiO_2 as a food additive.

In recognition of the fact that different oral dosing paradigms can alter the properties of TiO_2 particles and their interactions with biological substrates, Blevins *et al.* (2019) attempted to replicate the findings of Bettini *et al.* (2017) using a dietary model. Six-week-old male Wistar Han IGS (Crl:WI (Han)) rats (n = 16 per group) were fed a diet containing E171 at nominal concentrations of 0, 40, 400 or 5000 ppm for 100 days (equivalent to 1.1-1.5 (basal diet), 3.0-4.1, 19.0-25.7 or 236-300 mg/kg bw/d based on food consumption, measured Ti concentrations in the diet, and body weight data) in the presence or absence of the colon carcinogenesis initiator DMH*2HCl (180 mg/kg i.p.). The E171 sample used in this study (E171-E, anatase) was supplied by the Titanium Dioxide Manufacturers Association (TDMA) and was characterized by two independent contract laboratories. Analysis by SEM indicated the E171-E particles had an average diameter of 110-115 nm with approximately 36% of particles < 100 nm. The physicochemical properties of E171-E have also been described extensively in several published reports (see EFSA 2019b; Verleysen *et al.* 2020). The diets were analyzed in triplicate from the top, middle, and bottom for homogeneity and E171 concentrations and were reported to be very homogeneous (ECHA 2022a). The E171 concentrations in the diets were approximately the anticipated concentrations with the exception of the highest dose, which was approximately 10% less than the nominal 5000 ppm (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022). At the end of the 100-day treatment period, tissue samples from the colon and small intestine were evaluated for histopathological abnormalities, changes in mean gland length and mean number of goblet cells per gland as well as the development of ABCs/ACF in a blinded manner (except for gland length and goblet cell number) by an experienced, board-certified pathologist. Additional experiments were performed by Blevins *et al.* (2019) and are described elsewhere in this report (see 'Immunotoxicity, Inflammation and Effects on the GIT' section).

For the histopathological assessment, sections from the duodenum, jejunum, and ileum as well as a 1 cm transverse segment from the distal colon was removed, fixed in 10% buffered formalin, and then paraffin embedded. Tissue sections approximately 4-5 µm in thickness were stained with hematoxylin and eosin and examined for histopathological abnormalities. The transverse segments of the distal colon were also analyzed for changes in the number of goblet cells and gland length. An ocular micrometer was used to measure the length of two randomly chosen glands from each colon (from epithelial luminal surface to base of crypt) and the number of goblet cells in each measured gland was counted. For the ABC and ACF evaluation, three 2 cm sections from the colon (one each from the proximal, mid, and distal colon) were removed, fixed in 10% buffered formalin, and then stained with 0.5% toluidine blue in 0.5% sodium borate. The location of the colon samples were standardized such that comparable locations in the proximal, mid, and distal colon were examined for each rat (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022). Each colon sample was divided in approximately 4 mm^2 subsections and the number of ABCs and ACF in each subsection was counted using a light microscope at 40x magnification. The prolonged tissue preservation time required for standardization may have contributed to a technical limitation (autolysis) that prevented the entire surface of the colon samples from being evaluated, with approximately 25-50% of the surface area evaluated per rat (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022). The portion of the colon examined varied among rats, but was similar across groups (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022). To normalize the surface area of the colon samples that were analyzed, the number of ABCs and ACF per cm^2 was estimated for the observable colon with no differences detected between groups with respect to the location of ABCs or ACF in the colon (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022). In addition, the expected increase in the mean number of ABCs and ACF per cm^2 of colon was observed in the rats pre-treated with DMH compared to rats that were

not pre-treated and the authors reported they were able to consistently identify the effects of DMH in a blinded fashion (Drs. Cohen & Kaminski, *pers. comm.* 15 February 2022).

In rats pre-treated with DMH, a total of four intestinal tumours were observed; these included two invasive adenocarcinomas in one rat treated with DMH-only, a single adenoma in one rat treated with DMH + low-dose E171, and a single adenoma in one rat treated with DMH + mid-dose E171. No tumours or histopathological abnormalities were observed in rats treated with DMH + high-dose E171 or in the absence of DMH pre-treatment, with or without E171 exposure. Tumours in the intestinal tract of rats treated with DMH, a potent genotoxic carcinogen, was not an unexpected finding at 100 days (e.g., Shetye and Rubio, 2004) and considering tumour incidence in DMH-treated rats exposed to low- or mid-dose E171 was not above background levels in concurrent DMH-only controls, this finding was not considered treatment-related. No differences in the mean number of goblet cells per gland or in mean colonic gland length across the E171 exposure groups, with or without DMH pre-treatment, with the exception of increased colonic gland length in the DMH-only controls compared to negative controls. Dietary exposure to E171 in the absence of DMH pre-treatment also had no effect on the mean number of ABCs or ACF per cm^2 of the colon compared to negative controls, with mean ABC/cm^2 for E171 exposure groups ranging from 0.2-2.7/cm^2 versus 1.9/cm^2 for controls and mean ACF/cm^2 for E171 exposure groups ranging from 0.1-0.9/cm^2 versus 0.8/cm^2 for controls. Similarly, dietary exposure to E171 had no effect on the mean number of ABCs or ACF per cm^2 of colon of DMH-treated rats compared to controls, with mean ABC/cm^2 for E171 exposure groups ranging from 14.8-28.4/cm^2 versus 17.1/cm^2 for controls and mean ACF/cm^2 for E171 exposure groups ranging from 5.3-10.1/cm^2 versus 5.4/cm^2 for controls. Overall, the findings by Blevins *et al.* (2019) indicate that when administered via the diet, E171 exposure at a dose ~24-30 times greater than the highest dose administered by Bettini *et al.* (i.e., 236-300 versus 10 mg/kg bw/d, respectively) did not initiate or promote the development of ABCs or ACF in the rat colon. Dietary exposure to E171 at doses up to 236-300 mg/kg bw/d, with or without DMH pre-treatment, also did not increase tumour incidence in the intestinal tract of rats (although this study was likely not long enough to assess whether tumours would eventually form) and no treatment-related differences in mean colonic gland length or number of goblet cells per gland in the colon were observed, with the exception of significant gland lengthening in the DMH-only rats compared to negative controls. The difference in findings between the Blevins *et al.* (2019) and Bettini *et al.* (2017) studies is likely attributable to mode of exposure and/or sample preparation method, with dietary delivery of unsonicated particles being more representative of human exposure to TiO_2 as a constituent of food preparations.

An unpublished, GLP- and OECD 443 guideline-compliant EOGRT study with additional parameters was commissioned by industry to address endpoints of concern raised by the EFSA ANS Panel (2016; 2018a) and NVWA (2019), including an evaluation of whether E171 administered in the diet initiated the formation of ACF in the colon (LPT 2020 as cited by EFSA 2021a and ECHA 2022b). For a summary of the other endpoints examined in the EOGRT study please see Table 13 in Appendix K. For the ACF evaluation, F0 satellite, male and female CD/Crl:CD Sprague Dawley rats (*n* = 10 per sex per group) were administered an anatase form of E171 (E171-E) at nominal doses of 0, 100, 300, or 1000 mg/kg bw/d in the diet for 10 weeks prior to mating until weaning of the F1 generation (~18-19 weeks). Analyses of the basal diet revealed low background levels of Ti ranging from 11 to 31 mg Ti/kg diet (mean: 17 mg Ti/kg diet) which was estimated by the EFSA FAF Panel (2021a) to be equivalent to a dose of approximately 1.4 mg TiO_2/kg bw/d. The pristine form of E171-E used in this study was supplied by the TDMA and had a median particle

diameter (SD) of 99.9 ± 2.0 nm with approximately 50-51% of particles < 100 nm as determined by SEM (LNE 2020). The number size distribution and median size of the constituent E171-E particles present in samples from the top, middle, bottom, and leftover diet preparations were similar to the pristine form with a median diameter (SD) ranging from 109.2 ± 1.4 to 113.7 ± 4.9 nm and approximately 31-43% of particles < 100 nm as determined by SEM (LNE 2021a,b,c,d). Based on these results, the TDMA concluded the primary particle sizes within the different parts of the diet were homogeneous and no significant deaggregation, aggregation or particle segregation occurred (TDMA 2022b). The physicochemical characteristics of E171-E have also been described previously in EFSA 2019b and Verleysen *et al.* 2020.

The F0 satellite animals were sacrificed at the same time as the F0 main study animals (after weaning of the F1 animals) to allow for comparisons to be drawn between the ACF evaluation for the F0 satellite animals and the histopathological analysis of the GIT for the F0 main study animals. The colons of the F0 satellite animals were removed, stained with 0.5% (w/v) methylene blue in water, and the entire epithelial surface was examined for the presence of ACF under a stereomicroscope at 50x magnification in a blinded fashion by the study pathologist based on the definition by Shwter *et al.* (2016)[26]. No evidence of ACF were observed in the colon of male or female E171-treated rats or controls. The morphological variability of crypts in two caudal regions of the colon was slightly increased in a few rats, but did not meet the Shwter *et al.* (2016) definition of an ACF and there were no significant differences in incidence of these crypts across E171 treatment groups in males (1/10, 0/10, 1/10, 1/10) or females (1/10, 0/10, 1/10, 2/10). Additional investigations of crypt morphology revealed similar incidences of mild increases in the morphological variability of crypts in rats randomly selected from the control group (4/4 for males and 4/5 for females) and high-dose E171 group (3/3 for males and 3/5 for females). Consistent with the above findings, no treatment-related histopathological changes were observed in male and female F0 main study animals (n = 20 per sex per group) exposed to 0, 100, 300 or 1000 mg E171/kg bw/d in the diet, including histopathological changes in the GIT. Overall, the results of the EOGRT study indicated E171 did not initiate the development of ACF in the colon of male or female rats when administered at doses up to 1000 mg E171/kg bw/d in the diet, which was 100 times greater than the highest dose used by Bettini *et al.* (10 mg/kg bw/d). The EOGRT study was also consistent with the study by Blevins *et al.* (2019) where unsonicated E171 was administered via the diet and no evidence of ACF was observed in the absence of a colon carcinogenesis initiator. The potential promoting effects of E171 were not evaluated in the EOGRT study.

Summary

Overall, the study results suggesting oral exposure to food-grade TiO_2 initiated or promoted the formation of preneoplastic lesions in the colon, including ABCs and ACF, were limited to a single non-guideline study in which E171 was administered after ultrasonication to disperse particles in simple matrices (Bettini *et al.* 2017). In this study, male rats exposed to E171 dispersed in drinking water by ultrasonication developed ABCs and ACF in the colon at doses of 10 mg/kg bw/d for 100 days, with and without DMH pre-treatment. However, the magnitude of effect was small and considering most ACF do not progress to neoplasia, the observation of ABCs and ACF in the presence and absence of a colon carcinogenesis initiator was not considered conclusive scientific evidence of a potential adverse effect of E171. In a subsequent

[26] ACF were defined as an aberrant focus with more than two ABCs (Shwter *et al.* 2016).

study conducted in accordance with OECD 408 guidelines (Han *et al.* 2020), no histopathological abnormalities were observed in the GIT in male or female rats at doses up to 1000 mg E171/kg bw/d for 90 days, which was 100-fold higher than the doses administered by Bettini *et al.* (2017). In the present assessment, these findings have unclear relevance for the hazard characterization of TiO_2 as a food additive due to the administration of ultrasonicated E171 particles via drinking water, which did not take into consideration how interactions with the dietary matrix and/or how the disruption of agglomerates in the test article may have influenced the potential toxicity of these particles. Two dietary studies with E171 were conducted following the publication of the Bettini *et al.* (2017) study, neither of which found evidence that E171 induced the formation of ACF in the colon (Blevins *et al.* 2019; LPT 2020 as cited in EFSA 2021a). Blevins *et al.* (2019) was a non-guideline study conducted to replicate the findings of Bettini *et al.* (2017) in a dietary model and found no evidence that dietary exposure to E171 for 100 days at the highest dose tested (236-300 mg/kg bw/d) initiated or promoted the development of ABCs or ACF in the colon of male rats. The highest dose tested in Blevins *et al.* in which no effects were reported was ~24-30 times greater than the dose at which effects were observed in Bettini *et al.* (236-300 versus 10 mg/kg bw/d, respectively), which supports the notion that the dietary matrix and/or the disruption of agglomerates in the test article may influence/mitigate the toxicity of food-grade TiO_2 particles. The lack of intestinal lesions observed in this study was consistent with results of a subsequent GLP- and guideline-compliant EOGRT study with additional parameters evaluating ACF in the colon, which reported null findings (LPT 2020 as cited in EFSA 2021a). In this study, no evidence of ACF were observed in male or female rats following dietary exposure to E171 for ~18-19 weeks at the highest dose tested (1000 mg/kg bw/d) in the absence of a colon carcinogenesis initiator. The highest dose tested in this study was 100-fold higher than the dose at which effects were observed in Bettini *et al.* (10 mg/kg bw/d). There was also no evidence of carcinogenicity (including intestinal lesions) in a two-year cancer bioassay when Unitane® 0-220 (a form of TiO_2 highly comparable to the current form of TiO_2 found in food) was administered at dietary concentrations up to 50,000 ppm (5% w/w) to male and female mice and rats (equivalent to doses of 2250 to 8359 mg/kg bw/d depending on sex and species; NCI 1979). Overall, the studies by Blevins *et al.* (2019), LPT (2020 as cited by EFSA 2021a), and NCI (1979) suggest the food matrix may attenuate the formation of intestinal lesions in the colon, including ACF. Since dietary studies are more representative of human exposure to food-grade TiO_2, these studies were accorded greater weight in this hazard characterization of TiO_2 as a food additive. Based on the overall weight of evidence that was conducted as part of this subject review, no consistent evidence of preneoplastic lesions in the colons of rodents following exposure to food-grade TiO_2 via the oral route was identified. There was also a low level of concern for carcinogenicity and chronic toxicity based on the available information.

Genotoxicity

Previous safety assessments and opinions of food-grade TiO_2 did not raise any concerns with respect to genotoxicity (EFSA 2016; 2018a; 2019a,b). However, with the publication of new scientific results suggesting TiO_2-NPs may be genotoxic along with new analytical research revealing food-grade TiO_2 can contain a higher percentage of primary particles within the nanoscale than previously believed, the genotoxic potential of food-grade TiO_2 has come into question. The EFSA FAF panel recently re-evaluated the available genotoxicity database for TiO_2, including an assessment of genotoxicity studies with TiO_2-NPs, which were not previously considered relevant to the hazard characterization of E171 (EFSA 2021a). While an immediate health concern was not identified by the FAF Panel, a number of genotoxicity studies

reporting positive results when TiO_2 particles were used as the test article were identified. As a result, the FAF Panel concluded the genotoxicity of TiO_2 particles that may be present in food-grade TiO_2 could not be ruled out and a cut-off value for genotoxicity in terms of particle size could not be determined. The FAF Panel considered that several modes of action for genotoxicity may be acting in parallel, but indicated there was uncertainty as to whether a threshold mode of action could be assumed (see 'Mode of Action' section below for discussion).

For the present assessment of the potential genotoxicity food-grade TiO_2, it was noted that the FAF Panel's 2021a conclusion on genotoxicity was largely based on studies that used TiO_2-NPs (of which many used TiO_2-NPs < 30 nm) to characterize the genotoxic potential of TiO_2 added to food. However, less than 1% of primary particles detected in pristine samples of food-grade TiO_2 as well as food products with TiO_2 on the European market were smaller than 30 nm (Verleysen *et al.* 2020; 2021). In addition, due to significant differences in physicochemical properties between food-grade TiO_2 and TiO_2-NPs (e.g., mean particle diameter, particle size distribution, surface area and composition, etc.), TiO_2-NPs and in particular, TiO_2-NPs < 30 nm, were not considered appropriate test articles for the hazard characterization of TiO_2 as a food additive (see 'Relevant Reference Materials for Studying Food-Grade TiO_2' section and Appendix D for more details). Finally, given that food-grade TiO_2 may already contain a percentage of TiO_2-NPs as high as ~30% on a particle mass basis and ~70% on a particle number basis (Verleysen *et al.* 2020; 2021), genotoxicity assays conducted with food-grade TiO_2 will simultaneously evaluate the genotoxic potential of any TiO_2-NPs that may be present and a separate assessment of studies with TiO_2-NPs is considered unnecessary. Therefore, the current review focussed on *in vivo* genotoxicity studies involving food-grade TiO_2. However, due to the limited number of *in vivo* genotoxicity studies with food-grade TiO_2 available, studies that used non-food-grade TiO_2 particles with a mean diameter > 100 nm were also considered relevant and included in the present assessment. Where possible, a determination was made about how closely these test articles resembled food-grade TiO_2 and those considered comparable (based on a similar mean primary particle size, particle size distribution, % of nanoparticles, isoelectric point, mass-specific surface area, surface composition, elemental composition, crystalline form, and purity) were given more weight. *In vivo* genotoxicity studies with TiO_2-NPs were also reviewed (summarized in Table 10 of Appendix H) since they formed the basis of the EFSA FAF Panel (2021a) opinion on genotoxicity, but were not considered in the weight of evidence in the current assessment of food-grade TiO_2 for the reasons outlined above and in the 'Relevant Reference Materials for Studying Food-Grade TiO_2' section. *In vitro* genotoxicity studies with food-grade TiO_2 were also reviewed. However, the known potential for false positive *in vitro* results is high (see Kirkland *et al.* 2005; 2007) and therefore, *in vivo* genotoxicity studies with food-grade TiO_2 as well as the two-year cancer bioassay with Unitane® 0-220 were accorded greater weight in the present assessment.

In Vitro Genotoxicity

The majority of *in vitro* genotoxicity studies identified in the published literature evaluated the potential for E171 to induce DNA damage in an *in vitro* comet assay (Brown *et al.* 2019; Dorier *et al.* 2017; 2019; Franz *et al.* 2020; Gea *et al.* 2019; Proquin *et al.* 2017) and have produced mixed results. In human Caco-2 intestinal epithelial cells, positive results were reported following 24-hour exposure to a single, non-cytotoxic concentration of E171 (0.143 µg/cm^2; equivalent to 1 µg/ml) dispersed by sonication with and without co-exposure to the genotoxicity carcinogen AOM (20 µg/ml) with similar levels of DNA damage observed in E171 and E171+AOM conditions (Proquin *et al.* 2017). However, only a single E171

concentration was tested in the comet assay and the cellular uptake of particles at this concentration was not confirmed. In another study, acute exposure to E171 dispersed by sonication at concentrations of 10 or 50 µg/ml for 6, 24, and 48 hours did not produce DNA damage in Caco-2 cells in the presence or absence of formamidopyrimidine DNA glycosylase (Fpg)[27], but repeated exposure to the same E171 concentrations twice a week for three weeks led to a slight increase in Fpg-sensitive sites (but not DNA strand breaks) at both doses (Dorier *et al.* 2017). No evidence of cytotoxicity was observed under acute or repeated exposure conditions and the authors reported E171 accumulation in the cells. However, the method used to determine Ti levels (ICP-MS of lysed cells) could not distinguish between Ti accumulation in cells and Ti bound to the cell membrane. The TEM images of cells were also two dimensional making it difficult to determine if the particles resided inside the cell or on the cell surface. Other issues were identified, namely the exposure concentrations used in this study were reported by the authors to exceed the estimated daily intake of TiO_2 in humans by approximately 10,000-fold, the concentration was expressed in µg/ml instead of µg/cm^2, and only the fold-change in % tail DNA from negative controls was reported (no absolute values for the treatment groups or negative or positive controls were presented), which make the results of this study difficult to interpret. In a third study with Caco-2 cells, acute exposure to E171 (dispersed by sonication) at non-cytotoxic concentrations of 3.9, 7.8 or 15.6 µg/cm^2 (equivalent to 12.5, 25, 50 µg/ml) for 4 hours did not increase DNA strand breaks or Fpg-sensitive sites compared to negative controls (Brown *et al.* 2019). A visual scoring method was used to assess the nuclei for DNA damage and it was unclear if it was conducted in a blinded manner. The cellular uptake of E171 particles was also not confirmed. In another intestinal cell line, no evidence of DNA strand breaks were observed in mucus-secreting HT29-MTX-E12 cells when E171 was administered at non-cytotoxic concentrations of 0.5, 5, or 50 µg/ml for 48 hours (Franz *et al.* 2020). The E171 suspensions in this study were intentionally prepared (bath sonicated for 10 minutes) to select for bigger agglomerates to resemble the particle size distribution of E171 in the food matrix; however agglomeration status of the particles in the exposure media was not confirmed. The cellular uptake of particles was also not evaluated.

Caco-2 and HT29-MTX-E12 cells are considered appropriate cell lines for evaluating the potential genotoxicity of dietary exposure to food-grade TiO_2; however, *in vitro* assays with only a single cell line may not accurately represent the complex environment of the gastrointestinal system. Two studies from the same group of authors evaluated the potential for E171 to induce DNA damage in co-cultures of Caco-2/HT29-MTX cells. In the first study, the authors assessed the potential for acute and repeated exposure to E171 (dispersed by sonication) at concentrations of 10 or 50 µg/ml to produce DNA damage in a mono-culture of Caco-2 cells (results also described in previous paragraph) and co-culture of Caco-2/HT29-MTX cells (Dorier *et al.* 2017). For the acute exposure experiment, the mono- and co-cultures produced negative results at both E171 concentrations with and without Fpg. Similarly, no differences in DNA strand breaks were reported in the mono- or co-cultures at either concentration following repeated exposure to the same E171 concentrations twice a week for three weeks; however, a slight increase in Fpg-sensitive sites was observed at 50 µg/ml in the co-culture, whereas both 10 and 50 µg/ml produced a slight increase in Fpg-sensitive sites in the mono-culture. These findings suggest that the co-culture of Caco-2/HT29-MTX cells may be less sensitive to E171 exposure compared to Caco-2 cells alone, which is consistent with previous research indicating the presence of a mucus layer produced by HT29 cells was essential for

[27] The alkaline comet assay detects strand breaks and alkali-labile sites; however, many DNA-damaging agents form DNA adducts or base alterations as opposed to directly inducing strand breaks (Azqueta *et al.* 2013). The use of certain DNA repair enzymes can overcome this limitation and enhance the sensitivity of the comet assay by converting lesions to breaks. Fpg specifically is a DNA repair enzyme used for detecting oxidative DNA damage, as the enzyme attacks 8-oxo-7,8-dihydroguanine (8-oxo-dGuo) and other purine oxidation products, removing the damaged base which is then converted to a break (Collins 2014).

decreasing cellular uptake of TiO_2-NPs in a complex *in vitro* intestinal epithelial model (Garcia-Rodriguez *et al.* 2018). However, as mentioned previously, the study findings reported by Dorier *et al.* (2017) are difficult to interpret. In a follow-up study by the same authors, no evidence of DNA double strand breaks (as measured by 53BP1 immunostaining and 8-oxo-dGuo levels) was observed in co-culture of Caco-2/HT29-MTX cells following 24-hour exposure to 50 µg/ml of E171 dispersed by sonication (Dorier *et al.* 2019). However, only a single concentration was tested in this study and as was the case in the previous experiment, the concentration tested was 10,000-fold higher than anticipated human dietary exposure. The cellular uptake of the E171 particles was also not evaluated.

Many of the *in vitro* studies described above used undifferentiated Caco-2 cells harvested after only a few days of culture to evaluate the potential genotoxicity of E171. Caco-2 cells undergo spontaneous differentiation after approximately two to three weeks in culture to resemble mature enterocyte-like cells and therefore, differentiated Caco-2 cells are considered more representative of *in vivo* conditions (Ude *et al.* 2017; Vila *et al.* 2018). The cellular uptake of TiO_2-NPs was reported to be increased in undifferentiated Caco-2 cells with TiO_2-NPs aggregates observed within the cell and nucleus whereas TiO_2-NPs were found primarily within the apical membrane of differentiated Caco-2 cells and only in low numbers within the cell and none in the nucleus (Vila *et al.* 2018). The Caco-2 cells used by Proquin *et al.* (2017) as well as those used in the mono- and co-cultures in the chronic exposure experiment by Dorier *et al.* (2017) were undifferentiated and both studies produced positive results. In contrast, negative results were reported in the acute exposure experiment by Dorier *et al.* (2017), which used differentiated Caco-2 cells in both the mono- and co-cultures. However, in a follow-up experiment by Dorier *et al.* (2019), a co-culture of undifferentiated Caco-2 cells and mucus-secreting HT29-MTX cells produced negative results. Brown *et al.* (2019) also used undifferentiated Caco-2 cells and the levels of DNA damage in the treatment groups were not statistically different from concurrent controls. Overall, no clear pattern was observed regarding the potential genotoxic effects of E171 in differentiated and undifferentiated Caco-2 cells.

Other cell lines have also been used to investigate the potential for E171 to induce DNA damage *in vitro*. In human HepG2 liver cells, DNA strand breaks and Fpg-sensitive sites were not significantly increased compared to negative controls following acute exposure for 4 hours to E171 dispersed by sonication at concentrations of 3.9, 7.8, or 15.6 µg/cm^2, equivalent to 12.5, 25, 50 µg/ml (Brown *et al.* 2019). Evidence of cytotoxicity was also reported at the highest dose tested (15.6 µg/cm^2) and a visual scoring method was used to analyze the nuclei for DNA damage (it is unclear if the scorer was blinded to the treatment condition). The uptake of particles into the cell was not evaluated. In mouse embryonic stem cells (mES), acute exposure to E171 (dispersed by sonication) for 4 hours at concentrations of 0.98, 1.95, 3.9, 7.8, or 15.6 µg/cm^2 (equivalent to 3.13, 6.25, 12.5, 25, 50 µg/ml) increased the level of DNA strand breaks (but not Fpg-sensitive sites) at the highest dose tested compared to negative controls, but with no obvious dose-response relationship (Brown *et al.* 2019). Cells were randomly selected for analysis of DNA damage by ImageJ software, but it is unclear if this was done in a blinded manner. In a separate ToxTracker reporter cell assay with mES cells, 24-hour exposure to E171 at concentrations of 0.98, 1.95, 3.9, 7.8, or 15.6 µg/cm^2 (equivalent to 3.13, 6.25, 12.5, 25, 50 µg/ml) did not result in any significant changes in DNA damage, oxidative stress, p53-mediated cellular stress, or unfolded protein response compared to negative controls (Brown *et al.* 2019); however, due to excessive levels of cytotoxicity observed (approximately 25 to 75% of control), these findings were difficult to interpret. Positive results were also reported in human BEAS-2B bronchial epithelial cells following exposure to E171 at concentrations up to 160 µg/ml (up to 41.6 µg/cm^2; Gea *et al.* 2019), but this assay lacked a positive control group and the relevance of E171 exposure to lung cells *in vitro* to human dietary exposure to food-grade TiO_2 is uncertain.

Two *in vitro* micronucleus assays with E171 were also identified in the published literature. In human colonic epithelial HCT116 cells, 24-hour exposure to E171 (dispersed by sonication) at concentrations of 5, 10, 50, or 100 µg/cm^2 (equivalent to 50, 100, 500, 1000 µg/ml) resulted in a concentration-dependent increase in the incidence of micronucleated binucleated cells per 1000 binucleated cells at 5, 10, and 50 µg/cm^2 (Proquin *et al.* 2017). No evidence of cytotoxicity was reported under the test conditions, but the cells exposed to the highest concentration (100 µg/cm^2) could not be assessed due to the presence of agglomerated particles, which interfered with the identification of micronuclei. The authors also reported "E171 [seemed] to interact with the centromere region of kinetochore poles during mitosis"; however, based on the published photomicrographs, it is difficult to determine whether the particles were located inside the cells or on the surface of the cells. This study also did not include a positive control group. In the second study, no evidence of micronuclei or hypodiploid nuclei were observed in HT29-MTX-E12 cells exposed to E171 (dispersed by sonication) at concentrations of 0.5, 5, or 50 µg/ml for 48 hours (Franz 2020). The concentrations tested were confirmed to be non-cytotoxic. As mentioned previously, the E171 suspensions used by Franz *et al.* (2020) were intentionally prepared to generate larger agglomerates that more closely resembled the particle size distribution of E171 in the food matrix. However, the presence of these agglomerates interfered with the detection of micronuclei in the flow cytometry-based scoring method used in this study, making the null findings reported difficult to interpret. The cellular uptake of particles was also not evaluated.

Four genotoxicity survey studies of multiple chemicals that evaluated the potential for TiO_2 to induce bacterial and mammalian gene mutations, chromosomal aberrations, and sister chromatid exchanges *in vitro* were also identified in the published literature (Dunkel *et al.* 1985; Ivett *et al.* 1989; Myhr and Caspary 1991; Tennant *et al.* 1987). In these studies, the TiO_2 material was sourced from the NTP[28] repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is highly likely that this test article was the same material used in the NCI two-year cancer bioassay (i.e., Unitane® 0-220; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food (see 'Carcinogenicity and Chronic Toxicity' section for more details). While all four studies reported negative results, these studies were conducted with outdated protocols, did not include an assessment of cytotoxicity or particle uptake, and some provided limited to no information about study methodology. In addition, the bacterial reverse mutation test used by Dunkel *et al.* (1985) and Tennant *et al.* (1987) may not be suitable for the assessment of nanomaterials (Unitane® 0-220 may contain up to 44% nanoparticles) or insoluble particles, in general, since they may not be readily taken up by the bacterial cells used in this assay (Doak *et al.* 2012; OECD 2014; Kumari *et al.* 2010). TiO_2-NPs are also known to have antibacterial/bacteriostatic properties (see Khashan *et al.* 2021; Lopez de Dicastillo *et al.* 2020), which further limits the suitability of the bacterial reverse mutation test for the assessment of these materials. The *in vitro* sister chromatid exchange assay in mammalian cells used by Ivett *et al.* (1989) and Tennant *et al.* (1987) was also deleted by the OECD in 2014 due to "a lack of understanding of the mechanism(s) of action of the effect detected by the test" (OECD 2017). As a result of these limitations, none of these studies were considered appropriate for inclusion in the present review.

Three unpublished *in vitro* genotoxicity assays with E171 commissioned by the TDMA were also reviewed (BioReliance 2021a,b,c). All three assays used E171-E as the test article (anatase, median particle diameter

[28] The NCI is the predecessor of the NTP.

(SD) of 99.9 ± 2.0 nm with approximately 50-51% of particles <100 nm as determined by SEM; LNE 2020) and were conducted in accordance with current OECD guidelines 471 (bacterial reverse mutation test), 476 (*in vitro* mammalian cell gene mutation test), and 487 (*in vitro* mammalian cell micronucleus test). However, as mentioned above, the uptake of nanomaterials and insoluble particles by bacterial cells may be limited and TiO_2-NPs are known to possess antibacterial/bacteriostatic properties; therefore, the bacterial reverse mutation test may not be suitable for assessing the potential mutagenicity of nanomaterials (Doak *et al.* 2012; OECD 2014; Kumari *et al.* 2010). Since E171-E is known to contain a significant percentage of TiO_2 particles within the nanoscale (50-51% < 100 nm), the results of the bacterial reverse mutation test with E171-E (BioReliance 2021a) were not considered further in the present review. For the *in vitro* mammalian cell forward gene mutation assay at the HPRT locus of Chinese hamster ovary (CHO) cells, E171-E did not induce forward mutations at concentrations up to 30 µg/ml (suspended in serum free medium by vortex mixing) in the presence or absence of metabolic activation (BioReliance 2021b). However, in a subsequent TEM imaging analysis, the cellular uptake of E171-E was not demonstrated in CHO cells exposed to 30 µg/ml, with or without metabolic activation (Evans *et al.* 2021a) and therefore, the negative result may be related to the lack of exposure to the test article. For the *in vitro* mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBLs), E171-E did not increase micronuclei induction at concentrations up to 30 µg/mL in the presence or absence of metabolic activation (BioReliance 2021c). Cell samples were collected for electron microscopy imaging analysis to confirm whether E171-E was taken up by HPBLs. The results of this analysis were not available at the time of this review and therefore, it was unclear if the absence of genotoxicity was related to the lack of cellular internalization of E171-E or a true negative result.

In summary, both positive and negative results were obtained from *in vitro* genotoxicity studies with food-grade TiO_2. However, there was low confidence in the reliability and/or relevance of the findings in many of these studies due to the use of inappropriate cell lines for assessing dietary exposure (e.g., lung cells), the use of outdated or inappropriate genotoxicity assays (e.g., bacterial reverse mutation test, sister chromatid exchange assay), uncertainty in the biological relevance of some positive results, poor experimental design, and lack of compliance with OECD test guidelines. Three guideline-compliant *in vitro* studies with E171-E commissioned by industry were submitted to Health Canada during the course of this assessment. The bacterial reverse mutation assay may not be suitable for assessing the potential mutagenicity of nanomaterials and therefore, was not considered in the weight of evidence. The *in vitro* mammalian cell forward gene mutation assay in CHO cells and *in vitro* mammalian cell micronucleus assay in HPBLs were both negative; however, the internalization of E171-E in CHO cells was not demonstrated in a subsequent imaging analysis and the uptake of E171-E in HPBLs had not been confirmed at the time of this review. Therefore, the lack of genotoxicity observed in both assays may be related to the lack of E171-E exposure. Overall, the inconsistent or unclear findings observed along with the questionable reliability/relevance of many of the available *in vitro* genotoxicity studies do not allow for a formal conclusion to be drawn regarding the potential genotoxic effects of food-grade TiO_2 *in vitro*. It is recommended that additional, guideline-compliant studies that evaluate the potential for food-grade TiO_2 to induce genotoxic effects *in vitro* are conducted, particularly studies that use complex *in vitro* intestinal models that more closely resemble *in vivo* conditions.

Additional details on the *in vitro* genotoxicity studies with food-grade TiO_2 discussed above may be found in Table 7 of Appendix H.

In Vivo Genotoxicity

DNA Damage

Two non-guideline studies that evaluated the potential for food-grade TiO_2 to induce DNA damage *in vivo* were identified in the published literature (Bettini *et al.* 2017; Jensen *et al.* 2019). Another two non-guideline studies that used non-food-grade TiO_2 with a mean particle diameter > 100 nm were also identified (Murugadoss *et al.* 2020; Sycheva *et al.* 2011). All four of these studies administered the test article via the oral route and have been described in more detail below as well as in Tables 8 and 9 of Appendix H.

Bettini *et al.* (2017) treated adult male Wistar rats (*n* = 10 per group) with E171 (anatase; D_{50} 118±53 nm, range 20 to 340 nm, with 44.7% of particles < 100 nm as determined by TEM) purchased from a French commercial supplier of food colouring by oral gavage at a dose of 10 mg/kg bw/d for 7 days[29]. Prior to administration, the test article was dispersed in water following the NANOGENOTOX dispersion protocol (i.e., sonication for 16 minutes at 20 kHz in a 0.05% w/v BSA). A concurrent negative control group was administered water via oral gavage. No positive control group was included. Rats were euthanized (timing not reported) and Peyer's patch cells were collected and analyzed for DNA strand breaks and alkali-labile sites using the alkaline comet assay (+/- Fpg). A total of 100 Peyer's patch cells were analyzed and the median % tail DNA was calculated, but it was unclear if the analysis was conducted in a blinded manner and the method did not indicate if the authors controlled for ambient light during slide preparation, lysis or electrophoresis. E171 particles were detected in Peyer's patch cells of treated rats confirming exposure. No evidence of DNA strand breaks or oxidative DNA damage was observed in Peyer's patch cells in treated or control rats with or without Fpg. The test article and tissue samples (i.e., Peyer's patch cells) evaluated in Bettini *et al.* (2017) were considered relevant to the assessment of TiO_2 as a food additive; although it was noted that the study deviated from OECD guideline 489 (*in vivo* mammalian alkaline comet assay) due to the use of only one dose group and lack of a positive control. Overall, the *in vivo* alkaline comet assay (+/- Fpg) was considered negative.

Jensen *et al.* (2019) investigated the potential for E171 (99.8% anatase, 0.2% rutile with three group sizes (135±6, 305±61 and 900±247 nm as determined by TEM) purchased from Bolsjehuset (a supplier of candy ingredients) to induce DNA strand breaks and oxidative DNA damage. Female lean Zucker (Crl:ZUC-Lepr^{fa}) rats (*n* = 10 per group) were exposed to food-grade TiO_2 at doses of 50 or 500 mg/kg bw/d by oral gavage once a week for 10 weeks. Stock solutions of food-grade TiO_2 were prepared the same day of use following the ENPRA dispersion protocol (i.e., particles were sonicated at 20 kHz for 16 minutes in filtered sterile water with 2% fetal bovine serum [FBS]) and used immediately. Negative controls were dosed with filtered sterile water with 2% FBS by oral gavage. Rats were euthanized 24 hours after the last dose and liver and lung tissue samples were analyzed in duplicate for DNA damage in an alkaline comet assay (+/- Fpg or hOGG1). KBrO3 (5 mM) exposed THP-1 cells served as the positive control. The method did not indicate if the authors controlled for ambient light during slide preparation, lysis or electrophoresis. Randomly chosen nucleoids (100 per slide) were visually scored with a fluorescence microscope using a five-class scoring system (score of 0-4 for each nucleoid) with reviewers blinded to dose groups to determine the degree of DNA damage (arbitrary score range 0-400). The level of DNA damage in liver and lung tissue (i.e., DNA strand breaks, Fpg- or hOGG1-sensitive sites) was not significantly elevated at any dose level relative to control. In addition, E171 exposure did not affect the repair of KBrO3-induced oxidative DNA

[29] Bettini et al. (2017) also evaluated a nano-sized TiO_2 material with a mean particle size of 22 nm (P25; NM-105), which is not representative of the particle size distribution of food-grade TiO_2 (see Table 10 in Appendix H for summary).

damage in lung tissue samples. However, systemic exposure to the test article in the liver and lung was not confirmed and DNA damage was not evaluated in an appropriate target tissue (i.e., GIT). The study protocol also deviated from OECD guideline 489 due to the use of only two dose groups, the analysis of DNA damage 24 hours after last exposure instead of 2-6 hours after last dose (which may have allowed DNA repair to occur), the use of an outdated dosing regime (once per week for 10 weeks instead of two or more consecutive days) as well as the use of a visual scoring method to determine the level of DNA damage (instead of using automated or semi-automated image analysis software). Overall, these limitations raised some questions as to the reliability of the study findings and make the negative results difficult to interpret.

Murugadoss *et al.* (2020) evaluated whether exposure to non-food-grade TiO_2 (117 nm, anatase, JRC NM10200a) induced DNA damage in peripheral blood cells from female C57BL/6JRj mice (*n* = 4-5 per group) following a single dose of ~10, 50, or 250 µg TiO_2/mouse[30] (equivalent to ~0.6, 2.9, 14.7 mg TiO_2/kg bw[31]) administered via oral gavage[32]. The TiO_2 particles were ultrasonicated using a probe sonicator (7056J) in suspension media designed to produce small or large agglomerates by using different pH conditions (pH 7.5 and pH 2, respectively). Particle suspensions were immediately stabilized with 0.25% BSA and the suspension dispersed at pH 2 was then readjusted to pH 7-7.5. The median equivalent circle diameter of the small and large agglomerates (measured using TEM) were 122 and 352 nm, respectively. Concurrent negative control groups were administered either the small or large agglomerates suspension medium via oral gavage. Mice were euthanized three days post-dosing and blood samples were collected and analyzed in an alkaline comet assay. Peripheral blood cells from untreated mice exposed to hydrogen peroxide (100 µM) for 15 minutes served as the positive control. The method did not indicate if the authors controlled for ambient light during slide preparation, lysis or electrophoresis. Fifty comets were analyzed using Casplab software and the mean % tail DNA was calculated, but it was unclear if the selection of comets for analysis was conducted in a blinded manner. A significant positive result was observed starting at 10 µg (0.6 mg/kg bw) with large agglomerates, and 50 µg (2.9 mg/kg bw) with small agglomerates, but there was no evidence of a dose-response. However, the TiO_2 particles would have had to be taken up by the cell nucleus to interact with the DNA to produce positive results and there is no evidence this occurs for particles of this size (see 'Mode of Action' section for details). There was also no reported change in blood Ti levels in response to TiO_2 treatment (suggesting low bioavailability), the study deviated from OECD guideline 489 due to the inclusion of only a single sampling time 72 hours post-dosing (instead of multiple sampling times at both 2-6 hours and 16-26 hours as recommended for single administration studies), and the positive control data was not reported, which raised some questions as to the reliability of the study findings. The biological significance of the dose-response relationship was also uncertain given that similar levels of DNA damage were observed at all doses for both sizes of agglomerates. On account of these limitations and uncertainties, the results of this study were considered equivocal. This study was also designed to evaluate the properties/behaviour of nanomaterials under specific experimental conditions to generate small and large agglomerates of which the relevance to

[30] Concentrations were estimated from graphical data (see Figure 4c,d in Murugadoss *et al.* 2020).
[31] Assuming an average body weight of 17 grams for an 8-week-old female C57BL/6JRj mouse (https://www.janvier-labs.com/en/fiche_produit/2_c57bl-6jrj_mouse/#onglet__2).
[32] A nano-sized TiO_2 material (17 nm; JRCNM10202a) was also evaluated by Murugadoss *et al.* (2020), which is not representative of the particle size distribution of food-grade TiO_2 (see Table 10 in Appendix H for summary).

human exposure to food-grade TiO_2 through the diet is uncertain. In addition, the relevance of this study for the assessment of TiO_2 as a food additive is questionable due to insufficient characterization of the test article administered (JRCNM10200a), which prevented reasonable comparison with food-grade TiO_2.

Sycheva *et al.* (2011) examined the potential for a cosmetic grade of TiO_2 (anatase; 160±59.4 nm as determined by electron microscopy) to induce DNA damage in liver, brain, and bone marrow cells[33]. Male CBAxB6 mice were exposed to doses of 40 or 200 mg TiO_2/kg bw/d for 7 days via oral gavage (*n* = 5 per group). The particles were dispersed in distilled water prior to administration. No other details were reported about the particle dispersion protocol. A concurrent negative control group was administered distilled water via oral gavage. No positive control group was included. Mice were euthanized 24 hours after the last dose and bone marrow (femur), brain, and liver cells were collected and analyzed for DNA damage (% tail DNA) in an alkaline comet assay. The authors noted that the preparation of the slides, lysis and electrophoresis were conducted under yellow light to prevent additional DNA damage. For each organ, 100 randomly chosen comets were analyzed using CASP software to determine mean % tail DNA, but it was unclear if the selection of comets for analysis was conducted in a blinded manner. For bone marrow cells, a statistically significant increase in mean % tail DNA was observed at both doses (3.66±0.84 in negative control versus 7.99±1.21 and 6.8±1.13 at 40 and 200 mg/kg bw/d). However, this increase had uncertain biological significance since it was small in magnitude and there was no evidence of a dose response. In addition, in order to produce positive results, the TiO_2 particles would have had to gain access to the nucleus and interact with DNA; there is no evidence this occurs for particles of this size (see 'Mode of Action' section for details). For liver and brain cells, no evidence of DNA damage was observed at either dose compared to controls. No information on organ toxicity was reported and systemic exposure to the test article in the bone marrow, liver, and brain was not confirmed. The study protocol also deviated from OECD guideline 489 due to the use of only two dose groups, the analysis of DNA damage 24 hours after last exposure instead of 2-6 hours after last dose (which may have allowed DNA repair to occur), as well as the lack of a positive control. These limitations raised some questions as to the reliability of the study findings and therefore, the results of this study were considered unclear. The relevance of this study for the assessment of TiO_2 as a food additive was also questionable since the test article administered was not sufficiently characterized to allow for comparison with the form of TiO_2 added to food. Sycheva *et al.* (2011) also evaluated the same test article (160±59.4 nm anatase) in an *in vivo* micronucleus assay, which has been summarized in the 'Micronuclei/Chromosomal Aberrations' section below.

In summary, a total of four non-guideline studies that assessed the potential for TiO_2 particles > 100 nm to induce DNA damage *in vivo* using the alkaline comet assay were identified in the published literature. Two of these studies administered non-food-grade TiO_2 particles > 100 nm to mice by oral gavage, with positive results reported in peripheral blood cells (Murugadoss *et al.* 2020) and bone marrow cells, but not liver or brain cells (Sycheva *et al.* 2011). However, not enough information was available to determine how closely the test articles used in these studies (a JRC nanomaterial and a cosmetic grade) resembled the current form of TiO_2 added to food and therefore, it is unclear whether either study was appropriate to evaluate the potential for food-grade TiO_2 to induce DNA damage *in vivo*. In addition, it was unclear whether the small and large agglomerates experimentally generated in Murugadoss *et al.* (2020) had similar properties and/or behaved similarly to TiO_2 agglomerates consumed as part of the diet and the

[33] A nano-sized TiO_2 material (33 nm) was also evaluated by Sycheva *et al.* (2011), which is not representative of the particle size distribution of food-grade TiO_2 (see Table 10 in Appendix H for summary).

biological significance of the small increase in mean % tail DNA reported in Sycheva *et al.* (2011) in bone marrow of mice exposed to a cosmetic grade of TiO_2 was uncertain, especially since this study also lacked a positive control. Finally, to induce DNA damage, the TiO_2 particles must first enter the cell nucleus to gain access to the DNA and no convincing evidence exists indicating this occurs for particles of this size (see 'Mode of Action' section for details). As a result of these uncertainties and limitations, neither study was considered appropriate for the assessment of the *in vivo* genotoxicity of food-grade TiO_2 in the present review. The remaining two studies administered food-grade TiO_2 to rats by oral gavage, with both producing negative results (Bettini *et al.* 2017; Jensen *et al.* 2019). However, Jensen *et al.* (2019) did not confirm systemic exposure of the test article in the liver and lung nor did they assess the level of DNA damage in the GIT, which is the most appropriate target tissue given that exposure to food-grade TiO_2 occurs via the diet. Therefore, the lack of genotoxic effects observed in Jensen *et al.* (2019) could be related to the lack of systemic tissue exposure to the test article on account of its low oral bioavailability, rather than a true negative finding. As a result, this study was not considered appropriate for the assessment of the *in vivo* genotoxicity of food-grade TiO_2. A few limitations were also identified with the Bettini *et al.* (2017) study (i.e., single dose test, lack of a positive control); however, a target tissue relevant to dietary exposure to food-grade TiO_2 was evaluated (i.e., Peyer's patch cells) and exposure of this tissue was confirmed. Therefore, the results of this study were considered valid.

Overall, the available evidence considered appropriate for evaluating the potential for food-grade TiO_2 to induce DNA damage *in vivo* was limited to a single, non-guideline comet assay (Bettini *et al.* 2017). While this assay did not provide any indication that oral exposure to food-grade TiO_2 induced DNA damage *in vivo*, it should be noted that the comet assay is only an indicator test for DNA damage that may be repaired or result in mutation or chromosomal damage. Additional guideline-compliant studies with food grade TiO_2 are recommended to confirm the negative results observed in Bettini *et al.* (2017).

Micronuclei/Chromosomal Aberrations

No studies addressing the potential for food-grade TiO_2 to produce micronuclei/chromosomal aberrations *in vivo* were identified in the published literature. However, two *in vivo* studies that used a form of TiO_2 that is highly comparable to the current form of TiO_2 found in food (Unitane® 0-220) as well as two *in vivo* studies with non-food-grade TiO_2 particles > 100 nm were identified. Three of the four studies investigated the potential for TiO_2 particles > 100 nm to induce micronuclei; two of which administered the test article via the oral route (Donner *et al.* 2016; Sycheva *et al.* 2011) and the third by intraperitoneal injection (Shelby *et al.* 1993). The fourth study evaluated the potential for TiO_2 particles > 100 nm to produce chromosomal aberrations administered via intraperitoneal injection (Shelby and Witt, 1995). Each of these studies have been summarized below and in Tables 8 and 9 of Appendix H.

Donner *et al.* (2016) examined whether oral exposure to three pigment-grades of TiO_2 (pg-1, pg-2, and pg-3; source unknown) induced micronuclei in rat bone marrow using an *in vivo* micronucleus assay conducted in compliance with GLP and OECD test guideline 474 (1997)[34]. The pg-1 material was described as anatase with a median particle diameter of 120 nm and 27% of particles < 100 nm by number as determined by TEM. The mass-specific surface area was reported to be 8.1 m^2/g, the isoelectric point was

[34] Donner et al. (2016) also evaluated three nano-sized TiO_2 materials with a mean particle size of 43-47 nm, which are not representative of the particle size distribution of food-grade TiO_2 (see Table 10 in Appendix H for summary).

around pH 4, and the whole particle and surface elemental compositions included K, P, and Nb. All of these properties were consistent with food grade TiO_2 and taken together, suggest that the anatase pg-1 material was highly comparable to food-grade TiO_2. The pg-2 and pg-3 materials were rutile with median particle diameters of 165 nm (11% of particles < 100 nm by number; TEM) and 132 nm (26% of particles < 100 nm by number; TEM), respectively, with pg-3 also reported to be coated (no additional information provided about coating). The mass-specific surface areas were reported to be 7.1 and 17.1 m^2/g with an isoelectric point around pH 6 and 4, respectively for pg-2 and pg-3. The whole particle and surface elemental compositions were reported to include Al for pg-2 and Al, Si, and Nb for pg-3. While both of these test articles were well characterized, only a single commercial rutile form of food-grade TiO_2 has been characterized to date (see EFSA 2019b and Verleysen *et al.* 2020) and therefore, not enough data was available on the current rutile forms of TiO_2 added to food to determine if the pg-2 and pg-3 materials used in this study were comparable to food-grade TiO_2. Male and female Crl:CD Sprague Dawley or Wistar Crl:WI(Han) rats (*n* = 5 per sex per dose; *n* = 7 per sex in the highest dose group) were treated with a single dose of 500, 1000, or 2000 mg/kg bw of one of the three test articles or negative control (sterile water) via oral gavage. The test articles were dispersed in sterile water via sonication for 3 hours at 50W prior to administration. A concurrent positive control group was administered 10 mg/kg bw of cyclophosphamide (CY) via intraperitoneal injection. Peripheral blood samples were collected from all rats 48 and 72 hours later and evaluated for the presence of micronuclei in peripheral blood reticulocytes (RETs) with at least 20,000 RETs analyzed per blood sample. Bone marrow toxicity was evaluated by comparing the frequency of immature to total erythrocytes in the peripheral blood samples. The potential uptake of the pg-1 material from the GIT was also evaluated in the blood and liver. No increases in the frequency of micronucleated RETs or changes in the ratio of immature to total erythrocytes were reported for any of the TiO_2 test articles. However, Ti concentrations measured in the blood (at 48 and 72 hours) and liver (at 72 hours) were consistently ≤1.14 µg/g blood and ≤0.316 µg/g liver, indicating low oral bioavailability of the test article regardless of dose. There was also no evidence of a dose-response, with similar Ti concentrations measured across all three pg-1 dose groups and negative controls. Therefore, the lack of genotoxic effects observed for the three pigment-grades of TiO_2 were considered to be related to the lack of exposure to the test articles on account of their low oral bioavailability.

Sycheva *et al.* (2011) investigated micronuclei induction by cosmetic grade TiO_2 particles (anatase; 160±59.4 nm as determined by electron microscopy) via the oral route[35]. Male CBAxB6 mice were exposed to doses of 40, 200, or 1000 mg TiO_2/kg bw/d for 7 days via oral gavage (*n* = 6 per group). The particles were dispersed in distilled water prior to administration. No other details were reported about the particle dispersion protocol. A concurrent negative control group was administered distilled water via oral gavage, but no positive control group was included. Mice were euthanized 24 hours after the last dose; bone marrow samples were collected from the femur and 1000 polychromatic erythrocytes (PCEs) were analyzed for the presence of micronuclei. Bone marrow toxicity was evaluated by comparing the frequency of PCEs among 200 erythrocytes. The forestomach and colon were also removed and 1000 epithelial cells in each tissue were analyzed for the presence of micronuclei, nuclear protrusions, and atypical nuclei as well as evidence of cytotoxicity. One thousand spermatids from the right testis were also analyzed for the presence of micronucleated cells and evidence of cytotoxicity. A statistically significant increase in micronuclei formation in bone marrow PCEs was reported at the highest dose tested

[35] A nano-sized TiO_2 material (33 nm) was also evaluated by Sycheva *et al.* (2011), which is not representative of the particle size distribution of food-grade TiO_2 (see Table 10 in Appendix H for summary).

(1000 mg/kg bw/d) with average frequencies of 6.0 per 1000 PCEs versus 3.0 per 1000 PCEs for control mice. However, it was noted that the analysis of micronuclei incidence in PCEs deviated from OECD test guidelines (474; 1997), which recommends that at least 2000 immature erythrocytes be scored[36]. No evidence of cytotoxicity was observed in bone marrow PCEs. In contrast, there was no evidence that the test article induced micronuclei, nuclear protrusions or atypical nuclei in epithelial cells of the forestomach or colon or induced micronuclei in spermatids. However, evidence of cytotoxicity was reported in the forestomach and colon epithelia, but with no apparent dose-response relationship. There was also evidence of cytotoxicity in spermatids at the highest dose tested. Overall, the increase in micronuclei incidence in bone marrow PCEs was considered small in magnitude and given that the study also lacked a positive control and deviated from OECD guidelines in the analysis of PCEs, the results of the *in vivo* micronuclei assay were considered equivocal. In addition, systemic exposure to the bone marrow was not confirmed and there was no evidence of genotoxic effects in the forestomach or colon, which are considered more appropriate target tissues for assessing oral exposure to TiO_2 on account of its low systemic oral bioavailability. The insufficient characterization of the test article administered also did not allow for reasonable comparison with food-grade TiO_2, and therefore, the relevance of this study for the assessment of TiO_2 as a food additive was considered questionable. Sycheva *et al.* (2011) also evaluated the same test article (160±59.4 nm anatase) in an *in vivo* alkaline comet assay, which has been summarized in the previous section.

In two separate genotoxicity survey studies of multiple chemicals (Shelby *et al.* 1993; Shelby and Witt 1995), the potential for TiO_2 to induce micronuclei and chromosomal aberrations was evaluated. The TiO_2 material used in both studies was sourced from the NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation). Therefore, it is likely that this test article was the same material used in the NCI two-year cancer bioassay (i.e., Unitane® 0-220; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number), which can be considered highly comparable to the current form of TiO_2 added to food (see 'Carcinogenicity and Chronic Toxicity' section for more details).

In the first survey study, Shelby *et al.* (1993) evaluated the potential for TiO_2 (Unitane® 0-220) to induce micronuclei. B6C3F1 male mice (n = 5 per group) were exposed to TiO_2 via intraperitoneal injection for three days at doses of 0, 250, 500, or 1000 mg/kg bw/d in the first experiment and doses of 0, 500, 1000, or 1500 mg/kg bw/d in the second experiment. All doses were prepared using corn oil as the test vehicle and particles were suspended in solution using a Tek-Mar Tissumizer® homogenizer. It is unclear what effect this instrument would have had on the agglomeration status of the test article. Dimethylbenzanthracene (12.5 mg/kg in corn oil, presumably by intraperitoneal injection based on previous descriptions of the testing protocol) was used as the positive control. Mice were euthanized 24 hours after the final dose and 2000 bone marrow and peripheral blood erythrocytes were analyzed for micronuclei in the first experiment and 2000 bone marrow erythrocytes in the second experiment. Cytotoxicity was evaluated by analyzing the percentage of PCEs among 200 erythrocytes. In the first experiment, a statistically significant positive trend was observed for bone marrow erythrocytes, with the highest dose tested (1000 mg/kg bw/d) showing a statistically significant increase in micronucleated cells compared to controls (3.50 versus 1.70 per 1000 PCEs, respectively), but no clear dose-response. In the

[36] Current OECD test guideline 474 (2016) recommends that at least 4000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes.

second experiment, a statistically significant increase in the frequency of micronuclei in bone marrow erythrocytes of a similar magnitude was observed at the intermediate dose (1000 mg/kg bw/d) compared to control (3.60 versus 1.50 per 1000 PCEs, respectively). Conversely, the levels of micronucleated bone marrow erythrocytes were not elevated compared to controls at the lowest (500 mg/kg bw/d; 2.60 per 1000 PCEs) and highest doses tested (1500 mg/kg bw/d; 2.00 per 1000 PCEs) and no overall linear trend was detected. The authors reported that when the highest dose group was removed from the trend analysis, a significant linear trend was observed. However, the magnitude of effect with respect to the increase in micronucleated bone marrow erythrocytes at the 1000 mg/kg bw/d dose level was small and there was no evidence of dose-response in either experiment. Furthermore, the elevated test results at the 1000 mg/kg bw/d dose level (i.e., 3.50 and 3.60 micronuclei per 1000 PCEs for experiments 1 and 2, respectively) were within the range of control data for the same sex and strain reported by the same authors (i.e., 1.10 to 3.70 micronuclei per 1000 PCEs; Shelby and Witt, 1995). For peripheral blood erythrocytes, the overall trend was negative and no statistically significant differences were observed between any of the treatment groups and controls. Therefore, the results of the *in vivo* micronucleus assay were considered negative.

In the second survey study, Shelby and Witt (1995) evaluated the potential for TiO_2 (Unitane® 0-220) to produce chromosomal aberrations. B6C3F1 male mice (*n* = 8 per group) were administered a single intraperitoneal injection of 0, 625, 1250, or 2500 mg TiO_2/kg bw in corn oil (no details were provided with respect to the preparation of the TiO_2/corn oil solution). Based on previous descriptions of the testing protocol, it was presumed that dimethylbenzanthracene (12.5 mg/kg in corn oil, intraperitoneal injection) was used as the positive control, although these data were not presented. To select for first metaphase cells for scoring, mice were subcutaneously implanted with a BrdU tablet 18 hours before tissue harvesting. To arrest cells in metaphase, mice were injected (i.p.) with colchicine two hours prior to tissue harvesting. Mice were euthanized at 17 and 36 hours post-dosing and bone marrow (femur) samples were collected and analyzed for the presence of chromosome aberrations. The frequency of chromosomal aberrations in bone marrow erythrocytes was not significantly elevated at any dose level relative to control at either sampling time. Therefore, the results of the *in vivo* chromosomal aberration assay were considered negative.

In summary, a total of four studies using five different test articles that evaluated the potential for TiO_2 particles > 100 nm to induce micronuclei/chromosomal aberrations *in vivo* were identified in the published literature. A cosmetic grade of TiO_2 administered via the oral route increased micronuclei formation in bone marrow PCEs at the highest dose tested (Sycheva *et al.* 2011); however, this increase was small in magnitude and of questionable biological relevance, systemic exposure of the test article to the bone marrow was not confirmed, and the study protocol deviated from OECD guideline 474 (i.e., not enough PCEs were analyzed, no positive control). Therefore, this finding was considered equivocal. Moreover, there was no evidence that the test article induced micronuclei, nuclear protrusions or atypical nuclei in the forestomach and colon epithelia, which are more appropriate target tissues for evaluating exposure to TiO_2 particles via the diet. The cosmetic grade of TiO_2 used in this study was also insufficiently characterized to allow for reasonable comparison with food-grade TiO_2. As a result of these limitations and uncertainties, this study was not considered appropriate for the assessment of the *in vivo* genotoxicity of TiO_2 as a food additive in the present review. In another study, which was conducted in accordance with OECD guideline 474, three pigment grades of TiO_2 (anatase pg-1, rutile pg-2, and rutile pg-3) also

administered via the oral route did not induce micronuclei formation in bone marrow cells (Donner *et al.* 2016). While the physicochemical properties of the anatase pg-1 material was consistent with food-grade TiO_2, not enough information was available on the rutile forms of TiO_2 added to food to determine if the rutile pg-2 and pg-3 materials were comparable to food-grade TiO_2. However, the lack of genotoxic effects observed in this study was likely attributable to the lack of systemic exposure of the test articles at the target tissue and therefore, this study was not considered suitable for the assessment of the *in vivo* genotoxicity of food-grade TiO_2. The two remaining studies administered Unitane® 0-220 (which is highly comparable to the current form of TiO_2 added to food) via intraperitoneal injection and reported negative results in an *in vivo* micronucleus assay (Shelby *et al.* 1993) and an *in vivo* chromosomal aberration assay (Shelby and Witt, 1995). The administration of Unitane® 0-220 by intraperitoneal injection ensured target tissues were sufficiently exposed increasing the potential of identifying a genotoxic effect; however, some have argued that the intraperitoneal route does not always result in increased systemic exposure compared to the oral route, particularly for less soluble compounds (see Kirkland *et al.* 2019). Furthermore, the intraperitoneal administration of Unitane® 0-220 would have also resulted in the formation of a different particle corona when compared to particles administered orally, which may have affected the physicochemical properties and activity of this compound *in vivo*. Therefore, in the present review, due to the limited number of *in vivo* genotoxicity studies conducted with relevant test articles, studies that administered food-grade TiO_2 or test articles comparable to food-grade TiO_2 by intraperitoneal injection were still considered in the overall weight of evidence, but were accorded less weight compared to more physiologically relevant routes (i.e. oral).

Overall, only two of the four studies identified (Shelby *et al.* 1993; Shelby and Witt 1995) were considered relevant for evaluating the potential of food grade TiO_2 to induce micronuclei or chromosomal aberrations *in vivo*. These studies administered a test article highly comparable to the form of TiO_2 found in food (Unitane® 0-220) via intraperitoneal injection and both produced negative results. While there is some question as to the physiological relevance of exposure to TiO_2 particles via intraperitoneal injection, neither study provided any indication that food-grade TiO_2 induced micronuclei or chromosomal aberrations *in vivo*. Additional research is recommended to confirm these findings, specifically guideline-compliant studies that administer food grade TiO_2 via the oral route and examine appropriate target tissues for evaluating exposure to TiO_2 particles via the diet (e.g., colon epithelia).

Mutagenicity

No *in vivo* mutagenicity studies with food-grade TiO_2 or non-food-grade TiO_2 particles > 100 nm were identified in the published literature. Of the five *in vivo* mutagenicity studies that were identified, all used TiO_2–NPs with a mean particle diameter ranging from 12.1 to 22 nm (Louro *et al.* 2014; Sadiq *et al.* 2012; Suzuki *et al.* 2016; 2020; Trouiller *et al.* 2009) and all produced negative results (see Table 10 of Appendix H). However, none of these test articles were considered appropriate reference materials for investigating the potential mutagenicity of food-grade TiO_2 due to significant differences in their physicochemical properties. Therefore, no conclusions can be drawn regarding the potential for food-grade TiO_2 to induce gene mutations *in vivo*. Additional research is recommended to confirm whether food-grade TiO_2 is mutagenic *in vivo*.

Summary

Some positive genotoxicity results with food-grade TiO_2 have been observed *in vitro*; however, confidence in the reliability and relevance of these findings was low due to poor study design, non-compliance with OECD test guidelines, the use of inappropriate cell lines, and excessively high exposure concentrations tested, among other deficiencies. The potential for false positive results in *in vitro* genotoxicity studies is also known to be high (Kirkland *et al.* 2005; 2007), which underlines the importance of considering the results of *in vivo* genotoxicity and carcinogenicity studies when evaluating the genotoxic potential of TiO_2 used as a food additive. A review of the *in vivo* genotoxicity literature identified a few studies with non-food-grade TiO_2 particles > 100 nm that produced positive results (Murugadoss *et al.* 2020; Sycheva *et al.* 2011); however, the relevance of these studies was uncertain due to the insufficient characterization of the test articles administered, which prevented reasonable comparison to the form of TiO_2 added to food. There was also low confidence in the reliability of these results due to the lack of adherence to OECD test guidelines, poor experimental design, low systemic exposure to the test article, and questionable biological relevance of the positive genotoxic effects. The remaining *in vivo* genotoxicity studies identified in the literature produced negative results: two with food-grade TiO_2 (Bettini *et al.* 2017; Jensen *et al.* 2019), two with Unitane® 0-220 – a test article highly comparable to the current form of TiO_2 added to food (Shelby *et al.* 1993; Shelby and Witt, 1995), and one that tested both food- and pigment-grades of TiO_2 (Donner *et al.* 2016). The null findings reported by Donner *et al.* (2016) and Jensen *et al.* (2019) were considered dubious given that tissue exposure to the test article was not demonstrated and therefore, these studies were not considered in the overall weight of evidence assessment of genotoxicity. The remaining three studies were considered appropriate to inform on the potential genotoxicity of the forms of TiO_2 added to food. Based on the studies by Shelby *et al.* (1993) and Shelby and Witt (1995), there was no indication that food-grade TiO_2 induced micronuclei/chromosomal aberrations *in vivo*; however, the test article in these studies was administered by intraperitoneal injection, which was considered to be a less physiologically relevant route of exposure. There was also no indication that oral exposure to food-grade TiO_2 induced DNA damage *in vivo* based on the results of a single, non-guideline study (Bettini *et al.* 2017). Finally, there was no evidence genotoxicity was expressed in the form of an apical endpoint for food-grade TiO_2. A two-year cancer bioassay in mice and rats using very high dietary concentrations of Unitane® 0-220 (50,000 ppm or 5% w/w) did not show any evidence of carcinogenicity (NCI 1979). Similarly, a recent GLP- and OECD 443 guideline-compliant EOGRT study commissioned by industry to address endpoints of concern[37], found no evidence of ACF (which some consider a preneoplastic lesion for colorectal cancer[38]) in the colon of male or female rats exposed to food-grade TiO_2 (LPT 2020 as cited in EFSA 2021a). When taken together, the above findings indicate there is no immediate concern for the genotoxicity of the current form of TiO_2 added to food. However, due to the limited number of available studies with food-grade TiO_2 or test articles comparable to food-grade TiO_2, additional research is recommended, particularly well-conducted studies that adhere to modern OECD test guidelines for genotoxicity.

Immunotoxicity, Inflammation and Effects on the GIT

It is evident from both human and animal studies that TiO_2 particles are taken up in the intestinal lymphoid follicles known as Peyer's patches. However, as noted by Barreto da Silva *et al.* (2020), the cells in Peyer's patches appear to be "both immunologically and metabolically sluggish," and therefore despite

[37] For a summary of the other endpoints examined in the EOGRT study please see Appendix K.

[38] Information on the use of ACF as a potential biomarker of colorectal cancer in laboratory animals and humans can be found in Appendix G.

substantial particle burdens in these tissues there is no evidence of pro-inflammatory or otherwise undesirable signalling by affected cells. Less clear is the potential for TiO_2 particles to induce other local adverse effects in the gut, including effects mediated by dendritic cells or other components of the intestinal inflammasome, as well as those on or by gut microbiota. Various subpopulations of dendritic cells are present throughout the intestinal epithelium and play a key role in the first line of defence against enteric pathogens (Coombes and Powrie 2008). These specialized cells constitutively express class II major histocompatibility (MHC) complexes, which stimulate T-helper cells, but also have the ability to secrete inflammatory cytokines, thus bridging the innate and adaptive immune systems (Banchereau and Steinman 1998). They are also capable of capturing both nano- and microparticles (Manolova *et al.* 2008) and *in vitro* studies have shown that murine dendritic cells produce pro-inflammatory cytokines when incubated with dispersed TiO_2-NPs (Winter *et al.* 2011). Several studies have demonstrated that TiO_2 particles, including food-grade TiO_2, may influence cytokine production as well as alterations in T-cell subsets in the GIT, especially when dispersed in simple matrices (e.g. Bettini *et al.* 2017; Talamini *et al.* 2019). However, these are considered to be intermediate effects and they were not replicated in studies where E171 was administered via the diet (Blevins *et al.* 2019; Riedle *et al.* 2020; LPT 2020 as cited in EFSA 2021a).

In their recent assessment, the EFSA FAF Panel concluded that "some findings regarding immunotoxicity and inflammation with E171 …may be indicative of adverse effects" (EFSA 2021a). The FAF Panel identified 30 studies considered relevant to the assessment of inflammation and immunotoxicity, of which nine were of sufficient quality and relevant for hazard characterization of E171 while several others provided supporting evidence (the criteria used to establish quality and relevance are described in Appendix C of the EFSA FAF Panel 2021a assessment and relevant studies are summarized in Appendix I of this review). Notably, just three studies used E171 in a dietary dosing paradigm, which reflects the mode of human exposure to this substance and were given the highest weight. No adverse effects on inflammation or immunotoxicity were observed in any of these dietary studies where E171 was administered (Riedle *et al.* 2020; Blevins *et al.* 2019; LPT 2020 as cited in EFSA 2021a). Six additional studies administered food-grade TiO_2 well dispersed in water, two of which were drinking water studies (Pinget *et al.* 2019; Bettini *et al.* 2017), three were gavage studies (Urrutia-Ortega *et al.* 2016, Han *et al.* 2020; Mortensen *et al.* 2021) and one in which the suspended particles were dripped into the mouth with a pipette to be swallowed (Talamini *et al.* 2019).

Pinget *et al.* (2019) exposed male C67Bl/6JAusb mice (n = 5-6 per group) to E171 via drinking water at doses of 0, 2, 10 or 50 mg/kg bw/d for 4 weeks. TiO_2 was dispersed in drinking water using sonication (no further details provided) and the dose was calculated based on water intake measured per cage. TiO_2 was found to have a minor impact on gut microbiota composition at the highest dose tested and alterations in bacterial metabolites were observed from 10 mg/kg bw/d. Decreased *Muc2* gene expression, a key component of the intestinal mucus layer, and increased expression of *Defb3*, a gene associated with antimicrobial defense, were also observed at doses of 10 mg/kg bw/d and above, suggesting TiO_2 exposure is associated with alterations in gut homeostasis, although these changes were not associated with any histopathological alterations and expression 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. The changes in *Muc2* and *Defb3* expression were reported to be accompanied by a reduction in colonic crypt length, an increase in colon macrophages and

$CD8^+$ T cells, as well as increases in mRNA transcripts for the cytokines interleukin (IL)-10, tumor necrosis factor (TNF)-α, and IL-6, suggestive of an adaptive immune response to E171 exposure, although no consistent dose-response relationships were apparent.

Talamini *et al.* (2019) also exposed mice to food-grade TiO_2 in water (E171, anatase, D_{50} = 201.2 ± 8.5 nm) without sonication or other de-agglomeration methods to better simulate realistic conditions of exposure. In this study, 8 week-old male NFR mice (4 per group) were dosed 3 days/week for 3 weeks with E171 freshly dispersed in water, with the test article slowly dripped into the mouth via pipette. Mice received either 0 or 5 mg/kg bw/d (equivalent to an average daily dose of ~2 mg/kg bw/d) and were euthanized on day 21 (3 days after last dose). Based on quantitative real-time polymerase chain reaction (qRT-PCR), a statistically significant increase in IL-1b (but not TNF-α or IL-10) mRNA transcripts were reported in stomach (~ 75%) and whole intestine tissues (~ 75%), but not liver tissues. A statistically significant reduction in liver IL-10 mRNA transcript expression was observed (~40%). The study was limited to one dose and in the opinion of the EFSA FAF Panel (2021a) these changes are indicative of an adaptive response to oxidative stress and not evidence of adversity.

In addition to the 100-day studies described above, Bettini and colleagues (2017) exposed adult male Wistar rats (*n*=10) to 10 mg/kg bw/d E171 via gavage for 7 days to investigate acute immunotoxicity. The test article was obtained from an unspecified French commercial supplier and was primarily composed of TiO_2 anatase with a particle size distribution ranging from 20 to 340 nm (D_{50} 118±53 nm by TEM-EDX); 44.7% of particles by number were <100 nm in diameter. The dose was selected to correspond to a level that humans may be exposed to through the diet. After 7 days of treatment with E171 only via oral gavage, a decrease in regulatory T cells (Tregs) was observed that was concomitant with a decrease in T helper (Th) cells; the same findings were also observed after 100 days of E171 administration via drinking water. An increase in the frequency of GI resident $CD103^+$ dendritic cells (DC) was also observed after 7 days, however effects on DC cells were transient and not observed after 100 days of treatment. The authors suggested the increase in GI resident DC populations may be compensatory to the local inflammation induced by TiO_2 exposure, although this is difficult to reconcile with the decreases in anti-inflammatory Tregs and inflammatory Th cells reported. The effects of E171 treatment for 7 days on mucosal inflammation and immune cell responses in Peyer's patches and the spleen were also investigated. Relative to control rats, E171 treatment did not induce changes in myeloperoxidase activity (a marker of neutrophil infiltration) or in cytokine levels (i.e., TNF-α, IL-10, IL-1β, interferon-γ (IFN-γ) and IL-17) in mucosa of the small and large intestine. In addition, cells were isolated from Peyer's patches and spleen and cultured with anti-CD3/CD28 antibodies to induce cytokine secretion in order to examine *ex vivo* immune cell responses. In cells cultured from rats exposed to E171 IFN-γ secretion decreased in cells isolated from Peyer's patches and increased in those from the spleen; an increase in the pro-inflammatory cytokine IL-17 was also reported in the spleen cells, which was interpreted as indicative of systemic effects on immune regulation. However, the variations in these parameters were small in magnitude and in the opinion of an expert panel convened by ANSES (2017) not sufficient to affirm an impairment of immune homeostasis. These findings also diverge somewhat from a previous report of TiO_2-induced inflammation in the small intestines (Nogueira *et al.* 2012) that observed increases in pro-inflammatory cytokines in the small intestine as well as increases in CD4+ Tregs and Th cells, although it is acknowledged that both studies are suggestive of an inflammatory response in the GIT as a result of TiO_2 exposure.

Blevins *et al.* (2019) attempted to replicate the findings of Bettini *et al.* (2017) using a dietary model. Male Wistar Han IGS (Crl:WI (Han)) rats (*n* = 15 per group) were fed a diet containing E171 at nominal concentrations of 0, 40, 400 or 5000 ppm for 7 and 100 days. Based on food consumption, measured Ti concentration in diet and body weight data, these concentrations corresponded to doses of 1.81 (basal diet), 4.76, 31.43 or 373.86 mg/kg bw/d for the 7 day study and doses of 1.1-1.5 (basal diet), 3.0-4.1, 19.0-25.7, or 236-300 mg/kg bw/d for the 100 day study. Actual TiO_2 concentration as well as homogeneity of particle distribution in the diet were measured by spICP-MS. In the 100 day study, as in Bettini *et al.* (2017), E171 was administered alone or following pretreatment with 1,2-dimethylhydrazine (DMH) to initiate colon carcinogenesis. In contrast to Bettini *et al.* (2017), dietary E171 exposure did not significantly alter CD4+ Th cells or activated Th cells (CD4+, CD25+) in Peyer's patches, spleen or peripheral blood mononuclear cells (PBMC), or systemic or GIT resident $CD103^+$ dendritic cell populations after either period of exposure, suggesting an absence of increased local inflammation. Similarly, neither acute nor subchronic dietary E171 exposure had an effect on Treg cell populations in these tissues nor did it significantly alter inflammatory cytokine levels in plasma, GIT tissue or in lymphocytes isolated from peripheral blood, spleen and Peyer's patches. The authors suggest their failure to replicate the findings of Bettini *et al.* (2017) is likely attributable to the mode of exposure, with dietary delivery being more typical of human exposure to E171 as a constituent of food preparations.

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

Urrutia-Ortega *et al.* (2016) also evaluated the effects of food-grade TiO_2 on biomarkers of immunity (IL-2, TNF-α, IFN-γ, IL-10 and GM-CSF) in colonic tissue. Male BALB/c mice were divided into 4 groups (*n* = 6 per group): (i) an untreated control group; (ii) an E171 only group; (iii) a carcinogen-induced colitis-associated cancer (CAC) model and (iv) a CAC + E171 group. In the CAC model, azoxymethane (AOM)/dextran sulfate sodium (DSS) was used to chemically induce colon carcinogenesis (single intraperitoneal injection of 12.5 mg/kg bw AOM on week 1 + 2% DSS in drinking water on weeks 1, 5, and 8); the control group received a single intraperitoneal injection of saline solution on week 1. Mice in the E171 group received 5 mg/kg bw/d TiO_2 (dispersed in water by sonication) via gavage five days per week for 10 weeks; the control group does not appear to have received a vehicle control via gavage. No changes were observed between the E171 only group and the untreated controls for any of these markers. However, the CAC group displayed a trend towards lower levels of all cytokines relative to controls which

was statistically significant in the CAC + E171 group for all markers except GM-CSF. This result is difficult to interpret, as mice in the CAC group showed perianal blood and purulent material discharge as well as behavioral indicators of systemic toxicity (body curvature, raised hackles) as a result of treatment with the genotoxic initiator. Although it appears E171 had an anti-inflammatory effect in the CAC model, this could potentially be explained by increased cytotoxicity in GI-resident cytokine-producing immune cells leading to immunosuppression. In the opinion of the EFSA FAF Panel (2021a), these changes are likely to be secondary to colon tissue damage produced by the initiator. This study was also reviewed previously by the EFSA ANS Panel (2016), who concluded that "the study cannot be used for risk assessment of TiO_2 (E 171) as a food additive" without further elaborating. Health Canada's Food Directorate agrees that changes in markers of inflammation in a CAC model only appear to be of limited relevance for risk assessment, particularly in consideration of the fact that only a single dose was administered in this study.

The effects of oral exposure to food-grade TiO_2 on inflammation and immunity in the GIT of male and female rat pups was investigated by Mortensen *et al.* (2021). Lactating Sprague Dawley rats with standardized litters of five male and five female pups at PND 2-3 were acclimated for 4-5 days prior to dosing. Between PND 7-10, pups received a daily dose of either 10 mg/kg bw/d of E171 (purchased from Pronto Foods Co., Chicago, IL, USA) dispersed in water or an equivalent volume of water by oral gavage (three litters per dose group for a total of 15 male and 15 female pups per group). Particles were dispersed by ultrasonication in deionized water and DLS was used to measure the hydrodynamic diameter every 2-5 minutes until the change in diameter was less than 5% (the critical delivered energy was determined to be 1690 J/ml); dosing solutions were prepared fresh each day. One male and one female from each group was sacrificed 4 h after the fourth and last dose on PND 10 and the duodenum, jejunum, ileum, and colon were harvested for histopathology. The remaining pups were sacrificed on PND 21 and the liver and brain were collected. The study also included a three-phase *in vitro* digestion model to investigate the stability of E171 during simulated digestion. DLS measurements of E171 showed increased hydrodynamic diameter and polydispersity index during simulated digestion, which was confirmed by SEM. Enhanced darkfield microscopy with hyperspectral imaging (EDM-HSI) was used to evaluate intestinal uptake of particles. The majority of particles were present in the lumen, although some particles were also detected in the gastric mucus and a smaller number still in the underlying epithelial tissue; the percentage of E171 in all areas of intestinal tissues was higher in female pups than in male pups. There was no increase in liver Ti concentration based on ICP-MS. Histopathological analyses included an evaluation of changes in the number of intraepithelial lymphocytes (IEL) and granulocytes in the duodenum and colon. Following E171 administration, a significant increase in the number of IEL was observed in the duodenum but not the colon in both sexes, while the number of granulocytes increased in both the duodenum and the colon. No sign of active inflammation was observed. The authors concluded that oral exposure to E171 well-dispersed in water leads to the recruitment of immune cells in young rats, with the strongest effect observed in the small intestine, although whether this early life exposure is associated with long-term effects on intestinal homeostasis is unknown.

In a previous assessment of TiO_2 as a food additive, the EFSA ANS Panel (2016) identified concerns related to a lack of data on reproductive and developmental toxicity and recommended that a multigeneration or EOGRT study be conducted according to the current OECD guidelines. A GLP- and OECD 443 guideline-compliant EOGRT study (LPT 2020 as cited in EFSA 2021a) was therefore commissioned by industry with slight modifications to the protocol to accommodate the investigation of immunotoxicity (additional

details of this study are described in Table 13 in Appendix K). The material tested was a commercial formulation of E171 that was administered in the diet. The F0 generation was exposed to E171 via the diet at doses of 0, 100, 300 or 1000 mg/kg bw/d for 10 weeks prior to mating until the F1 generation was weaned. The F1 generation received the same diets until PND 4 or 8 of the F2 generation. Effects on developmental immunity in the F1 cohort (10/sex per group) were assessed by measuring the primary IgM antibody response following exposure to a sensitizing antigen, keyhole limpet haemocyanin (KLH). A small but statistically significant decrease in the antigen specific IgM level (–9%) was measured at the highest dose tested (1000 mg/kg bw/d) in males only with no apparent trend towards a decrease at intermediate doses. Satellite animals of the F1 cohort (10/sex) were KLH-immunised and also exposed to a known immunosuppressant (cyclophosphamide) to serve as a positive control for the KLH assay. However, the assay was not performed at the same time as the F1 cohort and there was no separate control for the cyclophosphamide response; therefore, in the view of the EFSA FAF Panel (2021a) the positive control was not considered valid and the sensitivity of the assay was not adequately demonstrated. This result, however, should be viewed in the context of an absence of treatment-related differences in weight or histopathology of spleen, thymus, and lymph nodes, as well as no changes in bone marrow histopathology, total and differential peripheral WBC count, or splenic lymphocyte subpopulation distribution in this same F1 cohort. In the splenic lymphocyte subpopulation analysis, there were no significant differences in the percentage of T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells in any of the treated groups relative to controls of both sexes and thus the deficiency identified in the KLH assay is considered to be of marginal significance in the present assessment.

The influence of dietary exposure to TiO_2-NPs was investigated in a mouse model by Huang *et al.* (2017). In this study C57BL/6J mice (*n* = 4–6; sex not specified) were administered either a control diet or one containing TiO_2-NPs at a concentration of 0.1% for approximately one month. The TiO_2-NPs are described as anatase with primary particle diameters of 10 or 50 nm (purchased from DK Nanotechnology Co., Beijing, China; method of manufacture not stated). Following exposure via the diet for one month, peritoneal macrophages were isolated and their activation state assessed by flow cytometry. In animals exposed to either of the TiO_2-NPs, pro-inflammatory cytokines (IL-1β, IL-6, IL-12α, TNF-α, and nitric oxide synthase-2) were elevated and anti-inflammatory cytokines (IL-4 and IL-10) were reduced relative to controls. The authors suggest these alterations may lead to impairments in host immune defense, although cytokine changes alone are considered an intermediate biomarker of effect that may be an adaptive response to ROS production and may or may not be of prognostic significance for toxicity as expressed in the form of an apical endpoint. It should also be noted that the dose of 0.1% in diet was selected to correspond to human dietary intake of 10–20 mg TiO_2/kg bw/d. However, as the test articles were wholly in the nanoscale, the equivalent dose on a mass basis would greatly exceed human exposure by any other metric (e.g. surface area, particle number).

Warheit *et al.* (2015) reported the outcomes of two repeated-dose studies of TiO_2 in rats. The first study was a subchronic 90-day study conducted in accordance with OECD TG 408. Male and female Crl:CD(SD) rats (10 per sex per group) were administered pigment-grade TiO_2 by oral gavage at doses of 0, 100, 300, or 1000 mg/kg bw/d for 92 (males) or 93 (females) days. The particles were suspended in 0.5% aqueous methylcellulose and fresh suspensions were prepared daily by ultrasonication at 20 kHz. The test article was a rutile-type TiO_2 particulate sample with an alumina surface coating that contained 21% NPs (< 100

nm) by particle number (D_{50} of 224 nm by mass and 145 nm by number[39]) which is comparable in size to food-grade TiO_2. Test-substance related findings were limited to the detection of TiO_2 particles within the digestive tract, draining lymph tissues and the nose, although there was no evidence of an adverse tissue response. There were no test article-related adverse effects on any parameter measured and the highest dose of 1000 mg/kg bw/d was determined to be the NOAEL. The second study conducted by this group was a 28-day repeated-dose oral toxicity study (OECD TG 407) in male rats (4-5 per group), presumably of the same strain. In this study, either vehicle control or one of two test articles was administered by oral gavage at a dose of 24,000 mg/kg bw/d for 29 days. The test articles were uncoated rutile (D_{50} = 173 nm) of two different purities, one considered "research-grade" and the other "commercial-grade", although the authors note that the two grades "were virtually identical in their physicochemical composition". Similar to the 90-d study, the test article was observed in intestinal lymphoid tissue although no treatment-related adverse effects on any endpoint were observed. The NOAEL for the 28-d study was determined to be 24,000 mg/kg bw/d.

Duan *et al.* (2021) exposed male ICR mice (*n* = 6 per group) to two different forms of TiO_2 via the diet for 1, 3 or 6 months. The particles are described as "food-grade" and were incorporated into a commercial pelleted diet at a concentration of 1% by mass; control animals received the basal diet (Ti content not reported). Based on JECFA conversion factors (JECFA 2000), a concentration of 1% w/w in food is equivalent to a dose of 1500 mg/kg bw/d in mice. The first particle type was a nano-sized anatase form with an average primary particle diameter of 38.3 ± 9.3 nm. The second particle type was a micro-sized rutile form with an average primary particle diameter of 128.0 ± 33.4 nm and both particle types were reported to be nearly spherical in shape based on TEM imaging. After receiving control or TiO_2-mixed feed for one, three or six months, animals were euthanized and blood and organs (liver, spleen, and kidney) were collected for histological and elemental analyses. Ti content in blood was higher in animals fed a TiO_2-containing diet for 6 months than those fed for 1 or 3 months, although the Ti content of organs was not significantly different from controls[40]. The authors evaluated the influence of TiO_2 exposure on the homeostasis of 22 trace elements in blood, liver, kidney and spleen (17 of which were above the LOD). The results are reported as ratios of these elements relative to controls and a number of minor alterations were observed that are of unclear toxicological significance. Moreover, the number of statistical comparisons was large (4 tissues x 17 elements x 3 dosing conditions x 3 time points = 612 comparisons) and the authors do not appear to have controlled for the familywise error rate (i.e., Bonferroni correction or similar); with an alpha level for significance of 0.05, the approximately 18 statistically significant observations reported does not exceed that which would be expected based on chance alone. Histopathological examination revealed that after six months of feeding, mice exposed to the micro-sized particles showed occasional protein casts in kidney canaliculus but no significant effects in liver or spleen. Mice exposed to the nanoform showed some liver pathology, described as punctate cell necrosis and infiltration of mononuclear lymphocytes, as well as macrophages engulfing particle aggregates in the spleen, the latter of which could be viewed as an adaptive response. It should be noted that the equivalent dose on a mass basis would result in far greater exposure on a particle number basis to those animals

[39] Apparent discrepancies between the text and table 1a were noted with respect to particle size. The values in the table are reported here.

[40] The reliability of this finding is uncertain. The authors measured Ti in tissues using quadrupole ICP-MS. However, according to Swiatkowska *et al.* (2019), this method cannot reliably quantify Ti in biological samples due to a range of polyatomic and isobaric interferences.

receiving the nanoform. As only one dose was tested, no indications of a dose-response could be gleaned from this study.

Kampfer and colleagues (2021) also exposed male and female mice C57BL6/J to TiO_2-NPs via the diet in order to evaluate effects on intestinal tissue. The particles were P25 (a mixed phase catalytic material consisting of spherical particles ~85% anatase/15% rutile, average primary diameter 21 nm) and were incorporated into pelleted diet at either 0.2% or 1% TiO_2 by mass, corresponding to an estimated dose of 400 or 2000 mg/kg bw/d. Mice (*n* = 10 per sex per group) were exposed to a control diet (basal Ti content not stated) or one of the two TiO_2-enriched diets for 28 days. Following exposure, intestinal tissue was harvested and analyzed for DNA damage via the alkaline comet assay in freshly isolated colonocytes as well as for markers of inflammation and gene expression analysis. No increase in DNA damage was observed in animals exposed to TiO_2-NPs, there was no enhancement in proinflammatory cytokines and none of the investigated genes related to DNA repair, oxidative stress or inflammation were differentially expressed. The authors concluded there is an absence of major local adverse effects in the intestines of mice following repeated exposure to TiO_2-NPs via the diet. They note that incorporating TiO_2-NPs in the feed allows for a highly realistic manner of exposure that mimics human exposure via the diet, although the results may not be consistent with studies that employ bolus dosing by gavage.

Gao and colleagues (2020) exposed male Sprague Dawley rats (*n* = 10 per group) to two types of uncoated anatase TiO_2, a nano-sized form or a micro-sized form, intended to represent the two fractions of food-grade TiO_2. The nano-sized particles had a mean primary particle diameter of 24 ± 5 nm, whereas the micro-sized particles averaged 120 ± 30 nm in diameter. The particles were dispersed in water by ultrasonication and administered via oral gavage at doses of 2, 10, or 50 mg/kg bw/d for 30 consecutive days; control rats were treated with an equal volume of ultrapure water. Following exposure for 30 days, rats were fasted overnight and anesthetized in order to examine the influence of TiO_2 particles on nutrient absorption and metabolism through an *in situ* intestinal loop experiment. Blood samples were collected at baseline as well as at 1 and 2 h following the introduction of a mixed nutrient solution to the closed intestinal loop. Following the *in situ* closed-loop experiment, animals were euthanized for histopathological examination of the small intestine and the ultrastructure of the small intestine mucosa was further analyzed by TEM. Sparse and short microvilli and inflammation of the small intestine was observed in the 50 mg/kg bw/d groups exposed to either nano- or micro-sized particles, although the effects were more pronounced in the nano-sized group. In the intestinal absorption experiment, six amino acids (Thr, Met, Val, His, Lys, and Trp), three metal elements (Mg, Mn, Zn), and glucose were injected into the *in situ* intestinal loop. Some inhibition of added histidine absorption was observed, particularly in the groups exposed to TiO_2-NPs, although there was no clear indication of a dose-response relationship. There was no influence on the remaining amino acids, glucose levels or metal elements. Ti levels were elevated in blood after exposure to TiO_2-NPs but not microscale particles.

The effect of food-grade TiO_2 on digestion was also investigated by Dudefoi *et al*. (2021) in an *in vitro* static digestion model that consisted of three steps representing salivary, gastric and intestinal compartments. Simulated digestion was performed at 37°C under agitation and in the dark. Food-grade TiO_2 particles were found to be chemically stable and insoluble following *in vitro* digestion, with no significant change in oxidation state although the presence of proteins and ions in the simulated fluids induced large agglomerates. The enzymes α-amylase (from the salivary phase) and pepsin (from the

gastric phase) were the first enzymes to adsorb to TiO_2 particles; structural changes in these enzymes were also observed suggesting that the presence of TiO_2 could potentially alter their activities. No effects of amylase activity were observed in the presence of TiO_2 particles at a concentration of 0.5 mg/ml. However, when the amylase concentration was increased, a dose-dependent decrease in activity was observed at 0.7 mg/ml (20%) and 1.0 mg/ml (34%) at pH 7 but not at pH 3, suggesting food-grade TiO_2 may have a concentration-dependent inhibitory effect on carbohydrate metabolism in the mouth but not the stomach. The activity of pepsin was not modified in the presence of TiO_2 particles. The authors noted that these results need to be confirmed with a human salivary α-amylase and under realistic consumption conditions.

The potential for oral exposure to food-grade TiO_2 to produce systemic oxidative stress outside the GIT was also examined by Jensen and colleagues (2018). These authors first conducted an *ex vivo* study in which aorta rings from naïve female Sprague Dawley rats (*n* = 11) were exposed for 30 minutes to food-grade TiO_2 (E171) at concentrations of 14 µg/ml or 140 µg/ml and vasomotor function assessed using wire myographs. Particles were dispersed in either 0.45 µm filtered sterile water with 2% added FBS or in cell culture medium at a concentration of 1.4 mg/ml by continuous sonication at 20 kHz for 16 minutes. The stock solutions were used immediately after sonication and dilution to the final concentrations. Following exposure to E171, the vessel rings were exposed to various concentrations of vasoactive compounds to assess vasorelaxative and vasoconstrictive responses. A slight increase in the maximal effect value of acetylcholine (ACh)-induced endothelium-dependent vasorelaxation was observed in aorta rings exposed to E171 (56.3%, 95% CI: 52.5–61.3%) compared to controls (42.9% (95% CI: 38.7–48.6%). No difference in endothelium-independent vasorelaxation was observed and co-incubation with a nitrous oxide synthase inhibitor abolished the ACh-mediated response, confirming the endothelium-dependence of the result. Incubation with E171 also increased the maximal effect value of tryptophan-mediated vasocontraction. In a second experiment, female lean Zucker rats (*n* = 10 per group) were exposed to either a low-dose (50 mg/kg bw/wk) or high-dose (500 mg/kg bw/wk) of E171 by oral gavage once per week for 10 weeks; the control group received an equivalent volume of sonicated dispersion media. Plasma levels of systemic oxidative stress and imbalances of nitrous oxide bioavailability demonstrated no changes between particle-exposed and control rats. The effects on vasomotor function observed following the *in vivo* exposure were generally consistent with those observed in the *ex vivo* experiment, although they were limited to the proximal left anterior descending artery and there was no response in the aorta. The effects on vasomotor function did not occur in a dose-dependent fashion. Although statistically significant, the effects on the coronary arteries were considered by the authors to be modest in magnitude, and without evidence of systemic oxidative stress.

Effects of TiO_2 particles on gut microbiota that may potentially be associated with adverse outcomes have also been reported (see Appendix J for a non-exhaustive list of studies; for review see Baranowska-Wójcik 2021; Rinninella *et al.* 2021). However, Health Canada's Food Directorate concurs with the EFSA FAF Panel's statement (2021a) that, "there is currently no consensus on when changes in GIT microbiota should be considered adverse". Moreover, Dudefoi *et al.* (2017b) point out that the gut environment may attenuate the toxicity of food-grade TiO_2 particles to human gut microbiota and overall the potential impact of dietary TiO_2 on the gut microbiome remains controversial (Ghebretatios *et al.* 2021). Regardless, this remains an area of active investigation and further research is required to fully elucidate the relationship between insoluble TiO_2 particles in contact with or residing in intestinal mucosa or lymphatic

tissue and the role of the food matrix in mediating particle-induced inflammation and/or alterations in gut flora.

Several studies have evaluated the disposition of insoluble particles in the human GIT. Hummel *et al.* (2014) investigated the fate of endogenous pigments in the GIT of children who were suspected of having inflammatory bowel disease (IBD). A total of 151 children underwent both ileocolonoscopy and upper gastrointestinal tract endoscopy: 62 were diagnosed with Crohn's disease, 26 with ulcerative colitis, and 63 were non-IBD. Only children in whom the terminal ileum was intubated were included in the study and at least one but typically two or more biopsies were taken from each segment of the colon, small intestine, stomach and esophagus. Biopsy specimens were assessed by light microscopy and the amount of pigment was scored on a semiquantitative scale by a pathologist who was blinded to the clinical condition. In 63 children (42%), fine black pigment was observed in the Peyer's patches, although pigmented cells were not observed in lymphoid follicles of the duodenum, colon or elsewhere in the GIT. A significant correlation was observed between age and relative pigment density. The authors suggest the particles primarily consist of aluminosilicates, TiO_2, and a small proportion of nonaluminum-containing silicate although this was not analytically determined. Pigment cells were observed in just 26% of patients with Crohn's disease, compared to 62% of patients with ulcerative colitis and 49% with non-IBD. As Peyer's patches in the terminal ileum are the "classical" site of inflammation in Crohn's disease, the authors hypothesize that pigment cells may be involved in the inflammatory process. However, any potential contribution of TiO_2 cannot be surmised as the identity and relative proportion of particles was not established; the authors do note that dietary intake of aluminosilicates is an order of magnitude higher than TiO_2 intake in the United Kingdom.

Shepherd *et al.* (1987) also examined tissue sections from intestinal resections of 35 patients (ages 2 days to 75 years) with various gastrointestinal disorders and seven post-mortem samples (56 – 75 years) with no clinical, radiologic nor pathological evidence of gastrointestinal disease (total *n* = 42, 21 males and 21 females). The presence of dark brown, granular pigment was reported in macrophages found in the Peyer's patches in all patients and cadaveric donors greater than six years of age (*n* = 34), but in no patients below that age (*n* = 8). As was the case of Hummel *et al.* (2014), pigment deposits were observed at the base of Peyer's patches but not in the GALT of other parts of the GIT. SEM with secondary and backscattered electron imaging and x-ray energy spectroscopy was performed on routine histologic sections and the pigmented sections were observed to contain aluminum and silicon diffusely scattered throughout the cytoplasm as well as discrete foci of Ti. However, no obvious correlation was observed between the degree of pigmentation and the presence or type of gastrointestinal pathology. Similarly, Thoree *et al.* (2008) examined pigment cells of the human Peyer's patch in ilieal sections obtained from patients with adenocarcinoma (*n* = 10), colonic Crohn's disease (*n* = 23) or non-Crohn's disease colitis (*n*= 10). Pigment cells were identified by darkfield microscopy and tissue sections were scored for inflammation by a histopathologist. The identity of the particles was not established but they were assumed by the authors to consist of silicates and TiO_2. The authors note that particles were mainly found in mature macrophages in Peyer's patches that are of low metabolic and immunological activity, and likely represent inert "storage sites" for those particles that are taken up by Peyer's patches. There was no evidence of differential cell phenotype or activation and no apparent association between the presence of particles and inflammation or disease state. The authors conclude it is unlikely that particles have a role

in Crohn's disease, which is consistent with previous observations that a reduced microparticle diet offered no advantage in the resolution of Crohn's disease (Lomer *et al.* 2005).

Summary

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.

Allergenicity

Food allergy is an umbrella term for several clinical entities that bear in common a breakdown in the acquired immunological tolerance to food antigens (Renz *et al.* 2018). Cow's milk protein allergy is the most common food allergy in young children, with an estimated prevalence of 2–7.5% (Castillo and Cassola 2017). Protein conformation is an important determinant of the allergenicity of milk proteins (Madsen *et al.* 2014) and binding to TiO_2 particles is known to cause conformational changes in protein structure. As TiO_2 is permitted in dairy products, Phue and colleagues (2022) hypothesized that interaction of cow's milk proteins with TiO_2 particles may alter their antigenic and allergenic properties by either destroying certain epitopes, creating novel ones or changing their accessibility. The authors obtained two forms of food-grade TiO_2; the first test article was described as "food grade titanium dioxide nanoparticles" and was purchased from CNMI industrial corporation, China (primary particle size ~120 nm by TEM, crystalline form not stated), whereas the second test article was described as E171 and obtained from Minerals-Water, UK (primary particles 50 – 300 nm by TEM, crystalline form not stated). Particles were dispersed in deionized water at 10 mg/ml (dispersion protocol not stated) and aliquots of particles were incubated with either skim milk or in 20 mM PBS (pH 6.5) as a control. After 1 h of incubation, the suspensions were centrifuged and the supernatant discarded to remove the loosely bound proteins that form the soft corona. The antigenicity of milk proteins in the presence of dietary particles was identified using an indirect-ELISA assay. The authors reported that the antigenicity of the milk proteins β-lactoglobulin and casein increased in the presence of E171 in comparison with non-interacted proteins but decreased in the case of the 120 nm particles. A human mast cell degranulation assay (LAD2) was used as a proxy for allergenicity, and interaction of either TiO_2 particle with milk caused a statistically significant increase in LAD2 degranulation, as indicated by the release of β-hexosaminidase. This result held true

when milk in the presence of particles was first passed through a 3-compartment *in vitro* simulated digestive system prior to use in the mast cell degranulation assay. The authors concluded that TiO_2 particle-mediated alterations in protein structure may enhance the allergenicity of milk proteins.

The study by Phue *et al.* (2022) clearly demonstrated that TiO_2 particles bind to milk proteins and modify their structure to some extent. While the changes in antigenicity based on the ELISA experiment could be the result of new epitopes being created or existing epitopes being either masked or unmasked due to conformational changes in protein structure, it is difficult to draw inferences given that the response to the two food-grade test articles differed in direction. With regards to allergenicity, the mast cell degranulation assay does not appear to have a particle-only control for β-hexosaminidase release and as only a single concentration was tested, no evidence of a dose-response could be established. Although the changes in allergenicity were consistent in direction between the two forms of TiO_2, the magnitude is relatively small and the biological significance is unclear. Extrapolation of the results from *in vitro* studies of NP allergenicity to toxicological responses *in vivo* is complex and Health Canada's Food Directorate agrees with the conclusion of the study authors that additional studies using animal models are warranted to better understand any potential implications of these findings to food allergy. For example, Lefebvre and colleagues (2014) demonstrated that carbon black NPs displayed adjuvant-like properties[41] *in vitro* by significantly increasing allergy-related (T helper 2) pathways during the immune cell response to a model allergen (ovalbumin). However, when the same particles were administered to a strain of allergen-prone mice (D011.10) by oral gavage in a follow-up study, no significant changes in the development of allergy markers in response to the allergen were observed (Fine *et al.* 2016).

Summary

TiO_2 does not appear to be intrinsically immunogenic but may modulate immune responses to allergenic proteins due to adjuvant-like properties *in vitro*. The potential human relevance of these results are unclear at this time, and further research is required to elucidate the relevance of these findings to the prevalence of food allergy in humans.

Reproductive and Developmental Toxicity

Exposure to TiO_2-NPs has been associated with various adverse effects on steroidogenesis, spermatogenesis and fertility in male rodents, with toxicity inversely related to particle size (for review see Souza *et al.* 2021). In a previous review of TiO_2 as a food additive, the EFSA ANS Panel (2016) was unable to establish an acceptable daily intake due in part to the lack of an extended 90-day study, multigenerational or EOGRT study with food-grade TiO_2. In response, a GLP- and OECD 443 guideline-compliant EOGRT study in CD/Crl:CD(SD) rats was commissioned by industry with slight modifications to the protocol in order to accommodate the investigation of additional endpoints of concern raised by the EFSA ANS Panel (2016; 2018a) and NVWA (2019), notably induction of ACF (LPT 2020 as cited in EFSA 2021a). The material tested was a commercial formulation of E171 that was administered in the diet (anatase, D_{50} = 100 nm, 50% of the individual particles at the nanoscale). The F0 generation was exposed to E171 via the diet at doses of 0, 100, 300 or 1000 mg/kg bw/d for 10 weeks prior to mating until the F1

[41] Adjuvants accelerate, prolong, or enhance immune responsiveness but do not necessarily provide any specific antigenic stimulus (Dodds 2016).

generation was weaned (20/sex/dose with 30/sex/dose in a F0 satellite group). The F1 generation received the same diets until PND 4 or 8 of the F2 generation. There was no evidence of adverse effects on reproduction or development up to the highest dose tested, and a NOAEL of 1000 mg/kg bw/d 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; additional details of the results of the EOGRT study may be found in Appendix K.

One study was identified in which E171 administered via the diet was reported to produce testicular toxicity in mice (Rodríguez-Escamilla *et al.* 2019). Male BALB/c mice (*n* = 4 per group) were administered a basal diet, or a diet containing E171 at concentrations of 0.1, 0.5 and 1% (equivalent to 100, 680 or 1300 mg/kg bw/d) for 7 weeks. In order to evaluate the effect of the food matrix, two additional groups of 4 mice were administered either a vehicle control or E171 suspended in water at a dose of 5 mg/kg bw/d by oral gavage for 10 weeks. The authors noted that similar effects, which included an increase in germ cell sloughing and the infiltration of inflammatory cells in seminiferous tubules, together with disruption of the Sertoli cell barrier (often referred to as the "blood-testis barrier"), were observed among mice receiving E171 at a dose of 5 mg/kg bw/d in liquid suspension and those administered E171 via the diet at doses of 860 or 1300 mg/kg bw/d (i.e. 136- to 260-fold higher doses), suggesting the food matrix attenuates the toxicity associated with TiO_2 when exposure occurs via the diet. The adverse effects on the testes observed in this study, however, were not replicated when the same authors administered a similar dose (5 mg/kg bw/d) of E171 suspended in drinking water to male C57BL/6 mice for 16 weeks (Medina-Reyes *et al.* 2020). These findings were also not observed in the larger, guideline-compliant EOGRT study nor in any of a number of subchronic studies (several of which were OECD guideline-compliant) that administered similar or higher doses of food-grade TiO_2 in drinking water, by oral gavage or via the diet.

Neurotoxicity

In their recent opinion, the EFSA FAF Panel (2021a) raised concerns regarding observations of potential neurotoxicity of TiO_2-NPs. The majority of studies with indications of neurotoxicity were performed using TiO_2-NPs < 30 nm, and there is convincing evidence from rodent studies that TiO_2-NPs as well as other NPs in this size range can produce various neurotoxic effects (for review see Prüst *et al.* 2020). These findings, however, are considered to be of limited relevance to human exposure via the diet given that < 1% of particles by number in food-grade TiO_2 are smaller than 30 nm, most of which are bound in aggregates.

In the study of Kandeil *et al.* (2020), TiO_2 particles obtained from Sigma (size, crystalline form not stated) were prepared using the high-energy ball mill (HEBM) technique, which is a mechanical deformation process that grinds solids to obtain NPs via high-energy collisions. According to Yang (2015), this technique "has been successfully used to produce metals with minimum particle sizes from 4 to 26 nm" although contamination from the milling media (balls and vial) is considered a serious limitation. The authors report that the characterization of TiO_2-NPs was done using TEM, and the average particle size was 90 nm (range 40–140 nm), although no mention of sample preparation is made (e.g. dispersion protocols). Male albino rats (*n* = 20 per group, strain and age not reported) were exposed by oral gavage to either vehicle control or 500 mg/kg bw/d TiO_2-NPs freshly dispersed in distilled water by ultrasonic vibration (15 minutes, power and frequency not stated) once daily for 14 days. It is uncertain whether the particle size distribution of

the ultrasonically dispersed test article corresponded to that of the raw material characterized following HEBM. Exposure to TiO_2-NPs induced lipid peroxidation in brain tissues and a dramatic increase in markers of ROS production, inflammation and apoptosis as well as a decrease in antioxidant parameters in the brain, histopathological alterations in the cerebellum and DNA damage, which was ameliorated by prior treatment with a botanically-derived antioxidant (Moringa seed extract). Unfortunately, as the TiO_2-NPs were prepared in the laboratory by ball milling of presumably bulk material, they are likely to have characteristics that are distinct from food-grade TiO_2, which precludes extrapolation of these findings to E171.

A second neurotoxicity study is referenced by the FAF EFSA Panel (2021a) in their recent assessment (Ebrahimzadeh Bideskan *et al.* 2017), in which female Wistar rats (*n* = 6 per group) were exposed to vehicle control or 100 mg/kg bw/d of suspended TiO_2-NPs in water (anatase, particle size less than 100 nm, SA >150 m^2/g, dispersion method not stated) via oral gavage during gestation (GD 2 to 21) or lactation (PND 2 to 21). Following treatment, two male pups from each litter were randomly sampled and one pup was used for histological examination while the other was used for evaluating the expression of *Bax* and *Bcl-2* (pro- and anti-apoptotic markers, respectively) in the hippocampus by RT-PCR. Results of a TUNEL assay indicted that both exposure protocols resulted in an increase in apoptotic cells and a reduction in neurogenesis in the hippocampus of male offspring (female offspring were not examined). Increased and decreased expression of *Bax* and *Bcl-2* transcripts, respectively, were also observed in hippocampus of offspring of treated dams. However, the TiO_2-NPs used are described as anatase with a particle size of "less than 100 nm" and a surface area of >150 m^2/g, as compared to E171 with a surface area of approximately 8 to 10 m^2/g (Dudefoi *et al.* 2017; Geiss *et al.* 2020). It has been shown that anatase NPs with a surface area as high as 150 m^2/g have an average particle size of 12 nm or less (Kubiak *et al.* 2011); therefore, it is highly unlikely the average particle size of the test article was greater than 30 nm in this study and the relevance to human exposure to food-grade TiO_2 is unclear.

Zhang and colleagues (2020) studied the effects of TiO_2 particles on the microbiota–gut–brain axis. Thirty male C57BL/6J mice were treated with either vehicle control or 150 mg/kg bw/d TiO_2NPs (reported primary particle size 21 nm, crystalline form not stated) for 30 days by oral gavage (group sizes not reported but presumably *n* = 15 per group). Particles were dispersed by sonication in 2% heat-inactivated mouse serum and controls were dosed with sonicated vehicle. The endpoints examined included small intestine and brain histopathology, effects on gut microbiota, gut and cerebral cortex transcriptomics, as well as locomotor activity (open field test) and spatial learning and memory ability (Morris water maze). Significant perturbations in gut microbial community composition were observed in the TiO_2-treated group that were not accompanied by histopathological changes. Oral exposure to TiO_2-NPs was also associated with enteric nervous system activation, as well as markers of serotonergic activity in the gut but not the brain. Levels of inflammatory cytokines, neurotransmitters and neuropeptides were not changed significantly in TiO_2NPs-treated group. Treatment was reported to reduce centre field activity in the open field test that was indicative of anxiety-like behaviour, although spatial learning and memory were not affected. The open field test, however, was only conducted for 5 minutes, which may have been an insufficient test duration to reliably distinguish a true effect from normal background variability.

Only one study was identified in which neurobehavioral endpoints were assessed following administration of food-grade TiO_2 (Medina-Reyes *et al.* 2020). In this study, E171 (purchased from Mark Al Chemical de

Mexico) was administered in drinking water to six-week-old male C57BL/6 mice (*n* = 9/group) at a dose of 5 mg/kg bw/d for 16 weeks. In order to achieve a dose of 5 mg/kg bw/d, E171 was formulated in drinking water at a concentration of 23 µg/ml, based on the mean of water consumption (5 ml/mouse/d) and the body weight at the first week (23 g/mouse). Particles were dispersed by sonication and administered in drinking water "to avoid protein corona formation" which can "modify the E171 absorption, distribution, and biological effects." Mice were fed either a regular or a high fat diet, with or without E171, and anxiety was measured via the elevated plus maze test as described by Komada *et al.* (2008) in weeks 1, 8 and 16. Both the high-fat diet alone as well as the regular diet plus E171 produced similar increases in anxiety-like behaviour in mice, as indicated by reduced entries into open arms and less time spent in open arms. However, the addition of E171 to the high-fat diet appeared to attenuate anxiety. While Medina-Reyes *et al.* (2020) report that a high fat diet significantly increased anxiety in male C57BL/6 mice as measured in an elevated plus maze test, others have shown the opposite (e.g. Yoshizaki *et al.* 2020). Moreover, in the study by Medina-Reyes *et al.* (2020), animals in the regular diet group spent nearly half of their time in open arms of the maze at baseline, versus approximately 15% observed by Yoshizaki *et al.* (2020) for this sex and strain, which is similar to the 17-23% reported by Tucker and McCabe (2017). These factors, combined with the observation that E171 increased anxiety in mice on a regular diet but had a seemingly anxiolytic effect in mice on a high fat diet, makes it difficult to draw inferences from this study.

Subsequent to the EFSA FAF Panel (2021a) opinion, an OECD guideline-compliant study (424; 1997) of the neurotoxic effects of TiO_2 in mice was published (Sofranko *et al.* 2021). Male and female C57Bl/6J mice (*n* = 10 per sex per group) were fed *ad libitum* with 1% TiO_2 (JRC reference nanomaterial NM105, mean particle size of 26.2 nm ± 10.7) incorporated into the feed pellets for 28 days, with or without a 14 day recovery period. Based on a body weight of 20 g and an estimated daily food consumption of 4 g this corresponds to a daily dose of approximately 2000 mg/kg bw/d. In the last week of the exposure period and second week of the recovery period, a battery of behavioral tests were conducted to evaluate any effects of TiO_2 on motor function, anxiety, learning and memory. In addition to the behavioural tests, following terminal sacrifice the effects of TiO_2 exposure on neuroinflammation, oxidative stress in the brain, kinase activity in cortical tissues and blood brain barrier (BBB) integrity were examined and ICP-MS was used to quantify Ti in the small intestine, blood, brain, liver, kidney and spleen. No neurological effects of TiO_2 exposure were identified in the behavioral battery. Similarly, there were no significant treatment-related neuropathological changes and no effect of TiO_2 on markers of oxidative stress, neuroinflammation, kinase activity or BBB disruption. The authors conclude that subacute dietary exposure to foodborne TiO_2 does not produce neurotoxicity in mice.

Summary

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 1000 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.

Mode of Action

TiO_2 has been classified as possibly carcinogenic to humans (IARC Group 2B) via the inhalation route (IARC, 2010), based on sufficient evidence in animal studies and insufficient evidence in human epidemiological studies. Most of the available evidence is consistent with lung tumours arising via a secondary genotoxic mechanism involving particle overload that results in an inflammation-dependent increase in cell proliferation and oxidative stress (Schins and Knaapen 2007; ECETOC 2013; Warheit *et al.* 2016; Bevan *et al.* 2018). In studies of poorly soluble low toxicity particles including TiO_2, the particle surface area dose was most predictive of pulmonary inflammation and tumour response in rats (Driscoll 1997; Dankovic *et al.* 2007). As noted previously, the inhalation route of exposure is not the focus of the state of the science on TiO_2 as a food additive and therefore, the mode of action for lung carcinogenicity will not be discussed further.

The biological mechanism(s) by which TiO_2 particles may produce toxicity via the oral route are less well understood. Several rodent studies in which food-grade TiO_2 was administered in liquid dispersions (in drinking water or by gavage) have reported that TiO_2 particles generate ROS and inflammatory responses in the GIT (e.g. Bettini *et al.* 2017; Pinget *et al.* 2019; Mortensen *et al.* 2021); although these findings could not be replicated in dietary studies (Blevins *et al.* 2019; Riedle *et al.* 2020; LPT 2020 as cited in EFSA 2021a). Evidence of altered patterns of gene expression in the distal colon of mice following gavage administration of dispersed food-grade TiO_2 in water have also been reported, including changes in the expression of genes involved in immune responses, oxidative stress, the development of colorectal cancer, and DNA repair, among others (Urrutia-Ortega *et al.* 2016; Proquin *et al.* 2018a,b). However, due to limitations in study design (e.g., low sample size and use of a dosing paradigm with unclear relevance to human dietary exposure to TiO_2); it is uncertain whether similar changes in differentially expressed genes and/or their products would be observed in colonic tissue following dietary exposure to food-grade TiO_2.

The potential adverse outcome pathways following TiO_2 exposure via the oral route were recently evaluated by Brand *et al.* (2020) and Braakhuis *et al.* (2021). For effects in the GIT (neoplastic changes in intestinal epithelia), it was postulated that following cellular uptake, TiO_2 particles produce ROS, oxidative stress and chronic inflammation leading to tissue damage, DNA damage and regenerative hyperplasia. In addition to intestinal tissue, Brand *et al.* (2020) investigated potential mechanisms of TiO_2 toxicity on human liver after oral exposure, although these hypothesized effects were also based on similar key events (ROS, oxidative stress, inflammation). These authors also noted inconsistent results among studies and suggested that formulation of test article (liquid dispersions versus diet) and protein corona formation greatly affect the toxicity of TiO_2 particles and may explain the discrepancies. The association of chronic inflammation with neoplastic transformation is well-established and considered to be a threshold mechanism. Braakhuis *et al.* (2021) noted that the available data show inconsistent results with respect to induction of intestinal epithelial hyperplasia following TiO_2 ingestion and that tumour formation was not observed in the two-year rodent bioassay (NCI 1979).

The EFSA FAF Panel (2021a) suggested there is "evidence for several modes of action for genotoxicity that may operate in parallel" as TiO_2-NPs may directly interact with DNA or directly generate ROS on account of their intrinsic properties. Although there are no studies that indicate E171 directly binds DNA and/or interferes with the mitotic apparatus (ANSES 2019), the EU Scientific Committee on Consumer Safety (SCCS 2018) observed that TiO_2-NPs have been reported to have been localised within the nucleus in several studies (citing Andersson *et al.* 2011; Lankoff *et al.* 2012; Ahlinder *et al.* 2013) and therefore a primary genotoxic mechanism by direct interaction with DNA cannot be excluded. The Committee stated that very small particles (<10 nm) may enter the nucleus through a receptor-regulated nuclear pore transport mechanism, whereas larger particles could gain access during cell division when the nuclear membrane is dissolved (SCCS 2018). All three studies referenced were conducted *in vitro* using A549 cells, which is an immortalized cell line derived from human adenocarcinomic alveolar. In the first study cited (Andersson *et al.* 2011), TiO_2-NPs (anatase and rutile, varying diameters ranging from 5 to 60 nm) were taken up by A549 cells when added to culture media at concentrations ranging from 5 to 200 µg/ml. While TiO_2-NPs were found in the vicinity of the nucleus, they were not found within the nucleus using Raman microspectroscopy and TEM. In the study by Lankoff *et al.* (2012), single silver (Ag) NPs (20 nm diameter) were found inside the nucleus of A549 cells but not HepG2 (liver-derived) or THP-1 cells (monocytic cell line); TiOs-NPs (anatase/rutile, 21 nm) were not found in the nucleus of any cells by TEM or SEM. In the study by Ahlinder *et al.* (2013), which was conducted by the same authors as Andersson *et al.* (2011), A549 cells were exposed to TiO_2-NPs (anatase, 21 nm primary particle size) in culture media at a concentration of 10 µg/ml. After 4 or 48 h of incubation, particle uptake was assessed using Raman spectroscopy. In contrast to the previous study by the same group and despite using a similar methodology, a "remarkably high fraction" of the voxels classified as belonging to the cell nucleus were observed with hyperspectral Raman image analysis (37%, or 21 out of 57 observations). Using TEM, however, TiO_2-NPs "seemed to have entered the cell nucleus" in just 2 of 30 images studied. The authors recommended that further TEM studies employ EDX in order to resolve this discrepancy.

Although not cited by SCCS (2018), Singh *et al*. (2007) also examined the distribution of "fine" (40 – 300 nm) and "ultrafine" (20 – 80 nm) TiO_2 particles in A549 cells. Similar to the results of Andersson *et al*. (2011), particles "were often observed next to the nucleus but never inside the nucleus". Hackenberg *et al.* (2011) exposed human peripheral blood lymphocytes from 10 male donors to TiO_2-NPs (anatase, 15-30 nm diameter) at concentrations ranging from 20 to 200 µg/ml for 24 h. Out of 100 cells analyzed by TEM, intranuclear particle deposition was reportedly observed in one cell. However it is not stated how intranuclear deposition was distinguished from perinuclear deposition. In an *in vivo* study where male WKY/NCrl BR rats (n = 10) were exposed via endotracheal intubation to an aerosol containing ultrafine TiO_2-NPs (the material consisted mainly of agglomerates with a median diameter of 22 nm and estimated primary particle size 4 nm, crystalline form not stated) at a mass concentration of 0.11 mg/m^3 for one hour, particle localization by TEM coupled with parallel electron energy-loss spectroscopy (parallel-EELS), electron spectroscopic imaging, and image-EELS was performed 24 h following exposure (Geiser *et al.* 2005). The authors report that particles were mainly localized in the cytoplasm and only rarely within the nucleus. Notably, any particles administered in this study that are not present in the form of agglomerates are within the size range that the SCCS (2018) reported may pass through nuclear membrane pores.

The *in vitro* cellular uptake of TiO_2 particles in Caco-2 cells was investigated by Vila *et al.* (2018). Although derived from a human colon adenocarcinoma, the Caco-2 cell line retains the ability to differentiate into

a monolayer with many of the properties typical of absorptive enterocytes of the small intestine (Leah 2015). In this study, NM-100 (anatase D_{50} = 104.01 ± 39.42 nm) particles were dispersed in 0.05% BSA according to the NANOGENOTOX dispersion protocol and Caco-2 cells were incubated with 100 µg/ml TiO_2 for 24 hours. After exposure, cells were stained and particles visualized using confocal laser scanning microscopy. The authors report that undifferentiated Caco-2 cells internalized a great number of TiO_2 aggregates within cells, including within the nucleus. In differentiated cells, however, much less uptake was observed and particles were found mainly in the apical cell membrane but not associated with or inside the nucleus. In a follow-up study, these authors used a tri-culture of Caco-2/HT29/Raji-B cells to generate a more realistic three-dimensional model, with the HT29 cells being goblet cell clones and the Raji-B cells differentiating into M-like cells that are capable of transcytosing particles. The co-culture was seeded and allowed to establish for 21 days, at which time 100 µg/ml TiO_2 was introduced and cells were incubated for 24 hours prior to confocal microscopy analysis. In this test system, particles were again observed within cells and although shown to be associated with the nucleus, they do not appear to have gained entry to the nucleus.

Several other studies have also reported observations of TiO_2-NPs within the nucleus of cells. The *in vitro* toxicity of uncoated anatase particles (D_{50} = 25 nm) was investigated by Chan *et al.* (2011) in HaCaT cells, which are derived from an immortalized keratinocyte cell line established from adult human skin cells. TiO_2-NPs were sonicated for 30 minutes before adding to cells at concentrations of 10, 50, 100, 200 or 300 µg/ml. After 4 h of incubation, cells were washed and fixed before being dehydrated, embedded in Epon812 epoxy resin and microtomed into 60 nm sections. Intracellular accumulation and distribution of TiO_2-NPs was assessed using TEM and the majority of particles were observed to be present in the cytoplasm in the form of agglomerates. At the highest concentration of 300 µg/ml, however, particles were reported to be present in the nucleus of cells. Shukla *et al.* (2011) also exposed human epidermoid carcinoma cells (A431 cells) to TiO_2-NPs, specifically a non-spherical anatase with an average primary particle size by TEM of ~ 50 nm (source and method of manufacture not reported) and examined subcellular distribution using TEM. Particles were dispersed in culture media by sonication and applied to cells at concentrations ranging from 0.0025 - 25 µg/cm^2 (volume not reported for comparison with the concentrations used by Chan *et al.* 2011). Cells were incubated along with particles for 6 h prior to being harvested, fixed and prepped for TEM analysis. The authors reported that TiO_2-NPs 30-100 nm were mostly in the cytoplasm but some were also found within the nucleus, while agglomerates > 500 nm remained outside cells (no details on the effects of concentration were reported).

In both Chan *et al*. (2011) and Shukla *et al.* (2011), the particles applied to cells are too large to have gained access to the interior of the nucleus by transport through receptor-regulated nuclear pores, a route the SCCS (2018) suggests may be applicable to particles < 10 nm in diameter. Therefore, these particles could presumably only gain access to the nucleus by accidental enclosure when the nuclear envelope disassembles and then reassembles in the course of mitosis. The doubling time for immortalized cell lines varies as a function of culture conditions but for HaCaT and A431 cells is typically on the order of 24 h. The cells were unlikely to be confluent under the conditions of the assay and thus were likely to be proliferating, although the exposure periods (4 and 6 hours for Chan *et al*. (2011) and Shukla *et al.* (2011), respectively) are well below the cell cycle time of the cell lines used. Moreover, both these studies determined that particles were inside the nucleus through the use of TEM imaging. TEM images are two-dimensional projections and therefore it may be difficult to discern with confidence whether a particle is

within the nucleus as opposed to being associated with the nuclear envelope on the basis of standard TEM imaging alone (i.e. without the use of a tilt series or similar specialized methods to render three-dimensional images or examine multiple focal planes). Particles may also be situated in nuclear folds, which can result in misleading images in which it appears they are inside the nucleus when they are not, as described by Pan *et al.* (2009).

Recently, the *in vitro* cellular uptake of 5 types of TiO_2 including E171 was investigated in TK6 (human lymphoblasts) and A549 cells (Evans *et al.* 2021b). The E171 was identified as E171-E, which had a median particle diameter (SD) of 99.9 ± 2.0 nm contained approximately 50-51% of constituent particles in the nanoscale (LNE 2020). The other four particles were identified as G6-3 (a rutile TiO2-NP coated with alumina and hydrophobic organic, D_{50} = 9.2 ± 2.0 nm), G2-5 (uncoated anatase TiO2-NP, D_{50} = 5.5 ± 2.0 nm), G3-1 (uncoated pigmentary rutile TiO_2, D_{50} = 146.9 ± 5.9 nm) and G4-19 (pigmentary rutile TiO_2 coated with alumina and polyol, D_{50} = 177.5 ± 3.9 nm). All particles were ultrasonically dispersed according to the NANOGENOTOX dispersion protocol. A cytotoxicity assessment was undertaken in order to determine the most appropriate concentration for cellular internalization assessment by TEM. Cell viability was assessed following exposure of cells to the test materials at concentrations ranging from 1 – 100 µg/cm^2 for 24 or 72 h. No statistical significant decrease in cell viability was observed for any of the test materials at any concentration over 24 or 72 h of exposure. Based on these results, the highest dose was initially selected for cellular uptake analysis but it was subsequently determined that the high concentration of particles resulted in structural abnormalities when cells were sectioned for TEM. To reduce this artifact, a concentration of 10 µg/cm^2 was used. In the A549 cell line, all test materials were identified in the cytoplasm but no nuclear uptake of any material was observed. In the TK6 cell line, only G3-1 was observed to have been internalized and was always localized in the cytoplasm. The authors noted that some TEM images suggested possible cell nucleus uptake of particles but upon defocusing the sample it was evident they were on different focal planes. The authors suggest that the sample preparation, such as sectioning by ultramicrotomy, may also produce artifacts by transferring particles from the resin and depositing them on the top or bottom of sections. The *in vitro* cellular uptake of E171 was also investigated in CHO cells by TEM imaging (Evans *et al.* 2021a). No evidence of cellular internalization was observed in CHO cells treated with E171-E (properties described above) at a concentration of 30 µg/ml, with or without metabolic activation.

Summary

Taken together, the available evidence suggests that even ultrafine TiO_2-NPs (< 30 nm) are not readily taken up by the cell nucleus in *in vitro* studies, and no convincing evidence exists for larger particles or for TiO_2-NPs in a food matrix. Therefore, there is only very limited evidence to support the biological plausibility of an alternative genotoxic hypothesis involving direct interaction of TiO_2-NPs with DNA, and no evidence to suggest a direct genotoxic mechanism is relevant to food-grade TiO_2 exposure via the oral route. The preponderance of evidence is consistent with secondary genotoxicity as a result of ROS generation and persistent inflammation.

8. Knowledge Gaps and Considerations for Future Research

Several knowledge gaps were identified in the process of conducting this review and are outlined below with the objective of encouraging further research.

Health Canada's Food Directorate requested additional information from stakeholders regarding food industry practices in dispersing TiO_2 and were informed that sonication or other intensive methods that break down agglomerates into their constituent particles are not used to disperse TiO_2 in food or beverage manufacture (International Association of Color Manufacturers, *pers. comm.* 11 March 2022; Titanium Dioxide Manufacturers Association, *pers. comm.* 13 May 2022). However, in order to characterize the human health risk associated with exposure to TiO_2 as used as a food colour additive in Canada, it will be important to determine how closely the TiO_2 test articles used in future research studies (in terms of particle size distribution, sample preparation techniques, concentrations, etc.) resemble the form(s) of food-grade TiO_2 that Canadians are exposed to. In addition, well-designed studies intended to examine the fate of TiO_2 particles in the GIT and the role of the food matrix in attenuating potential site-of-contact effects such as inflammation under biologically-relevant conditions are needed.

Studies that disperse food-grade TiO_2 in simple liquid matrices have unclear relevance to the hazard characterization of non-dispersed TiO_2 as a constituent of food. These dispersion methods are intended for hazard identification of the material's constituent particles (both primary particles and reduced agglomerates) and are considered to represent a "worst case scenario" as realistically humans are exposed to these constituent particles in the form of larger agglomerates and in the presence of a food matrix. Under certain conditions, the methods used to break up agglomerates and form homogeneous dispersions may also produce unintended alterations in the test article and/or dispersion vehicle, such that the material used in test systems may possess properties not found in the pristine, non-sonicated form. Therefore, the extent to which sample preparation contributes to the elicitation of biological responses represents a gap in interpreting the evidence base concerning the safety of TiO_2 in food.

No immediate concerns for the genotoxicity of the current form of TiO_2 added to food were identified in this review. However, given the limited number of available *in vivo* genotoxicity studies conducted with food-grade TiO_2, more research is recommended to confirm these findings. Specifically there is a need for GLP- and OECD-guideline-compliant *in vivo* genotoxicity assays to confirm food-grade TiO_2 lacks the potential to induce gene mutations, chromosomal aberrations, micronucleus formation, and DNA damage at site of contact (i.e., GIT tissue).

There were also no immediate concerns that food-grade TiO_2 may be intrinsically immunogenic, but it may modulate immune responses to allergenic proteins by acting as an adjuvant *in vitro*. A recent publication by Phue *et al.* (2020) demonstrated that food-grade TiO_2 particles are capable of binding to milk proteins and modifying their structure to some extent *in vitro*; although, the magnitude is relatively small and the biological significance is unclear. More research is required to confirm any potential significance of this finding *in vivo.*

9. Summary of Findings

The evidence base upon which the hazard characterization of TiO_2 depends is complex; a range of factors collectively contribute to the poor reproducibility of test results and may account for the existence of contradictory findings for virtually all endpoints examined. Among these, high variability among test articles has resulted in substances with vastly different physicochemical properties, bioavailability and inherent toxicity all having been evaluated under the mantle of TiO_2[42]. For example, 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, generally have a substantial fraction of particles <30 nm in size, and may be manufactured by various processes that lead to different surface properties and are thus not representative of the food-grade material. Adding further complexity is the fact that in many studies, the test article is not adequately characterized or is poorly described if at all, making it difficult to determine the similarity or relevance to food-grade TiO_2. It is also well established that TiO_2 particles are prone to form agglomerates, with smaller particles having a greater propensity to aggregate than larger particles (Zhou *et al.* 2013). Moreover, TiO_2 particles avidly and rapidly bind other macromolecules such as proteins to their surface, which alters their size, agglomeration state and bioaccessibility (Winkler *et al.* 2018). Therefore, the dose formulation and dosing paradigm are perhaps as significant as the identity of the test article in determining outcomes in both *in vivo* and *in vitro* toxicity assays.

Potential toxicity concerns of food-grade TiO_2 appear to be largely driven by studies that were designed for hazard identification of the material's constituent particles (both primary particles and reduced agglomerates) as opposed to the intact material as encountered in the diet. As dietary studies best reflect how humans are exposed to TiO_2 in food and given evidence of a significant food matrix effect, the results of dietary studies were accorded the greatest weight in this review. Food-grade TiO_2 also contains a significant fraction of particles in the nanoscale and therefore, studies conducted with food-grade TiO_2 will simultaneously evaluate the toxicity of any TiO_2-NPs that may be present. In addition, GLP- and OECD guideline-compliant studies were deemed the most reliable and of the highest quality; therefore, these studies were provided the highest weight in this review.

Overall, Health Canada's Food Directorate found:

- 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

[42] These variabilities include but are not limited to particle size and size distribution, agglomeration state, morphology, crystalline structure, coatings and functionalization.

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.

- Evidence of low Ti concentrations detected in human term placentas and meconium of neonates and *ex vivo* studies demonstrated E171 particles were capable of crossing the placental barrier to a small extent (Guillard *et al.* 2020).
- TiO_2 particles had a very low acute oral toxicity in mice and rats, with an estimated acute oral LD_{50} of greater than 5000 mg/kg bw based on two OECD guideline-compliant studies (Wang *et al.* 2007; Warheit *et al.* 2007).
- No consistent evidence of preneoplastic lesions in the colons of rodents exposed to food-grade TiO_2 via the oral route. While evidence of ABCs and ACF were identified in 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/d for 100 days (Bettini *et al.* 2017), these results could not be replicated in any subsequent dietary studies when considerably higher doses were administered, up to ~236-300 mg/kg bw/d for 100 days in a non-guideline study (Blevins *et al.* 2019) and up to 1000 mg/kg bw/d for ~18-19 weeks in a GLP- and OECD guideline-compliant study (LPT 2020 as cited in EFSA 2021a). In addition, an OECD guideline-compliant study that administered food-grade TiO_2 dispersed in water to rats via oral gavage at doses up to 1000 mg/kg for 90 days demonstrated no treatment-related effects in gross or histopathological endpoints, including histopathological changes in the GIT (Han *et al.* 2020).
- No evidence of carcinogenicity, chronic toxicity, or other non-neoplastic lesions of the GIT in a well-conducted, two-year cancer bioassay in male and female mice and rats using very high dietary concentrations (up to 50,000 ppm or 5% w/w) of a form of TiO_2 that is highly comparable to TiO_2 currently used in food (NCI 1979). This study was conducted in accordance with the 1976 NCI guidance for chronic toxicity and carcinogenicity in small rodents, which is very similar to current recommendations and therefore, this study is considered reliable.
- No immediate concern for the genotoxicity of food-grade TiO_2 was identified, with the three studies considered the most reliable and relevant producing negative results *in vivo* (Bettini *et al.* 2017; Shelby *et al.* 1993; Shelby and Witt 1995). While some positive genotoxicity results with food-grade TiO_2 and non-food-grade TiO_2 materials have been reported both *in vitro* and *in vivo*, there was low confidence in the reliability and relevance of these findings due to poor study design, non-compliance with OECD test guidelines, the use of inappropriate cell lines or test articles, as well as uncertainty in the biological relevance of the positive genotoxic effects, among other deficiencies. There was also no evidence that genotoxicity was expressed in the form of an apical endpoint based on the absence of carcinogenicity in a two-year cancer bioassay with mice and rats exposed to very high dietary concentrations (50,000 ppm or 5% w/w) of a test article highly comparable to food-grade TiO_2.
- 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 (LPT 2020 as cited in EFSA 2021a), 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 (LPT 2020 as cited in EFSA 2021a) or chronic bioassay (NCI 1979). Similarly, no treatment-related changes in hematology or gross or histopathological abnormalities in lymphoid organs were observed in rats following the gavage administration of food-grade 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).

- No evidence that food-grade TiO_2 was intrinsically immunogenic *in vitro*; however, it may modulate immune responses to allergenic proteins by acting as an adjuvant *in vitro* (Phue *et al.* 2022). More research is require to confirm any potential significance of this finding *in vivo.*
- No evidence of reproductive or developmental toxicity or gross or histopathological abnormalities in male or female reproductive organs in a recent GLP- and OECD guideline-compliant EOGRT study in rats following dietary exposure to food-grade TiO_2 at doses up to 1000 mg/kg bw/d (LPT 2020 as cited in EFSA 2021a). No other reliable studies with food-grade TiO_2 were identified in the literature.
- No evidence of neurotoxicity, developmental neurotoxicity, or behavioural changes in rodents exposed to food-grade TiO_2 in the diet. Concerns related to the potential neurotoxicity of TiO_2 particles pertain to non-guideline studies that administered test articles that do not correspond to food-grade TiO_2 and used oral dosing paradigms that do not represent human dietary exposure. In a recent GLP- and OECD guideline-compliant EOGRT study, no treatment-related effects were observed in any neurodevelopmental or neurofunctional endpoint and no gross or histopathological abnormalities were detected in the brain or peripheral nerves in male and female rats exposed to food-grade TiO_2 at doses up to 1000 mg/kg bw/d (LPT 2020 as cited in EFSA 2021a). Notably, when non-food grade TiO_2-NPs were administered via the diet in an OECD guideline-compliant study, no evidence of neurotoxicity, behavioural abnormalities, or neuropathological changes in the brain were observed in male and female mice at doses up to ~2000 mg/kg bw/d for 28 days (Sofranko *et al.* 2020).

In summary, based on a review of the available scientific data relevant to food uses of TiO_2, Health Canada Food Directorate's position is that there is no conclusive scientific evidence that the food additive TiO_2 is a concern for human health. While some uncertainties in the database were identified that would benefit from further research, the weight of available evidence suggests these data gaps are not significant enough to warrant a more precautionary approach at present. 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 as a food additive and these conclusions may be revisited should new scientific information become available.

10. References

Adler DG, Gostout CJ, Sorbi D, Burgart LJ, Wang L, Harmsen WS. 2002. Endoscopic identification and quantification of aberrant crypt foci in the human colon. Gastrointestinal Endoscopy. 56(5):657-662.

Ahlinder, L., Ekstrand-Hammarström, B., Geladi, P. and Österlund, L., 2013. Large uptake of titania and iron oxide nanoparticles in the nucleus of lung epithelial cells as measured by Raman imaging and multivariate classification. *Biophysical journal*, *105*(2), pp.310-319.

Akhter, M.H., Khalilullah, H., Gupta, M., Alfaleh, M.A., Alhakamy, N.A., Riadi, Y. and Md, S., 2021. Impact of Protein Corona on the Biological Identity of Nanomedicine: Understanding the Fate of Nanomaterials in the Biological Milieu. *Biomedicines*, *9*(10), p.1496.

Akpan, E.I., Shen, X., Wetzel, B. and Friedrich, K., 2019. Design and synthesis of polymer nanocomposites. In *Polymer composites with functionalized nanoparticles* (pp. 47-83). Elsevier.

Ali SA, Rizk MZ, Hamed MA, Aboul-Ela EI, El-Rigal NS, Aly HF and Abdel-Hamid AZ. 2019. Assessment of titanium dioxide nanoparticles toxicity via oral exposure in mice: effect of dose and particle size. *Biomarkers.* 24: 492–498.

Alrawi SJ, Schiff M, Carroll RE, Dayton M, Gibbs JF, Kulavlat M, Tan D, Berman K, Stoler DL and Anderson GR. 2006a. Aberrant crypt foci. *Anticancer Research.* 26: 107-120.

Alrawi SJ, Carroll RE, Hill HC, Gibbs JF, Tan D, Brenner BM, Nowak NJ, Swede H, Stoler DL and Anderson GR. 2006b. Genomic instability of human aberrant crypt foci measured by inter-(simple sequence repeat) PCR and array-CGH. *Mutation Research*. 601: 30-38.

Ammendolia MG, Iosi F, Maranghi F, Tassinari R, Cubadda F, Aureli F, Raggi A, Superti F, Mantovani A and De Berardis B, 2017. Short-term oral exposure to low doses of nano-sized TiO_2 and potential modulatory effects on intestinal cells. Food and Chemical Toxicology, 102, 63–75.

Anderson JC, Swede H, Rustagi T, Protiva P, Pleau D, Brenner BM, Rajan TV, Heinen CD, Levine JB and Rosenberg DW. 2012. Aberrant crypt foci as predictors of colorectal neoplasia on repeat colonoscopy. *Cancer Causes & Control*. 23: 355–361.

Andersson, P.O., Lejon, C., Ekstrand-Hammarström, B., Akfur, C., Ahlinder, L., Bucht, A. and Österlund, L., 2011. Polymorph-and size-dependent uptake and toxicity of TiO_2 nanoparticles in living lung epithelial cells. *Small*, *7*(4), pp.514-523.

ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail) 2017. Groupe d'expertise collective en urgence « TiO2». Expert Committee commissioned by ANSES. Procès-verbal de la reunion du « 24 mars 2017». Available online at https://www.anses.fr/fr/system/files/GECU-TiO2-PV-2017-03-24.pdf

ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), 2019. Relatif aux risques liès à l'ingestion de l'additif alimentaire E 171. Referral No. 2019-SA-0036.

Arruda, T.R., Vieira, P., Silva, B.M., Freitas, T.D., do Amaral, A.J.B., Vieira, E.N.R. and Leite Júnior, B.R.D.C., 2021. What are the prospects for ultrasound technology in food processing? An update on the main effects on different food matrices, drawbacks, and applications. *Journal of Food Process Engineering*, *44*(11), p.e13872.

Arvanitoyannis, I.S., Kotsanopoulos, K.V. and Savva, A.G., 2017. Use of ultrasounds in the food industry–Methods and effects on quality, safety, and organoleptic characteristics of foods: A review. *Critical reviews in food science and nutrition*, *57*(1), pp.109-128.

Asare N, Duale N, Slagsvold HH, Lindeman B, Olsen AK, Gromadzka-Ostrowska J, Meczynska-Wielgosz S, Kruszewski M, Brunborg G and Instanes C. 2016. Genotoxicity and gene expression modulation of silver and titanium dioxide nanoparticles in mice. *Nanotoxicology.* 10: 312–321.

Auffan, M., Rose, J., Bottero, J.-Y., Lowry, G.V., Jolivet J.-P., and Wiesner, M.R., 2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nature Nanotechnology, 4,* pp.634-641.

Azqueta, A., Arbillaga, L., López de Cerain, A. and Collins, A., 2013. Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG. *Mutagenesis*, *28*(3), pp.271-277.

Azim SA, Darwish HA, Rizk MZ, Ali SA and Kadry MO. 2015. Amelioration of titanium dioxide nanoparticles -induced liver injury in mice: possible role of some antioxidants. *Experimental and Toxicologic Pathology*. 67: 305–314.

Balcaen, L., Bolea-Fernandez, E., Resano, M. and Vanhaecke, F., 2014. Accurate determination of ultra-trace levels of Ti in blood serum using ICP-MS/MS. *Analytica chimica acta*, *809*, pp.1-8.

Banchereau, J. and Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature*, *392*(6673), pp.245-252.

Baranowska-Wójcik, E., 2021. Factors Conditioning the Potential Effects TiO_2 NPs Exposure on Human Microbiota: a Mini-Review. *Biological Trace Element Research*, pp.1-8.

Barui, A.K., Oh, J.Y., Jana, B., Kim, C. and Ryu, J.H., 2020. Cancer-targeted nanomedicine: Overcoming the barrier of the protein corona. *Advanced Therapeutics*, *3*(1), p.1900124.

Bellmann, S., Carlander, D., Fasano, A., Momcilovic, D., Scimeca, J.A., Waldman, W.J., Gombau, L., Tsytsikova, L., Canady, R., Pereira, D.I. and Lefebvre, D.E., 2015. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, *7*(5), pp.609-622.

Berardi, A. and Baldelli Bombelli, F., 2019. Oral delivery of nanoparticles-let's not forget about the protein corona. *Expert opinion on drug delivery*, *16*(6), pp.563-566.

Bettencourt, A., Gonçalves, L.M., Gramacho, A.C., Vieira, A., Rolo, D., Martins, C., Assunção, R., Alvito, P., Silva, M.J. and Louro, H., 2020. Analysis of the characteristics and cytotoxicity of titanium dioxide nanomaterials following simulated In Vitro digestion. *Nanomaterials*, *10*(8), p.1516.

Bettini, S., Boutet-Robinet, E., Cartier, C., Coméra, C., Gaultier, E., Dupuy, J., Naud, N., Taché, S., Grysan, P., Reguer, S. and Thieriet, N., 2017. Food-grade TiO 2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. *Scientific reports*, *7*(1), pp.1-13.

Bevan RJ, Kreiling R, Levy LS, Warheit DB. 2018. Toxicity testing of poorly soluble particles, lung overload and lung cancer. Regul Toxicol Pharmacol. 100:80-91.

Bhagyaraj, S.M., Oluwafemi, O.S., Kalarikkal, N. and Thomas, S. eds., 2018. Synthesis of inorganic nanomaterials: Advances and key technologies.

Bi, Y. and Westerhoff, P., 2019. High-throughput analysis of photocatalytic reactivity of differing TiO2 formulations using 96-well microplate reactors. *Chemosphere*, *223*, pp.275-284.

BioReliance. 2021a. Bacterial Reverse Mutation Assay. Titanium dioxide E171-E. Unpublished study report from BioReliance Corporation. Study Number: AG28TA.502ICH.BTL. 08/03/2021, submitted to Health Canada by the TDMA.

BioReliance. 2021b. *In Vitro* Mammalian Cell Forward Gene Mutation (CHO/HPRT) Assay with Duplicate Cultures. Titanium dioxide E171-E. Unpublished study report from BioReliance Corporation. Study Number: AG28TA.783.BTL. 21/10/2021, submitted to Health Canada by the TDMA.

BioReliance. 2021c. *In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL). Titanium dioxide E171-E. Unpublished study report from BioReliance Corporation. Study Number: AG28TA.348.BTL. 19/10/2021, submitted to Health Canada by the TDMA.

Bird, R.P., 1987. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer letters*, *37*(2), pp.147-151.

Blevins, L.K., Crawford, R.B., Bach, A., Rizzo, M.D., Zhou, J., Henriquez, J.E., Khan, D.I.O., Sermet, S., Arnold, L.L., Pennington, K.L. and Souza, N.P., 2019. Evaluation of immunologic and intestinal effects in rats administered an E171-containing diet, a food grade titanium dioxide (TiO_2). *Food and Chemical Toxicology*, *133*, p.110793.

Borgognoni, C.F., Mormann, M., Qu, Y., Schäfer, M., Langer, K., Öztürk, C., Wagner, S., Chen, C., Zhao, Y., Fuchs, H. and Riehemann, K., 2015. Reaction of human macrophages on protein corona covered TiO_2 nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine*, *11*(2), pp.275-282.

Braakhuis, H.M., Gosens, I., Heringa, M.B., Oomen, A.G., Vandebriel, R.J., Groenewold, M. and Cassee, F.R., 2021. Mechanism of Action of TiO2: Recommendations to reduce uncertainties related to carcinogenic potential. *Annual review of pharmacology and toxicology*, *61*, pp.203-223.

Brand, W., Peters, R.J., Braakhuis, H.M., Maślankiewicz, L. and Oomen, A.G., 2020. Possible effects of titanium dioxide particles on human liver, intestinal tissue, spleen and kidney after oral exposure. *Nanotoxicology*, *14*(7), pp.985-1007.

Brayton, C.F., Treuting, P.M. and Ward, J.M., 2012. Pathobiology of aging mice and GEM: background strains and experimental design. *Veterinary pathology*, *49*(1), pp.85-105. Breznica, P., Koliqi, R. and Daka, A., 2020. A review of the current understanding of nanoparticles protein corona composition. *Medicine and Pharmacy Reports*, *93*(4), p.342.

Breznica, P., Koliqi, R. and Daka, A., 2020. A review of the current understanding of nanoparticles protein corona composition. *Medicine and Pharmacy Reports*, *93*(4), p.342.

Brown DM, Danielsen PH, Derr R, Moelijker N, Fowler P, Stone V, Hendriks G, Moller P, Kermanizadeh A. 2019. The mechanism-based toxicity screening of particles with use in the food and nutrition sector via the ToxTracker reporter system. *Toxicology in Vitro*. 61: 104594.

Buettner, K.M. and Valentine, A.M., 2012. Bioinorganic chemistry of titanium. *Chemical reviews*, *112*(3), pp.1863-1881.

Caderni G, Giannini A, Lancioni L, Luceri C, Biggeri A and Dolara P. 1995. Characterisation of aberrant crypt foci in carcinogen-treated rats: association with intestinal carcinogenesis. *British Journal of Cancer.* 71: 763-769.

Cao, X., Han, Y., Li, F., Li, Z., McClements, D.J., He, L., Decker, E.A., Xing, B. and Xiao, H., 2019. Impact of protein-nanoparticle interactions on gastrointestinal fate of ingested nanoparticles: Not just simple protein corona effects. *NanoImpact*, *13*, pp.37-43.

Cao, X., Zhang, T., DeLoid, G.M., Gaffrey, M.J., Weitz, K.K., Thrall, B.D., Qian, W.J. and Demokritou, P., 2020. Evaluation of the cytotoxic and cellular proteome impacts of food-grade TiO_2 (E171) using simulated gastrointestinal digestions and a tri-culture small intestinal epithelial model. *NanoImpact*, *17*, p.100202.

Carp, O., Huisman, C.L. and Reller, A., 2004. Photoinduced reactivity of titanium dioxide. *Progress in solid state chemistry*, *32*(1-2), pp.33-177.

Carrière M, Arnal M-E and Douki T. 2020. TiO_2 genotoxicity: An update of the results published over the last six years. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* 854-855: 503198.

Carrière, M., Pigeot-Rémy, S., Casanova, A., Dhawan, A., Lazzaroni, J.C., Guillard, C. and Herlin-Boime, N., 2014. Impact of titanium dioxide nanoparticle dispersion state and dispersion method on their toxicity towards A549 lung cells and Escherichia coli bacteria. *Journal of Translational Toxicology*, *1*(1), pp.10-20.

Carvalho B, Sillars-Hardebol AH, Postma C, Mongera S, Terhaar Sive Droste J, Obulkasim A, van de Wiel M, van Criekinge W, Ylstra B, Fijneman RJ and Meijer GA. 2012. Colorectal adenoma to carcinoma progression is accompanied by changes in gene expression associated with ageing, chromosomal instability, and fatty acid metabolism. *Cellular oncology (Dordrecht)*. *35*(1): 53-63.

Castillo, D.S. and Cassola, A., 2017. Novel sensitive monoclonal antibody based competitive enzyme-linked immunosorbent assay for the detection of raw and processed bovine beta-casein. *PLoS One*, *12*(7), p.e0182447.

Cedervall, T., Lynch, I., Foy, M., Berggård, T., Donnelly, S.C., Cagney, G., Linse, S. and Dawson, K.A., 2007. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. *Angewandte Chemie International Edition*, *46*(30), pp.5754-5756.

Chakrabarti S, Goyary D, Karmakar S and Chattopadhyay P. 2019. Exploration of cytotoxic and genotoxic endpoints following sub-chronic oral exposure to titanium dioxide nanoparticles. *Toxicology and Industrial Health*. 35: 577–592.

Chan, J., Ying, T., Guang, Y.F., Lin, L.X., Kai, T., Fang, Z.Y., Ting, Y.X., Xing, L.F. and Ji, Y.Y., 2011. In vitro toxicity evaluation of 25-nm anatase TiO2 nanoparticles in immortalized keratinocyte cells. *Biological trace element research*, *144*(1), pp.183-196.

Chao L, Zuo D, Yin L, Lin Y, Li C, Liu T & Wang L. 2018. Prognostic value of MUC2 expression in colorectal cancer: a systemic review and meta-analysis. 2018. *Gastroenterology Research and Practice.* 6986870.

Charles S, Jomini S, Fessard V, Bigorgne-Vizade E, Rousselle C and Michel C. 2018. Assessment of the in vitro genotoxicity of TiO_2 nanoparticles in a regulatory context. *Nanotoxicology*. 12: 357–374.

Chen, Z., Zheng, P., Han, S., Zhang, J., Li, Z., Zhou, S. and Jia, G., 2020. Tissue-specific oxidative stress and element distribution after oral exposure to titanium dioxide nanoparticles in rats. *Nanoscale*, *12*(38), pp.20033-20046.

Chen, Z., Wang, Y., Zhuo, L., Chen, S., Zhao, L., Chen, T., Li, Y., Zhang, W., Gao, X., Li, P. and Wang, H., 2015. Interaction of titanium dioxide nanoparticles with glucose on young rats after oral administration. *Nanomedicine: Nanotechnology, Biology and Medicine*, *11*(7), pp.1633-1642.

Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J and Jia G. 2014. Genotoxic evaluation of titanium dioxide nanoparticles *in vivo* and *in vitro*. *Toxicology Letters*. 226: 314–319.

Cho, W.S., Kang, B.C., Lee, J.K., Jeong, J., Che, J.H. and Seok, S.H., 2013. Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. *Particle and fibre toxicology*, *10*(1), pp.1-9.

Cho NL, Redston M, Zauber AG, 2008. Aberrant crypt foci in the adenoma prevention with celecoxib trial. *Cancer Prevention Research.* 1(1): 21-31.

Choi JW, Kim P, Kim JK, Kim YR, Fukumura D, Yun SH. 2015. Longitudinal tracing of spontaneous regression and anti-angiogenic response of individual microadenomas during colon tumorigenesis. *Theranostics*. 5(7):724–32.

Chu LY, Guo DM, Chen JT, Fang WK, Xie JJ, Peng YH, Xu YW & Li XX. 2020. The Diagnostic Value of Serum L1CAM in Patients with Colorectal Cancer. *Technology in Cancer Research & Treatment.* 19: 1533033820920971.

Clapper, M.L., Chang, W.C.L. and Cooper, H.S., 2020. Dysplastic aberrant crypt foci: biomarkers of early colorectal neoplasia and response to preventive intervention. *Cancer prevention research*, *13*(3), pp.229-240.

Collins, A.R., 2014. Measuring oxidative damage to DNA and its repair with the comet assay. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1840*(2), pp.794-800.

Colucci, P., Turgeon, J. and Ducharme, M.P., 2011. How critical is the duration of the sampling scheme for the determination of half-life, characterization of exposure and assessment of bioequivalence?. *Journal of Pharmacy & Pharmaceutical Sciences*, *14*(2), pp.217-226.

Coméra, C., Cartier, C., Gaultier, E., Catrice, O., Panouille, Q., El Hamdi, S., Tirez, K., Nelissen, I., Théodorou, V. and Houdeau, E., 2020. Jejunal villus absorption and paracellular tight junction permeability are major routes for early intestinal uptake of food-grade TiO 2 particles: an in vivo and ex vivo study in mice. *Particle and fibre toxicology*, *17*(1), pp.1-15.

Conteduca V, Sansonno D, Russi S and Dammacco F. 2013. Precancerous colorectal lesions (review). *International Journal of Oncology.* 43(4): 973-984.

Coombes, J.L. and Powrie, F., 2008. Dendritic cells in intestinal immune regulation. *Nature reviews immunology*, *8*(6), pp.435-446.

Coreas, R., Cao, X., DeLoid, G.M., Demokritou, P. and Zhong, W., 2020. Lipid and protein corona of food-grade TiO_2 nanoparticles in simulated gastrointestinal digestion. *NanoImpact*, *20*, p.100272.

COT 2022. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment Interim position paper on titanium dioxide. Available online at https://cot.food.gov.uk/sites/default/files/2022-01/TiO2%20COT%20Interim%20position%20paper.pdf

Dankovic, D., Kuempel, E. and Wheeler, M., 2007. An approach to risk assessment for TiO_2. *Inhalation toxicology*, *19*(sup1), pp.205-212.

de Dicastillo CL, Correa MG, Martínez FB, Streitt C, Galotto MJ. 2020. Antimicrobial Effect of Titanium Dioxide Nanoparticles. In: Mareș M, Lim SHE, Lai K, Cristina R, editors. Antimicrobial Resistance - A One Health Perspective. London: IntechOpen. Available online at: https://www.intechopen.com/chapters/70919 [Accessed 19 April 2022].

Dekanski D, Spremo-Potparevic B, Bajic V, Zivkovic L, Topalovic D, Sredojevic DN, Lazic V and Nedeljkovic JM. 2018. Acute toxicity study in mice of orally administrated TiO_2 nanoparticles functionalized with caffeic acid. *Food and Chemical Toxicology*. 115: 42–48.

Dekker E, Tanis PJ, Vleugels JLA, Kasi PM and Wallace MB. 2019. Colorectal cancer. *The Lancet.* 394(10207): 1467-1480.

Delmaar, C.J., Peijnenburg, W.J., Oomen, A.G., Chen, J., de Jong, W.H., Sips, A.J., Wang, Z. and Park, M.V., 2015. A practical approach to determine dose metrics for nanomaterials. *Environmental toxicology and chemistry*, *34*(5), pp.1015-1022.

DeLoid, G.M., Wang, Y., Kapronezai, K., Lorente, L.R., Zhang, R., Pyrgiotakis, G., Konduru, N.V., Ericsson, M., White, J.C., De La Torre-Roche, R. and Xiao, H., 2017. An integrated methodology for assessing the impact of food matrix and gastrointestinal effects on the biokinetics and cellular toxicity of ingested engineered nanomaterials. *Particle and fibre toxicology*, *14*(1), pp.1-17.

Di Bucchianico, S., Cappellini, F., Le Bihanic, F., Zhang, Y., Dreij, K. and Karlsson, H.L., 2017. Genotoxicity of TiO_2 nanoparticles assessed by mini-gel comet assay and micronucleus scoring with flow cytometry. *Mutagenesis*, *32*(1), pp.127-137.

Di Gregorio C, Losi L, Fante R, Modica S, Ghidoni M, Pedroni M, Tamassia MG, Gafa L, Ponz de Leon M and Roncucci L. 1997. Histology of aberrant crypt foci in the human colon. *Histopathology.* 30: 328-334.

Dillon, A. and Lo, D.D., 2019. M cells: intelligent engineering of mucosal immune surveillance. *Frontiers in immunology*, *10*, p.1499.

Doak SH, Manshian B, Jenkins GJS, Singh N. (2012). *In vitro* genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. *Mutation Research.* 745(1-2):104-111.

Dobrzynska MM, Gajowik A, Radzikowska J, Lankoff A, Dusinska M and Kruszewski M. 2014. Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats *in vivo*. *Toxicology.* 315: 86–91.

Dodds, W.J., 2016. Adjuvants and additives in human and animal vaccines. *Medical Research Archives*, (3).

Donner EM, Myhre A, Brown SC, Boatman R and Warheit DB. 2016. *In vivo* micronucleus studies with 6 titanium dioxide materials (3 pigment-grade & 3 nanoscale) in orally-exposed rats. *Regulatory Toxicology and Pharmacology*. 74: 64–74.

Dorier M, Beal D, Marie-Desvergne C, Dubosson M, Barreau F, Houdeau E, Herlin-Boime N, Carriere M. 2017. Continuous *in vitro* exposure of intestinal epithelial cells to E171 food additive causes oxidative stress, inducing oxidation of DNA bases but no endoplasmic reticulum stress. *Nanotoxicology*. 11(6): 751–761.

Dorier M, Tisseyre C, Dussert F, Beal D, Arnal ME, Douki T, Valdiglesias V, Laffon B, Fraga S, Brandao F, Herlin-Boime N, Barreau Rabilloud T and Carriere M. 2019. Toxicological impact of acute exposure to E171 food additive and TiO2 nanoparticles on a co-culture of Caco-2 and HT29-MTX intestinal cells. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis.* 845: 402980.

Drew DA, Mo A, Grady JJ, Stevens RG, Levine JB, Brenner BM, Anderson JC, Forouhar F, O'Brien MJ, Devers TJ and Rosenberg DW. 2018. Proximal aberrant crypt foci associate with synchronous neoplasia and are primed for neoplastic progression. *Molecular Cancer Research*. 16: 486–495.

Driscoll, K.E., Deyo, L.C., Carter, J.M., Howard, B.W., Hassenbein, D.G. and Bertram, T.A., 1997. Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. *Carcinogenesis*, *18*(2), pp.423-430.

Duan, S.M., Zhang, Y.L., Gao, Y.J., Lyu, L.Z. and Wang, Y., 2021. The influence of long-term dietary intake of titanium dioxide particles on elemental homeostasis and tissue structure of mouse organs. *Journal of Nanoscience and Nanotechnology*, *21*(10), pp.5014-5025.

Dudefoi, W., Rabesona, H., Rivard, C., Mercier-Bonin, M., Humbert, B., Terrisse, H. and Ropers, M.H., 2021. In vitro digestion of food grade TiO_2 (E171) and TiO_2 nanoparticles: physicochemical characterization and impact on the activity of digestive enzymes. *Food & Function*.

Dudefoi, W., Terrisse, H., Richard-Plouet, M., Gautron, E., Popa, F., Humber, B., and Ropers, M.-H., 2017a. Criteria to define a more relevant reference sample of titanium dioxide in the context of food: a multiscale approach. *Food Additives & Contaminants: Part A*, 34(5), pp.653-665.

Dudefoi, W., Moniz, K., Allen-Vercoe, E., Ropers, M.H. and Walker, V.K., 2017b. Impact of food grade and nano-TiO_2 particles on a human intestinal community. *Food and Chemical Toxicology*, *106*, pp.242-249.

Dunkel VC, Zeiger E, Brusick D, McCoy E, McGregor D, Mortelmans K, Rosenkranz HS and Simmon VF, 1985. Reproducibility of microbial mutagenicity assays: II. Testing of carcinogens and noncarcinogens in Salmonella typhimurium and Escherichia coli. *Environmental Mutageneis.* 7(Suppl 5): 1–248.

Dumon, J.C. and Ernst, W.H.O., 1988. Titanium in plants. *Journal of Plant Physiology*, *133*(2), pp.203-209.

Ebrahimzadeh Bideskan, A, Mohammadipour, A., Fazel, A., Haghir, H., Rafatpanah, H., Hosseini, M. and Rajabzadeh, A., 2017. Maternal exposure to titanium dioxide nanoparticles during pregnancy and lactation alters offspring hippocampal mRNA BAX and Bcl-2 levels, induces apoptosis and decreases neurogenesis. *Experimental and toxicologic pathology*, *69*(6), pp.329-337.

EBRC 2022. EBRC Consulting GmbH. Summary Report - Relative oral bioavailability of 5 different TiO2 grades. LPT Report No. 36219, Fraunhofer Report No. U-EBR-250/6-27. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

ECETOC. European Centre for Ecotoxicology and Toxicology of Chemicals. 2013. Poorly Soluble Particles/Lung Overload. Brussels, Belgium. Technical Report number 122.

ECHA (European Chemicals Agency). 2022a. Reach registration dossier for titanium dioxide. Repeated dose toxicity: oral. Available online. https://echa.europa.eu/fr/registration-dossier/-/registered-dossier/15560/7/6/2/?documentUUID=4dee57a2-3cd7-4dcb-8b53-a34c53c06569 [Accessed 22 March 2022].

ECHA (European Chemicals Agency). 2022b. Reach registration dossier for titanium dioxide. Toxicity to reproduction. Available online. https://echa.europa.eu/fr/registration-dossier/-/registered-dossier/15560/7/9/2 [Accessed 23 March 2022].

EFSA Scientific Committee. 2012. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. *EFSA Journal.* 10(3):2579, 32 pp. https://doi.org/10.2903/j.efsa.2012.2579

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food), 2016. Re-evaluation of titanium dioxide (E171) as a food additive. *EFSA Journal.* 14(9):4545, 83 pp.

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food). 2018a. Evaluation of four new studies on the potential toxicity of titanium dioxide used as a food additive (E 171). *EFSA Journal*. 16 (7): 5366. https://doi.org/10.2903/j.efsa.2016.4545 [Accessed 18 May 2021].

EFSA (European Food Safety Authority) Scientific Committee. 2018b. Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health. EFSA Journal 2018;16(7):5327, 95 pp.

EFSA (European Food Safety Authority), 2019a. EFSA statement on the review of the risks related to the exposure to the food additive titanium dioxide (E 171) performed by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES). *EFSA Journal*, *17*(6), p.e05714.

EFSA FAF Panel (EFSA Panel on Food Additive and Flavourings). 2019b. Scientific opinion on the proposed amendment of the EU specification for titanium dioxide (E 171) with respect to the inclusion of additional parameters related to its particle size distribution. *EFSA Journal*. 17(7): 5760.

EFSA FAF Panel (European Food Safety Authority Panel on Food Additives and Flavourings). 2020. Re-evaluation of sodium aluminium silicate (E 554) and potassium aluminium silicate (E 555) as food additives. EFSA J. 18:e06152.

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), Younes M, Aquilina G, Castle L, Engel K-H, Fowler P, Frutos Fernandez MJ, Furst P, Gundert-Remy U, Gurtler R, Husøy T, Manco M, Mennes W, Moldeus P, Passamonti S, Shah R, Waalkens-Berendsen I, Wolfle D, Corsini E, Cubadda F, De Groot D, FitzGerald R, Gunnare S, Gutleb AC, Mast J, Mortensen A, Oomen A, Piersma A, Plichta V, Ulbrich B, Van Loveren H, Benford D, Bignami M, Bolognesi C, Crebelli R, Dusinska M, Marcon F, Nielsen E, Schlatter J, Vleminckx C, Barmaz S, Carfi M, Civitella C, Giarola A, Rincon AM, Serafimova R, Smeraldi C, Tarazona J, Tard A and Wright M. 2021a. Scientific Opinion on the safety assessment of titanium dioxide (E171) as a food additive. *EFSA Journal.* 19(5):6585, 130 pp. https://doi.org/10.2903/j.efsa.2021.6585

EFSA Scientific Committee, More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Hougaard Bennekou, S., Koutsoumanis, K., Lambré, C. and Machera, K., 2021b. Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. *EFSA Journal*, *19*(8), p.e06768.

El-Bassyouni GT, Eshak MG, Barakat IAH and Khalil WKB. 2017. Immunotoxicity evaluation of novel bioactive composites in male mice as promising orthopaedic implants. *Central European Journal of Immunology*. 42: 54–67.

El-Din EAA, Mostafa HE, Samak MA, Mohamed EM and El-Shafei DA. 2019. Could curcumin ameliorate titanium dioxide nanoparticles effect on the heart? A histopathological, immunohistochemical, and genotoxic study. *Environmental Science and Pollution Research*. 26: 21556–21564.

El-Ghor AA, Noshy MM, Gala A and Mohamed HRH. 2014. Normalization of nano-sized TiO_2-induced clastogenicity, genotoxicity and mutagenicity by chlorophyllin administration in mice brain, liver, and bone marrow cells. *Toxicological Sciences*. 142: 21–32.

Elnagar AMB, Ibrahim A and Soliman AM. 2018. Histopathological effects of titanium dioxide nanoparticles and the possible protective role of N-acetylcysteine on the testes of male albino rats. *International Journal of Fertility & Sterility*. 12: 249–256.

Evans S.J., Clift M.J.D and S.H. Doak. 2021a. Evaluating the *in vitro* cellular uptake of titanium dioxide. Unpublished study report from the *In Vitro* Toxicology Group, Institute of Life Science, Swansea University Medical School dated 29/01/2021, submitted to Health Canada by the TDMA.

Evans S.J., Clift M.J.D and S.H. Doak. 2021b. Evaluating the *in vitro* cellular uptake of titanium dioxide. Unpublished study report from the *In Vitro* Toxicology Group, Institute of Life Science, Swansea University Medical School dated 29/01/2021, submitted to Health Canada by the TDMA.

Fadda LM, Ali HM, Mohamed AM and Hagar H. 2019. Prophylactic administration of carnosine and melatonin abates the incidence of apoptosis, inflammation, and DNA damage induced by titanium dioxide nanoparticles in rat livers. *Environmental Science and Pollution Research International*. 27: 19142–19150.

Fadda LM, Hagar H, Mohamed AM and Ali HM. 2018. Quercetin and idebenone ameliorate oxidative stress, inflammation, DNA damage, and apoptosis induced by titanium dioxide nanoparticles in rat liver. *Dose-Response*. 16(4): 1-9.

Fadoju O, Ogunsuyi O, Akanni O, Alabi O, Alimba C, Adaramoye O, Cambier S, Eswara S, Gutleb AC and Bakare A. 2019. Evaluation of cytogenotoxicity and oxidative stress parameters in male Swiss mice co-exposed to titanium dioxide and zinc oxide nanoparticles. *Environmental Toxicology and Pharmacology*, 70: 103204.

Farrell, T.P. and Magnuson, B., 2017. Absorption, distribution and excretion of four forms of titanium dioxide pigment in the rat. *Journal of food science*, *82*(8), pp.1985-1993.

Fasoli, E., 2020. Protein corona: Dr. Jekyll and Mr. Hyde of nanomedicine. *Biotechnology and Applied Biochemistry*.

Filho, J.C., Moreira, R.A., Crocker, P.R., Levison, D.A. and Corrin, B., 1991. Identification of titanium pigment in drug addicts' tissues. *Histopathology*, *19*(2), pp.190-192.

Fine, J.H., Bondy, G.S., Coady, L., Pearce, B., Ross, N., Tayabali, A.F., Halappanavar, S., Caldwell, D., Curran, I. and Lefebvre, D.E., 2016. Immunomodulation by gastrointestinal carbon black nanoparticle exposure in ovalbumin T cell receptor transgenic mice. *Nanotoxicology*, *10*(10), pp.1422-1430.

Fleischer, C.C. and Payne, C.K., 2014. Secondary structure of corona proteins determines the cell surface receptors used by nanoparticles. *The Journal of Physical Chemistry B*, *118*(49), pp.14017-14026.

Foerster, M., Schreiver, I., Luch, A. and Schüz, J., 2020. Tattoo inks and cancer. *Cancer epidemiology*, *65*.

Franz P, Burkle A, Wick P, Hirsch C. 2020. Exploring flow cytometry-based micronucleus scoring for reliable nanomaterial genotoxicity assessment. *Chemical Research in Toxicology*. 33: 2538-2549.

Furukawa F, Nishikawa A, Kitahori Y, Tanakamaru Z, Hirose M. 2002. Spontaneous development of aberrant crypt foci in F344 rats. *Journal of Experimental & Clinical Cancer Research: CR.* 21(2):197-201.

Gao, Y., Ye, Y., Wang, J., Zhang, H., Wu, Y., Wang, Y., Yan, L., Zhang, Y., Duan, S., Lv, L. and Wang, Y., 2020. Effects of titanium dioxide nanoparticles on nutrient absorption and metabolism in rats: distinguishing the susceptibility of amino acids, metal elements, and glucose. *Nanotoxicology*, *14*(10), pp.1301-1323.

Garcia-Rodriguez A, Vila L, Cortes C, Hernandez A and Marcos R, 2018. Exploring the usefulness of the complex in vitro intestinal epithelial model Caco-2/HT29/Raji-B in nanotoxicology. *Food and Chemical Toxicology*. 113: 162–170.

Gea M, Bonetta S, Iannarelli L, Giovannozzi AM, Maurino V, Bonetta S, Hodoroaba VD, Armato C, Rossi AM, Schilirò T. 2019. Shape-engineered titanium dioxide nanoparticles (TiO2- NPs): cytotoxicity and genotoxicity in bronchial epithelial cells. *Food and Chemical Toxicology*. 127: 89–100.

Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schürch, S., Kreyling, W., Schulz, H., Semmler, M., Hof, V.I., Heyder, J. and Gehr, P., 2005. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environmental health perspectives*, *113*(11), pp.1555-1560.

Geiss O, Bianchi I, Senaldi C, Bucher G, Verleysen E, Waegeneers N, Brassinne F, Mast J, Loeschner K, Vidmar J, Aureli F, Cubadda F, Raggi A, Iacoponi F, Peters R, Undas A, Muller A, Meinhardt AK, Walz E, Graf V and Barrero-Moreno J, 2021. Particle size analysis of pristine food-grade titanium dioxide and E171 in confectionery products: Interlaboratory testing of a single-particle inductively coupled plasma mass spectrometry screening method and confirmation with transmission electron microscopy. Food Control, 120,107550.

Geiss, O., Ponti, J., Senaldi, C., Bianchi, I., Mehn, D., Barrero, J., Gilliland, D., Matissek, R. and Anklam, E., 2020. Characterisation of food grade titania with respect to nanoparticle content in pristine additives and in their related food products. *Food Additives & Contaminants: Part A*, *37*(2), pp.239-253.

Geraets, L., Oomen, A.G., Krystek, P., Jacobsen, N.R., Wallin, H., Laurentie, M., Verharen, H.W., Brandon, E.F. and de Jong, W.H., 2014. Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. *Particle and fibre toxicology*, *11*(30), pp.1-21.

Gerloff K, Albrecht C, Boots AW, Forster I, Schins RPF. 2009. Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. Nanotoxicology 3: 355–364.

Ghebretatios, M., Schaly, S. and Prakash, S., 2021. Nanoparticles in the food industry and their impact on human gut microbiome and diseases. *International Journal of Molecular Sciences*, *22*(4), p.1942.

Gilbert, J.D., Neubauer, K. and Byard, R.W., 2021. Macroscopic identification of visceral titanium pigment in an intravenous drug user. *Journal of Forensic Sciences*.

Grissa, I., El Ghoul, J., Mrimi, R., El Mir, L., Cheikh, H.B. and Horcajada, P., 2020. In deep evaluation of the neurotoxicity of orally administered TiO_2 nanoparticles. *Brain research bulletin*, *155*, pp.119-128.

Grissa I, Ezzi L, Chakroun S, Mabrouk A, Saleh AB, Braham H, Haouas Z and Cheikh HB. 2017. *Rosmarinus officinalis* L. ameliorates titanium dioxide nanoparticles and induced some toxic effects in rats' blood. *Environmental Science and Pollution Research*. 24: 12474–12483.

Grissa I, Elghoul J, Ezzi L, Chakroun S, Kerkeni E, Hassine M, El Mir L, Mehdi M, Ben Cheikh H and Haouas Z. 2015. Anemia and genotoxicity induced by sub-chronic intragastric treatment of rats with titanium dioxide nanoparticles. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis.* 794: 25–31.

Guillard, A., Gaultier, E., Cartier, C., Devoille, L., Noireaux, J., Chevalier, L., Morin, M., Grandin, F., Lacroix, M., Coméra, C. and Cazanave, A., 2020. Basal Ti level in the human placenta and meconium and evidence of a materno-foetal transfer of food-grade TiO 2 nanoparticles in an ex vivo placental perfusion model. *Particle and fibre toxicology*, *17*(1), pp.1-15.

Hackenberg, S., Friehs, G., Kessler, M., Froelich, K., Ginzkey, C., Koehler, C., Scherzed, A., Burghartz, M. and Kleinsasser, N., 2011. Nanosized titanium dioxide particles do not induce DNA damage in human peripheral blood lymphocytes. *Environmental and molecular mutagenesis*, *52*(4), pp.264-268.

Hamilton, L.E., 2013. Titanium Pigment Deposition in an Intravenous Drug User. *Academic Forensic Pathology*, *3*(4), pp.486-489.

Han, H.Y., Yang, M.J., Yoon, C., Lee, G.H., Kim, D.W., Kim, T.W., Kwak, M., Heo, M.B., Lee, T.G., Kim, S. and Oh, J.H., 2020. Toxicity of orally administered food-grade titanium dioxide nanoparticles. *Journal of Applied Toxicology*.

Hashem MM, Abo-El-Sooud K, Abd-Elhakim YM, Badr YA, El-Metwally AE and Bahy-El-Dien A. 2020. The long-term oral exposure to titanium dioxide impaired immune functions and triggered cytotoxic and genotoxic impacts in rats. *Journal of Trace Elements in Medicine and Biology.* 60: 126473.

Hassanein KM and El-Amir YO. 2017. Protective effects of thymoquinone and avenanthramides on titanium dioxide nanoparticles induced toxicity in Sprague-Dawley rats. *Pathology - Research and Practice.* 213: 13–22.

Haynes, V.N., Ward, J.E., Russell, B.J. and Agrios, A.G., 2017. Photocatalytic effects of titanium dioxide nanoparticles on aquatic organisms—Current knowledge and suggestions for future research. *Aquatic toxicology*, *185*, pp.138-148.

Hendrickson OD, Platonova TA, Piidvorova SM, Zherdev AV, Gmoshinsky IV, Vasilevskaya LS, Shumakova AA, Hotimchenko SA and Dzantiev BB, 2020. Electron-microscopic investigation of the distribution of titanium dioxide (rutile) nanoparticles in the rats' small intestine mucosa. Liver, and Spleen Current Nanoscience, 16, 268–279.

Hendrickson OD, Pridvorova SM, Zherdev AV, Klochkov SG, Novikova OV, Shevtsova EF, Bachurin SO and Dzantiev BB, 2016. Size-dependent differences in biodistribution of titanium dioxide nanoparticles after sub-acute intragastric administrations to rats. Current Nanoscience, 12, 228–236.

Hentz KL., 2010. 3.03 - Safety assessment of pharmaceuticals. In: CA McQueen (Ed.), Comprehensive Toxicology (Second Edition), Elsevier, Oxford, 2010, pp. 17–28.

Heo MB, Kwak M, An KS, Kim HJ, Ryu HY, Lee SM, Song KS, Kim IY, Kwon JH and Lee TG, 2020. Oral toxicity of titanium dioxide P25 at repeated dose 28-day and 90-day in rats. Part Fibre Toxicol, 17, 34.

Heringa, M.B., Peters, R.J.B., Bleys, R.L.A.W., Van Der Lee, M.K., Tromp, P.C., Van Kesteren, P.C.E., Van Eijkeren, J.C.H., Undas, A.K., Oomen, A.G. and Bouwmeester, H., 2018. Detection of titanium particles in human liver and spleen and possible health implications. *Particle and fibre toxicology*, *15*(1), p.15.

Hermsen M, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends J-W, Williams R, Giaretti W, de Goeij A, Meijer G. 2002. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. *Gastroenterology*. 123(4): 1109-1119.

Hielscher, K., 2011. Innovative use of ultrasound in the manufacture of paints and coatings. *Composites*, pp.2-4.

Huang, C., Sun, M., Yang, Y., Wang, F., Ma, X., Li, J., Wang, Y., Ding, Q., Ying, H., Song, H. and Wu, Y., 2017. Titanium dioxide nanoparticles prime a specific activation state of macrophages. *Nanotoxicology*, *11*(6), pp.737-750.

Huang, Y.Y. and Terentjev, E.M., 2012. Dispersion of carbon nanotubes: mixing, sonication, stabilization, and composite properties. *Polymers*, *4*(1), pp.275-295.

Hummel, T.Z., Kindermann, A., Stokkers, P.C., Benninga, M.A. and ten Kate, F.J., 2014. Exogenous pigment in Peyer patches of children suspected of having IBD. *Journal of pediatric gastroenterology and nutrition*, *58*(4), pp.477-480.

Humphries A and Wright NA. 2008. Colonic crypt organization and tumorigenesis. *Nature Reviews Cancer.* 8: 415-424.

Hurlstone DP, Karajeh M, Sanders DS, Drew SK, Cross SS. 2005. Rectal aberrant crypt foci identified using high-magnification-chromoscopic colonoscopy: biomarkers for flat and depressed neoplasia. The American Journal of Gastroenterology. 100(6):1283-1289.

IARC Work. Group Eval. Carcinog. Risks Hum. 2010. Carbon black, titanium dioxide, and talc. IARC Monogr. Eval. Carcinog. Risks Hum. 93:1–413

Ivett JL, Brown BM, Rodgers C, Anderson BE, Resnick MA and Zeiger E, 1989. Chromosomal aberrations and sister chromatid exchange tests in Chinese hamster ovary cells in vitro. IV. Results with 15 chemicals. *Environmental and Molecular Mutagenesis*. 14: 165–187.

Janer, G., Del Molino, E.M., Fernández-Rosas, E., Fernández, A. and Vázquez-Campos, S., 2014. Cell uptake and oral absorption of titanium dioxide nanoparticles. *Toxicology Letters*, *228*(2), pp.103-110.

JECFA 1969. Joint FAO/WHO Expert Committee on Food Additives. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anti-caking agents and certain other substances. FAO Nutrition Meetings Report Series No. 46A WHO/FOOD ADD/70.36. Available online at http://www.inchem.org/documents/jecfa/jecmono/v46aje19.htm [Accessed 17 May 2021].

JECFA 2000. Guidelines for the preparation of toxicological working papers for the Joint FAO/WHO Expert Committee on Food Additives. Available online at https://www.who.int/foodsafety/chem/jecfa/en/tox_guidelines.pdf [Accessed 03 February 2022].

JECFA 2012. Compendium of food additive specifications. 76th Meeting, Geneva, Switzerland, 5-14 June 2012. FAO JECFA Monograph 13. pg. 31-34.

Jensen DM, Lohr M, Sheykhzade M, Lykkesfeldt J, Wils RS, Loft S and Moller P. 2019. Telomere length and genotoxicity in the lung of rats following intragastric exposure to food-grade titanium dioxide and vegetable carbon particles. *Mutagenesis*. 34: 203–214.

Jensen, D.M., Christophersen, D.V., Sheykhzade, M., Skovsted, G.F., Lykkesfeldt, J., Münter, R., Roursgaard, M., Loft, S. and Møller, P., 2018. Vasomotor function in rat arteries after ex vivo and intragastric exposure to food-grade titanium dioxide and vegetable carbon particles. *Particle and fibre toxicology*, *15*(1), pp.1-18.

JRC. 2016. European Commission Joint Research Centre. JRC Nanomaterial Repository. List of Representative Nanomaterials. Available online at https://joint-research-centre.ec.europa.eu/system/files/2016-06/JRC%2520Nanomaterials%2520Repository-List%2520of%2520Representative%2520Nanomaterials-201606.pdf [Accessed 15 March 2022].

Jo, M.R., Yu, J., Kim, H.J., Song, J.H., Kim, K.M., Oh, J.M. and Choi, S.J., 2016. Titanium dioxide nanoparticle-biomolecule interactions influence oral absorption. *Nanomaterials*, *6*(12), p.225.

Jones, K., Morton, J., Smith, I., Jurkschat, K., Harding, A.H. and Evans, G., 2015. Human in vivo and in vitro studies on gastrointestinal absorption of titanium dioxide nanoparticles. *Toxicology letters*, *233*(2), pp.95-101.

Kämpfer, A.A., Busch, M., Büttner, V., Bredeck, G., Stahlmecke, B., Hellack, B., Masson, I., Sofranko, A., Albrecht, C. and Schins, R.P., 2021. Model Complexity as Determining Factor for In Vitro Nanosafety Studies: Effects of Silver and Titanium Dioxide Nanomaterials in Intestinal Models. *Small*, *17*(15), p.2004223.

Kandeil MA, Mohammed ET, Hashem KS, Aleya L and Abdel-Daim MM, 2020. Moringa seed extract alleviates titanium oxide nanoparticles (TiO_2-NPs)-induced cerebral oxidative damage, and increases cerebral mitochondrial viability. *Environmental Science and Pollution Research.* 27: 19169-19184.

Kazimirova A, Baranokova M, Staruchova M, Drlickova M, Volkovova K and Dusinska M. 2019. Titanium dioxide nanoparticles tested for genotoxicity with the comet and micronucleus assays *in vitro*, *ex vivo* and *in vivo*. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis*. 843: 57–65.

Keenan, C., Elmore, S., Francke-Carroll, S., Kemp, R., Kerlin, R., Peddada, S., Pletcher, J., Rinke, M., Schmidt, S.P., Taylor, I. and Wolf, D.C., 2009. Best practices for use of historical control data of proliferative rodent lesions. *Toxicologic pathology*, *37*(5), pp.679-693.

Khashan KS, Sulaiman GM, Abdulameer FA, Albukhaty S, Ibrahem MA, Al-Muhimeed T, AlObaid AA. 2021. Antibacterial Activity of TiO_2 Nanoparticles Prepared by One-Step Laser Ablation in Liquid. *Applied Sciences*. 11(10): 4623.

Kirkland D, Aardema M, Henderson L, Müller L. 2005. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 584(1-2): 1-256.

Kirkland D, Pfuhler S, Tweats D, Aardema M, Corvi R, Darroudi F, Elhajouji A, Glatt H, Hastwell P, Hayashi M, Kasper P, Kirchner S, Lynch A, Marzin D, Maurici D, Meunier JR, Müller L, Nohynek G, Parry J, Parry E, Thybaud V, Tice R, van Benthem J, Vanparys P, White P. 2007. How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 628(1): 31-55.

Kirkland D, Uno Y, Luijten M, Beevers C, van Benthem J, Burlinson B, Dertinger S, Douglas GR, Hamada S, Horibata K, Lovell DP, Manjanatha M, Martus HJ, Mei N, Morita T, Ohyama W, Williams A. 2019. In vivo genotoxicity testing strategies: Report from the 7th International workshop on genotoxicity testing (IWGT). *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis*. 847:403035.

Komada, M., Takao, K. and Miyakawa, T., 2008. Elevated plus maze for mice. *JoVE (Journal of Visualized Experiments)*, (22), p.e1088.

Kowalczyk M, Orłowski M, Klepacki Ł, Zinkiewicz K, Kurpiewski W, Kaczerska D, Pesta W, Zielinski E and Siermontowski P. 2020. Rectal aberrant crypt foci (ACF) as a predictor of benign and malignant neoplastic lesions in the large intestine. *BMC Cancer*. 20: 133.

Kreyling, W.G., Holzwarth, U., Schleh, C., Kozempel, J., Wenk, A., Haberl, N., Hirn, S., Schäffler, M., Lipka, J., Semmler-Behnke, M. and Gibson, N., 2017a. Quantitative biokinetics of titanium dioxide nanoparticles after oral application in rats: part 2. *Nanotoxicology*, *11*(4), pp.443-453.

Kreyling, W.G., Holzwarth, U., Haberl, N., Kozempel, J., Hirn, S., Wenk, A., Schleh, C., Schäffler, M., Lipka, J., Semmler-Behnke, M. and Gibson, N., 2017b. Quantitative biokinetics of titanium dioxide nanoparticles after intravenous injection in rats: Part 1. *Nanotoxicology*, *11*(4), pp.434-442.

Kristiansen E. 1996. The role of aberrant crypt foci induced by the two heterocyclic amines 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) in the development of colon cancer in mice. *Cancer Letters.* 110(1-2): 187-192.

Kubiak, P., Fröschl, T., Hüsing, N., Hörmann, U., Kaiser, U., Schiller, R., Weiss, C.K., Landfester, K. and Wohlfahrt-Mehrens, M., 2011. TiO_2 anatase nanoparticle networks: Synthesis, structure, and electrochemical performance. *Small*, *7*(12), pp.1690-1696.

Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, van de Velde CJH and Watanabe T. 2015. Colorectal cancer. *Nature Reviews Disease Primers. 1: 15065.*

Kumar S, Meena R and Paulraj R. 2016. Role of macrophage (M1 and M2) in titanium-dioxide nanoparticle-induced oxidative stress and inflammatory response in rat. *Applied Biochemistry and Biotechnology.* 180: 1257–1275.

Kumari S, Mg S, Mayor S. 2010. Endocytosis unplugged: multiple ways to enter the cell. *Cell Research*. 20(3): 256-75.

Lance P and Hamilton SR. 2008. Sporadic aberrant crypt foci are not a surrogate endpoint for colorectal adenoma prevention. *Cancer Prevention Research*. 1(1): 4-8.

Lankoff, A., Sandberg, W.J., Wegierek-Ciuk, A., Lisowska, H., Refsnes, M., Sartowska, B., Schwarze, P.E., Meczynska-Wielgosz, S., Wojewodzka, M. and Kruszewski, M., 2012. The effect of agglomeration state of silver and titanium dioxide nanoparticles on cellular response of HepG2, A549 and THP-1 cells. *Toxicology letters*, *208*(3), pp.197-213.

Lea, T., 2015. Caco-2 cell line. *The impact of food bioactives on health*, pp.103-111.

Lee, H., 2021. Molecular Modeling of Protein Corona Formation and Its Interactions with Nanoparticles and Cell Membranes for Nanomedicine Applications. *Pharmaceutics*, *13*(5), p.637.

Lefebvre, D.E., Pearce, B., Fine, J.H., Chomyshyn, E., Ross, N., Halappanavar, S., Tayabali, A.F., Curran, I. and Bondy, G.S., 2014. In vitro enhancement of mouse T helper 2 cell sensitization to ovalbumin allergen by carbon black nanoparticles. *Toxicological Sciences*, *138*(2), pp.322-332.

Lefebvre, D.E., Venema, K., Gombau, L., Valerio Jr, L.G., Raju, J., Bondy, G.S., Bouwmeester, H., Singh, R.P., Clippinger, A.J., Collnot, E.M. and Mehta, R., 2015. Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices. *Nanotoxicology*, *9*(4), pp.523-542.

Li, G., Lv, L., Fan, H., Ma, J., Li, Y., Wan, Y. and Zhao, X.S., 2010. Effect of the agglomeration of TiO2 nanoparticles on their photocatalytic performance in the aqueous phase. *Journal of colloid and interface science*, *348*(2), pp.342-347.

Li, B., Chua, S.L., Yu, D., Chan, S.H. and Li, A., 2021a. Separation and size characterization of highly polydisperse titanium dioxide nanoparticles (E171) in powdered beverages by using Asymmetric Flow Field-Flow Fractionation hyphenated with Multi-Angle Light Scattering and Inductively Coupled Plasma Mass Spectrometry. *Journal of Chromatography A*, *1643*, p.462059.

Li, Y., Jiang, K., Cao, H., Yuan, M. and Xu, F., 2021b. Influences of a standardized food matrix and gastrointestinal fluids on the physicochemical properties of titanium dioxide nanoparticles. *RSC Advances*, *11*(19), pp.11568-11582.

Li, X., Zhang, Y., Li, B., Cui, J., Gao, N., Sun, H., Meng, Q., Wu, S., Bo, J., Yan, L. and Wu, J., 2019. Prebiotic protects against anatase titanium dioxide nanoparticles-induced microbiota-mediated colonic barrier defects. *NanoImpact*, *14*, p.100164.

Li, J., Yang, S., Lei, R., Gu, W., Qin, Y., Ma, S., Chen, K., Chang, Y., Bai, X., Xia, S. and Wu, C., 2018. Oral administration of rutile and anatase TiO 2 nanoparticles shifts mouse gut microbiota structure. *Nanoscale*, *10*(16), pp.7736-7745.

Li, Q., Li, T., Liu, C., DeLoid, G., Pyrgiotakis, G., Demokritou, P., Zhang, R., Xiao, H. and McClements, D.J., 2017a. Potential impact of inorganic nanoparticles on macronutrient digestion: titanium dioxide nanoparticles slightly reduce lipid digestion under simulated gastrointestinal conditions. *Nanotoxicology*, *11*(9-10), pp.1087-1101.

Li Y, Yan J, Ding W, Chen Y, Pack LM and Chen T. 2017b. Genotoxicity and gene expression analyses of liver and lung tissues of mice treated with titanium dioxide nanoparticles. *Mutagenesis*. 32: 33–46.

Li N, Ma L and Wang J. 2010. Interaction Between Nano-Anatase TiO_2 and Liver DNA from Mice In Vivo. *Nanoscale Research Letters*. 5(108).

Lima, M.A.D., Santos, V.M.D., Demachki, S. and Lazo, J., 2004. Titanium pigment in tissues of drug addicts: report of 5 necropsied cases. *Revista do Hospital das Clinicas*, *59*(2), pp.86-88.

Liu, N., Tang, M. and Ding, J., 2020. The interaction between nanoparticles-protein corona complex and cells and its toxic effect on cells. *Chemosphere*, *245*, p.125624.

LNE (Laboratoire national de métrologie et d'essais). 2020. Test Report for titanium dioxide powder sample E171-E. File P201779 – Document DMSI/1. 18 June 2020. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

LNE (Laboratoire national de métrologie et d'essais). 2021a. Test Report for rat diet TW20-top. File P207941 – Document DMSI/1. 18 January 2021. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

LNE (Laboratoire national de métrologie et d'essais). 2021b. Test Report for rat diet TW20-middle. File P207941 – Document DMSI/2. 18 January 2021. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

LNE (Laboratoire national de métrologie et d'essais). 2021c. Test Report for rat diet TW20-bottom. File P207941 – Document DMSI/3. 18 January 2021. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

LNE (Laboratoire national de métrologie et d'essais). 2021d. Test Report for rat diet TW21-leftover. File P207941 – Document DMSI/4. 18 January 2021. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

Lomer, M.C., Grainger, S.L., Ede, R., Catterall, A.P., Greenfield, S.M., Cowan, R.E., Vicary, F.R., Jenkins, A.P., Fidler, H., Harvey, R.S. and Ellis, R., 2005. Lack of efficacy of a reduced microparticle diet in a multi-centred trial of patients with active Crohn's disease. *European journal of gastroenterology & hepatology*, *17*(3), pp.377-384.

Lotfi A, Zirak RG, Moghadam MS and Pazooki N. 2016. Effects of the interaction of nanorutile TiO_2 with vincristine sulfate on chromosomal abnormalities in vivo. *International Journal of Advanced Biotechnology and Research.* 7: 1083–1093.

Louro H, Tavares A, Vital N, Costa PM, Alverca E, Zwart E, de Jong W, Fessard V, Lavinha J and Silva MJ. 2014. Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using *LacZ* plasmid based transgenic mice. *Environmental and Molecular Mutagenesis*. 55: 500–509.

LPT (Laboratory Pharmacology and Toxicology), 2020. Interim Report - EOGRT study of Titanium dioxide E171-E in rats by oral administration via the diet. Report No. 36222. Submitted by the European Commission on 27 May 2020; updated version of the report submitted by Titanium Dioxide Manufacturers Association on 1st September 2020 [as cited in EFSA 2021a].

MacNicoll, A., Kelly, M., Aksoy, H., Kramer, E., Bouwmeester, H. and Chaudhry, Q., 2015. A study of the uptake and biodistribution of nano-titanium dioxide using in vitro and in vivo models of oral intake. *Journal of Nanopartide Research*, *17*(2), pp.1-20.

Madsen, J.L., Kroghsbo, S., Madsen, C.B., Pozdnyakova, I., Barkholt, V. and Bøgh, K.L., 2014. The impact of structural integrity and route of administration on the antibody specificity against three cow's milk allergens-a study in Brown Norway rats. *Clinical and Translational Allergy*, *4*(1), pp.1-11.

Magdolenova, Z., Bilaničová, D., Pojana, G., Fjellsbø, L.M., Hudecova, A., Hasplova, K., Marcomini, A. and Dusinska, M., 2012. Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. *Journal of Environmental Monitoring*, *14*(2), pp.455-464.

Magnuson BA, Carr I and Bird RP. 1993. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats few cholic acid. *Cancer Research.* 53:499-4504.

Manivannan J, Banerjee R and Mukherjee A. 2020. Genotoxicity analysis of rutile titanium dioxide nanoparticles in mice after 28 days of repeated oral administration. *The Nucleus*. 63: 17–24.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P. and Bachmann, M.F., 2008. Nanoparticles target distinct dendritic cell populations according to their size. *European journal of immunology*, *38*(5), pp.1404-1413.

Marín, R.R., Babick, F. and Stintz, M., 2017. Ultrasonic dispersion of nanostructured materials with probe sonication–practical aspects of sample preparation. *Powder technology*, *318*, pp.451-458.

Martínez-Negro, M., González-Rubio, G., Aicart, E., Landfester, K., Guerrero-Martínez, A. and Junquera, E., 2021. Insights into colloidal nanoparticle-protein corona interactions for nanomedicine applications. *Advances in Colloid and Interface Science*, p.102366.

Martins Jr ADC, Azevedo LF, de Souza Rocha CC, Carneiro MFH, Venancio VP, de Almeida MR, Antunes LMG, de Carvalho Hott R, Rodrigues JL, Ogunjimi AT, Adeyemi JA and Barbosa Jr F. 2017. Evaluation of distribution, redox parameters, and genotoxicity in Wistar rats co-exposed to silver and titanium dioxide nanoparticles. *Journal of Toxicology and Environmental Health. Part A.* 80: 1156–1165.

Marucco, A., Prono, M., Beal, D., Alasonati, E., Fisicaro, P., Bergamaschi, E., Carriere, M. and Fenoglio, I., 2020. Biotransformation of Food-Grade and Nanometric TiO_2 in the Oral–Gastro–Intestinal Tract: Driving Forces and Effect on the Toxicity toward Intestinal Epithelial Cells. *Nanomaterials*, *10*(11), p.2132.

McClements, D.J., DeLoid, G., Pyrgiotakis, G., Shatkin, J.A., Xiao, H. and Demokritou, P., 2016. The role of the food matrix and gastrointestinal tract in the assessment of biological properties of ingested engineered nanomaterials (iENMs): State of the science and knowledge gaps. *NanoImpact*, *3*, pp.47-57.

McClements, D.J. and Xiao, H., 2017. Is nano safe in foods? Establishing the factors impacting the gastrointestinal fate and toxicity of organic and inorganic food-grade nanoparticles. *npj Science of Food*, *1*(1), pp.1-13.

Medina-Reyes, E.I., Delgado-Buenrostro, N.L., Díaz-Urbina, D., Rodríguez-Ibarra, C., Déciga-Alcaraz, A., González, M.I., Reyes, J.L., Villamar-Duque, T.E., Flores-Sánchez, M.L., Hernández-Pando, R. and Mancilla-Díaz, J.M., 2020. Food-grade titanium dioxide (E171) induces anxiety, adenomas in colon and goblet cells hyperplasia in a regular diet model and microvesicular steatosis in a high fat diet model. *Food and Chemical Toxicology*, *146*, p.111786.

Meena R, Kajal K and Paluraj R. 2015a. Cytotoxic and genotoxic effects of titanium dioxide nanoparticles in testicular cells of male Wistar rat. *Applied Biochemistry and Biotechnology*, 175: 825–840.

Meena R, Kumar S and Paulraj R. 2015b. Titanium oxide (TiO_2) nanoparticles in induction of apoptosis and inflammatory response in brain. *Journal of Nanoparticle Research.* 17(49).

Minigalieva IA, Katsnelson BA, Privalova LI, Sutunkova MP, Gurvich VB, Shur VY, Shishkina EV, Valamina IE, Makeyev OH, Panov VG, Varaksin AN, Bushueva TV, Sakhautdinova RR, Klinova SV, Solovyeva SN and Meshtcheryakova EY. 2018. Combined Subchronic Toxicity of Aluminum (III), Titanium (IV) and Silicon (IV) Oxide Nanoparticles and Its Alleviation with a Complex of Bioprotectors. *International Journal of Molecular Sciences.* 19(3): 837.

Modrzynska J, Berthing T, Ravn-Haren G, Jacobsen NR, Weydahl IK, Loeschner K, Mortensen A, Saber AT and Vogel U. 2018. Primary genotoxicity in the liver following pulmonary exposure to carbon black nanoparticles in mice. *Particle and Fibre Toxicology.* 15(2).

Mohamed HR and Hussien NA. 2016. Genotoxicity studies of titanium dioxide nanoparticles (TiO_2NPs) in the brain of mice. *Scientifica.* 6710840.

Mohamed, H.R.H., 2015. Estimation of TiO_2 nanoparticle-induced genotoxicity persistence and possible chronic gastritis-induction in mice. *Food and Chemical Toxicology*, *83*, pp.76-83.

Moran-Martinez J, del Rio-Parra RB, Betancourt-Martinez ND, Garcia-Garza R, Jimenez-Villarreal J, Nino-Castaneda MS, Nava-Rivera LE, Umana JAF, Carranza-Rosales P and Perez-Vertti RDA. 2018. Evaluation of the coating with TiO_2 nanoparticles as an option for the improvement of the characteristics of NiTi archwires: histopathological, cytotoxic, and genotoxic evidence. *Journal of Nanomaterials.* 2585918.

Mortensen, N.P., Moreno Caffaro, M., Aravamudhan, S., Beeravalli, L., Prattipati, S., Snyder, R.W., Watson, S.L., Patel, P.R., Weber, F.X., Montgomery, S.A. and Sumner, S.J., 2021. Simulated gastric digestion and in vivo intestinal uptake of orally administered CuO nanoparticles and TiO2 E171 in male and female rat pups. *Nanomaterials 11(6)*, p.1487.

MRI 1974. Midwest Research Institute. Titanium Dioxide Manufacturers Association. Identity and purity report for TiO_2 (Unitane® 0-220) lot no: 402110C 46 used in the NCI 1979 cancer bioassay. Unpublished report dated 11 July 1974 provided to Health Canada by the National Toxicology Program (NTP).

MRI 1975a. Midwest Research Institute. Titanium Dioxide Manufacturers Association. Identity and purity report for TiO_2 (Unitane® 0-220) lot no: 402129A29 used in the NCI 1979 cancer bioassay. Unpublished report dated 06 March 1975 provided to Health Canada by the National Toxicology Program (NTP).

MRI 1975b. Midwest Research Institute. Titanium Dioxide Manufacturers Association. Identity and purity report for TiO_2 (Unitane® 0-220) lot no: 402129 B20 used in the NCI 1979 cancer bioassay. Unpublished report dated 26 March 1975 provided to Health Canada by the National Toxicology Program (NTP).

Mrowetz, M., Pirola, C. and Selli, E., 2003. Degradation of organic water pollutants through sonophotocatalysis in the presence of TiO_2. *Ultrasonics sonochemistry*, *10*(4-5), pp.247-254.

Mumtaz, A., Ibrahim, M.S., Siddiqui, N.R., Safdar, M.N., Munir, M., Qayyum, A., Shibli, S. and Ibrahim, M.K., 2019. Ultrasounds: A recent perspective in food industry. *Pakistan Journal of Agricultural Research*, *32*(2), p.334.

Murugadoss, S., Brassinne, F., Sebaihi, N., Petry, J., Cokic, S.M., Van Landuyt, K.L., Godderis, L., Mast, J., Lison, D., Hoet, P.H. and van den Brule, S., 2020. Agglomeration of titanium dioxide nanoparticles increases toxicological responses in vitro and in vivo. *Particle and fibre toxicology*, *17*(1), pp.1-14.

Myhr BC and Caspary WJ, 1991. Chemical mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells: results for 31 coded compounds in the National Toxicology Program. *Environmental and Molecular Mutagenesis*. 18: 51–83.

NANOGENOTOX 2013. Available online at https://www.anses.fr/en/content/nanogenotox-final-report

Nature Nanotechnology (unsigned editorial). 2020. The risks of nanomaterial risk assessment. *Nat. Nanotechnol.* 15, 163.

NCI (National Cancer Institute) 1979. Bioassay of titanium dioxide for possible carcinogenicity. *National Cancer Institute Carcinogenesis Technical Report Series.* 97: 1–123.

Nierenberg, D., Khaled, A.R. and Flores, O., 2018. Formation of a protein corona influences the biological identity of nanomaterials. *Reports of Practical Oncology and Radiotherapy*, *23*(4), pp.300-308.

Niu L, Shao M, Liu Y, Hu J, Li R, Xie H, Zhou L, Shi L, Zhang R and Niu Y. 2017. Reduction of oxidative damages induced by titanium dioxide nanoparticles correlates with induction of the Nrf2 pathway by GSPE supplementation in mice. *Chemico-Biological Interactions.* 275: 133–144.

Nogueira, C.M., de Azevedo, W.M., Dagli, M.L.Z., Toma, S.H., de Arruda Leite, A.Z., Lordello, M.L., Nishitokukado, I., Ortiz-Agostinho, C.L., Duarte, M.I.S., Ferreira, M.A. and Sipahi, A.M., 2012. Titanium dioxide induced inflammation in the small intestine. *World journal of gastroenterology: WJG*, *18*(34), p.4729.

NVWA (Netherlands Food and Consumer Product Safety Authority), 2019. Opinion of BuRO on possible health effects of the food additive titanium dioxide (E171). Trcvwa/2019/4476/EN.

OECD (Organisation for Economic Co-operation and Development). 2014. Genotoxicity of manufactured nanomaterials: Report of the OECD expert meeting. Series on the Safety of Manufacturered Nanomaterials. No. 43. ENV/JM/MONO(2014)34. Available online at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)34&doclanguage=en [Accessed 04 April 2022].

OECD (Organisation for Economic Co-operation and Development). 2017. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015. Report of the OECD expert meeting. Series on Testing & Assessment. No. 238 – 2nd edition. ENV/JM/MONO(2016)33/REV1. Available online at: https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-JM-MONO(2016)33/rev1&doclanguage=en [Accessed 09 April 2022].

Pan, Z., Lee, W., Slutsky, L., Clark, R.A., Pernodet, N. and Rafailovich, M.H., 2009. Adverse effects of titanium dioxide nanoparticles on human dermal fibroblasts and how to protect cells. *Small*, *5*(4), pp.511-520.

Parrino F and Palmisano F (eds). 2020. *Titanium Dioxide (TiO_2) and Its Applications*. Metal Oxides Series. Elsevier.

Parveen, R., Shamsi, T.N. and Fatima, S., 2017. Nanoparticles-protein interaction: Role in protein aggregation and clinical implications. *International journal of biological macromolecules*, *94*, pp.386-395.

Pele, L.C., Thoree, V., Bruggraber, S.F., Koller, D., Thompson, R.P., Lomer, M.C. and Powell, J.J., 2015. Pharmaceutical/food grade titanium dioxide particles are absorbed into the bloodstream of human volunteers. *Particle and fibre toxicology*, *12*(1), pp.1-6.

Pellegrino, F., Pellutiè, L., Sordello, F., Minero, C., Ortel, E., Hodoroaba, V.D. and Maurino, V., 2017. Influence of agglomeration and aggregation on the photocatalytic activity of TiO2 nanoparticles. *Applied Catalysis B: Environmental*, *216*, pp.80-87.

Peters, R.J., Oomen, A.G., van Bemmel, G., van Vliet, L., Undas, A.K., Munniks, S., Bleys, R.L., Tromp, P.C., Brand, W. and van der Lee, M., 2020. Silicon dioxide and titanium dioxide particles found in human tissues. *Nanotoxicology*, *14*(3), pp.420-432.

Petrier C, Jeunet A, Luche JL and Reverdy G. 1992. Unexpected frequency effects on the rate of oxidative processes induced by ultrasound. *J AM Chem Soc.* 114(8): 31-48-3150.

Phue, W.H., Xu, K. and George, S., 2022. Inorganic food additives nanomaterials alter the allergenicity of milk proteins. *Food and Chemical Toxicology*, p.112874.

Pinget, G., Tan, J., Janac, B., Kaakoush, N.O., Angelatos, A.S., O'Sullivan, J., Koay, Y.C., Sierro, F., Davis, J., Divakarla, S.K. and Khanal, D., 2019. Impact of the food additive titanium dioxide (E171) on gut microbiota-host interaction. *Frontiers in nutrition*, *6*, p.57.

Prasad R, Wallace YK, Daniel KM, Tennant AH, Zucker RM, Strickland J, Dreher K, Kligerman AD, Blackman CF and DeMarini.DM. 2013. Effect of treatment media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle. *ACS Nano.* 7(3): 1929–1942.

Pretlow TP, O'Riordan MA, Somich GA, Amini SB and Pretlow TG. 1992. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis (Lond.).* 13:1509-1512.

Pretlow TP, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA and Stellato TA. 1991. Aberrant crypt foci: Putative preneoplastic foci in human colonic mucosa. *Cancer Research*. 51:1564-1567.

Proquin H, Rodriguez-Ibarra C, Moonen CG, Urrutia Ortega IM, Briede JJ, de Kok TM, van Loveren H, Chirino YI. 2017. Titanium dioxide food additive (E171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions. *Mutagenesis*, 32, 139–149.

Proquin, H., Jetten, M.J., Jonkhout, M.C., Garduno-Balderas, L.G., Briede, J.J., de Kok, T.M., Chirino, Y.I. and van Loveren, H., 2018a. Gene expression profiling in colon of mice exposed to food additive titanium dioxide (E171). *Food and chemical toxicology*, *111*, pp.153-165.

Proquin, H., Jetten, M.J., Jonkhout, M.C., Garduño-Balderas, L.G., Briedé, J.J., de Kok, T.M., van Loveren, H. and Chirino, Y.I., 2018b. Transcriptomics analysis reveals new insights in E171-induced molecular alterations in a mouse model of colon cancer. *Scientific reports*, *8*(1), pp.1-16.

Prüst, M., Meijer, J. and Westerink, R.H., 2020. The plastic brain: neurotoxicity of micro-and nanoplastics. *Partide and fibre toxicology*, *17*, pp.1-16.

Quintanilla I, Lopez-Ceron M, Jimeno M, Cuatrecasas M, Zabalza M, Moreira L, Alonso V, Rodrıguez de Miguel C, Munoz J, Castellvi-Bel S, Llach J, Castells A, Balaguer F, Camps J and Pellise M. 2019. Rectal aberrant crypt foci in humans are not surrogate markers for colorectal cancer risk. *Clinical and Translational Gastroenterology*. 10:e00047.

Ramoju, S., Andersen, M.E., Nong, A., Karyakina, N., Shilnikova, N., Krishnan, K. and Krewski, D., 2020. Derivation of whole blood biomonitoring equivalents for titanium for the interpretation of biomonitoring data. *Regulatory Toxicology and Pharmacology*, *114*, p.104671.

Rampado, R., Crotti, S., Caliceti, P., Pucciarelli, S. and Agostini, M., 2020. Recent advances in understanding the protein corona of nanoparticles and in the formulation of "Stealthy" Nanomaterials. *Frontiers in bioengineering and biotechnology*, *8*.

Ranjan, S., Dasgupta, N., Verma, P. and Ramalingam, C., 2020. Acute and sub-chronic toxicity of titanium dioxide nanoparticles synthesized by microwave-irradiation-assisted hybrid chemical approach. *J Indian Chem Soc*, *97*, pp.483-491.

Ranjha, M.M.A., Irfan, S., Lorenzo, J.M., Shafique, B., Kanwal, R., Pateiro, M., Arshad, R.N., Wang, L., Nayik, G.A., Roobab, U. and Aadil, R.M., 2021. Sonication, a potential technique for extraction of phytoconstituents: a systematic review. *Processes*, *9*(8), p.1406.

Renz, H., Allen, K.J., Sicherer, S.H., Sampson, H.A., Lack, G., Beyer, K. and Oettgen, H.C., 2018. Food allergy. *Nature reviews Disease primers*, *4*(1), pp.1-20.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P. and Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology*, *2*(4), pp.361-367.

Riedle, S., Wills, J.W., Miniter, M., Otter, D.E., Singh, H., Brown, A.P., Micklethwaite, S., Rees, P., Jugdaohsingh, R., Roy, N.C. and Hewitt, R.E., 2020. A Murine Oral-Exposure Model for Nano-and Micro-Particulates: Demonstrating Human Relevance with Food-Grade Titanium Dioxide. *Small*, *16*(21), p.2000486.

Rinninella, E., Cintoni, M., Raoul, P., Mora, V., Gasbarrini, A. and Mele, M.C., 2021. Impact of Food Additive Titanium Dioxide on Gut Microbiota Composition, Microbiota-Associated Functions, and Gut Barrier: A Systematic Review of In Vivo Animal Studies. *International journal of environmental research and public health*, *18*(4), p.2008.

Rizk MZ, Ali SA, Kadry MO, Fouad GI, Kamel NN, Younis EA and Gouda SM. 2020. C-reactive protein signaling and chromosomal abnormalities in nanotoxicity induced via different doses of TiO_2 (80 nm) boost liver function. *Biological Trace Element Research*. 198: 157-167.

Rizk MZ, Ali SA, Hamed MA, El-Rigal NS, Aly HF and Salah HH. 2017. Toxicity of titanium dioxide nanoparticles: effect of dose and time on biochemical disturbance, oxidative stress and genotoxicity in mice. *Biomedicine & Pharmacotherapy*. 90: 466–472.

Rodrigues MAM, Silva LAG, Salvadori DMF, de Camargo JLV, Montenegro MR. 2002. Aberrant crypt foci and colon cancer: comparison between a short- and medium-term bioassay for colon carcinogenesis using dimethylhydrazine in Wistar rats. *Brazilian Journal of Medical and Biological Research.* 35: 351-355.

Rodríguez-Escamilla, J.C., Medina-Reyes, E.I., Rodríguez-Ibarra, C., Déciga-Alcaraz, A., Flores-Flores, J.O., Ganem-Rondero, A., Rodríguez-Sosa, M., Terrazas, L.I., Delgado-Buenrostro, N.L. and Chirino, Y.I., 2019. Food-grade titanium dioxide (E171) by solid or liquid matrix administration induces inflammation, germ cells sloughing in seminiferous tubules and blood-testis barrier disruption in mice. *Journal of Applied Toxicology*, *39*(11), pp.1586-1605.

Ropers, M.H., Terrisse, H., Mercier-Bonin, M. and Humbert, B., 2017. *Titanium dioxide as food additive* (pp. 1-22). Rijeka: InTech.

Rosenberg DW, Yang S, Pleau DC, Greenspan EJ, Stevens RG, Rajan TV, Heinen CD, Levine J, Zhou Y and O'Brien MJ. 2007. Mutations in BRAF and KRAS differentially distinguish serrated versus non-serrated hyperplastic aberrant crypt foci in humans. *Cancer Research.* 67: 3551-3554.

Runa, S., Lakadamyali, M., Kemp, M.L. and Payne, C.K., 2017. TiO_2 nanoparticle-induced oxidation of the plasma membrane: importance of the protein Corona. *The Journal of Physical Chemistry B*, *121*(37), pp.8619-8625.

Sadiq R, Bhalli JA, Yan J, Woodruff RS, Pearce MG, Li Y, Mustafa T, Watanabe F, Pack LM, Biris A, Khan QM and Chen T. 2012. Genotoxicity of TiO_2 anatase nanoparticles 383 in B6C3F1 male mice evaluated using *Pig-α* and flow cytometric micronucleus assays. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis.* 745: 65–72.

SCCS (Sci. Comm. Consumer Saf.). 2018. Opinion on titanium dioxide (nano form) as UV-filter in sprays. Opin. SCCS/1583/17, Sci. Comm. Consumer Saf., Eur. Comm., Luxembourg.

Schins, R.P. and Knaapen, A.M., 2007. Genotoxicity of poorly soluble particles. *Inhalation toxicology*, *19*(sup1), pp.189-198.

Schoen RE, Mutch M, Rall C, Dry SM, Seligson D, Umar A, and Pinsky P. 2008. The natural history of aberrant crypt foci. *Gastrointestinal Endoscopy*. 67(7):1097–102.

Schreiver, I., Hesse, B., Seim, C., Castillo-Michel, H., Villanova, J., Laux, P., Dreiack, N., Penning, R., Tucoulou, R., Cotte, M. and Luch, A., 2017. Synchrotron-based ν-XRF mapping and μ-FTIR microscopy enable to look into the fate and effects of tattoo pigments in human skin. *Scientific reports*, *7*(1), pp.1-12.

Sclafani A and Herrmann JM. 1996. Comparison of the photoelectronic and photocatalytic activities of various anatase and rutile forms of titania in pure liquid organic phases and in aqueous solutions. *Journal of Physical Chemistry.* 100(32): 13655-13661.

Selli, E., Bianchi, C.L., Pirola, C., Cappelletti, G. and Ragaini, V., 2008. Efficiency of 1, 4-dichlorobenzene degradation in water under photolysis, photocatalysis on TiO_2 and sonolysis. *Journal of Hazardous Materials*, *153*(3), pp.1136-1141.

Shelby MD and Witt KL. 1995. Comparison of results from mouse bone marrow aberration and micronucleus test. *Environmental and Molecular Mutagenesis*. 25: 302–313.

Shelby MD, Erexson GL, Hook GJ and Tice RR. 1993. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. *Environmental and Molecular Mutagenesis*. 21: 160–179.

Shepherd, N.A., Crocker, P.R., Smith, A.P. and Levison, D.A., 1987. Exogenous pigment in Peyer's patches. *Human pathology*, *18*(1), pp.50-54.

Shetye J, Rubio CA. 2004. The chronological appearance of flat colonic neoplasias in rats. *In Vivo.* 18(2): 197-202.

Shi Z, Niu Y, Wang Q, Shi L, Guo H, Liu Y, Zhu Y, Liu S, Liu C, Chen X and Zhang R. 2015. Reduction of DNA damage induced by titanium dioxide nanoparticles through Nrf2 in vitro and in vivo. *Journal of Hazardous Materials.* 298: 310–319.

Shpitz B, Hay K, Medline A, Bruce WR, Bull SB, Gallinger S, Stern H. 1996. Natural history of aberrant crypt foci. A surgical approach. *Diseases of the Colon and Rectum*. 39(7):763-767.

Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S. and Dhawan, A., 2011. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. *Toxicology in vitro*, *25*(1), pp.231-241.

Shukla RK, Kumar A, Vallabani NV, Pandey AK and Dhawan A. 2014. Titanium dioxide nanoparticle-induced oxidative stress triggers DNA damage and hepatic injury in mice. *Nanomedicine.* 9(9).

Shwter AN, Abdullah NA, Alshawsh MA, El-Seedi HR, Al-Henhena NA, Khalifa SA and Abdulla MA, 2016. Chemopreventive effect of Phaleria macrocarpa on colorectal cancer aberrant crypt foci in vivo. *Journal of Ethnopharmacology*. 193: 195–206.

Singh, G., Stephan, C., Westerhoff, P., Carlander, D. and Duncan, T.V., 2014. Measurement methods to detect, characterize, and quantify engineered nanomaterials in foods. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), pp.693-704.

Singh N, Dev I, Pal S, Yadav SK, Idris MM, Ansari KM. 2022. Transcriptomic and proteomic insights into patulin mycotoxin-induced cancer-like phenotypes in normal intestinal epithelial cells. *Molecular and Cellular Biochemistry*. https://doi.org/10.1007/s11010-022-04387-3

Singh, S., Shi, T., Duffin, R., Albrecht, C., van Berlo, D., Höhr, D., Fubini, B., Martra, G., Fenoglio, I., Borm, P.J. and Schins, R.P., 2007. Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO_2: role of the specific surface area and of surface methylation of the particles. *Toxicology and applied pharmacology*, *222*(2), pp.141-151.

Siskova A, Cervena K, Kral J, Hucl T, Vodicka P and Vymetalkova V. 2020. Colorectal adenomas-genetics and searching for new molecular screening biomarkers. International Journal of Molecular Sciences, 21, 3260.

Sofranko, A., Wahle, T., Heusinkveld, H.J., Stahlmecke, B., Dronov, M., Pijnenburg, D., Hilhorst, R., Lamann, K., Albrecht, C. and Schins, R.P., 2021. Evaluation of the neurotoxic effects of engineered nanomaterials in C57BL/6J mice in 28-day oral exposure studies. *NeuroToxicology*, *84*, pp.155-171.

Sohal IS., Cho YK., O'Fallon KS., Gaines P., Demokritou P., Bello D. (2018b) Dissolution Behavior And Biodurability Of Ingested Engineered Nanomaterials In The Gastrointestinal Environment. ACS Nano, 2018,12(8):8115-8128.

Sontag JM, Page NP, Saffiotti U, 1976. Guidelines for carcinogen bioassay in small rodent. NCI-CG-TR-1. DHEW Publication No. (NIH) 76-801.

Souza, M.R., Mazaro-Costa, R. and Rocha, T.L., 2021. Can nanomaterials induce reproductive toxicity in male mammals? A historical and critical review. *Science of The Total Environment*, p.144354.

Suzui M. Morioka T and Yoshimi N. 2013. Colon preneoplastic lesions in animal models. *Journal of Toxicologic Pathology.* 26(4): 335-341.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS. 2020. Genotoxicity assessment of titanium dioxide nanoparticle accumulation of 90 days in the liver of *gpt* delta transgenic mice. *Genes and Environment*. 42(7).

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS. 2016. Genotoxicity assessment of intravenously injected titanium dioxide nanoparticles in *gpt* delta transgenic mice. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis.* 802: 30–37.

Swiatkowska, I., Martin, N. and Hart, A.J., 2019. Blood titanium level as a biomarker of orthopaedic implant wear. *Journal of Trace Elements in Medicine and Biology*, *53*, pp.120-128.

Sycheva LP, Zhurkova VS, Iurchenkoa VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK and Durnev A. 2011. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. *Mutation Research*. 726:8–14.

Szakal, C., Tsytsikova, L., Carlander, D. and Duncan, T.V., 2014. Measurement methods for the oral uptake of engineered nanomaterials from human dietary sources: summary and outlook. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), pp.669-678.

Takayama T, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, Kato J, Kogawa K, Miyake H and Niitsu Y. 1998. Aberrant crypt foci of the colon as precursors of adenoma and cancer. *New England Journal of Medicine.* 339:1277-1284.

Talbot P, Radziwill-Bienkowska JM, Kamphuis JBJ, Steenkeste K, Bettini S, Robert V, Noordine ML, Mayeur C, Gaultier E, Langella P, Robbe-Masselot C, Houdeau E, Thomas M and Mercier-Bonin M, 2018. Food-grade TiO_2 is trapped by intestinal mucus in vitro but does not impair mucin O-glycosylatioand short-chain fatty acid synthesis in vivo: implications for gut barrier protection. Journal of Nanobiotechnology, 16, 53.

Talamini, L., Gimondi, S., Violatto, M.B., Fiordaliso, F., Pedica, F., Tran, N.L., Sitia, G., Aureli, F., Raggi, A., Nelissen, I. and Cubadda, F., 2019. Repeated administration of the food additive E171 to mice results in accumulation in intestine and liver and promotes an inflammatory status. *Nanotoxicology*, *13*(8), pp.1087-1101.

Tampakis A, Tampaki EC, Nonni A, Tsourouflis G, Posabella A, Patsouris E, Kontzoglou K, von Flüe M, Nikiteas N and Kouraklis G. 2020. L1CAM expression in colorectal cancer identifies a high-risk group of patients with dismal prognosis already in early-stage disease. *Medical Oncology.* 59(1): 55-59.

Tassinari R, Cubadda F, Moracci G, Aureli F, D'Amato M, Mauro Valeri M, De Berardis B, Raggi A, Mantovani A, Passeri D, Rossi M and Francesca Maranghi F, 2014. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology, 8, 654–662.

Taurozzi, J.S., Hackley, V.A. and Wiesner, M.R., 2011. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment–issues and recommendations. *Nanotoxicology*, *5*(4), pp.711-729.

TDMA 2019. Titanium Dioxide Manufacturers Association. Data on Particle Size and Particle Size Distribution for Titanium Dioxide (E171). Response by the Titanium Dioxide Manufacturers Association (TDMA) to the EFSA Call for Data published on 30th January 2017. June 2018. Updated March and June 2019. Unpublished report submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

TDMA 2022a. Titanium Dioxide Manufacturers Association. Comparison of current food grade titanium dioxide (E171) with historical samples of Unitane O-220. Unpublished draft TDMA|175a dated 03 February 2022 provided to Health Canada by the TDMA.

TDMA 2022b. Titanium Dioxide Manufacturers Association. Characterization of the feed matrix in the extended one-generation reproductive toxicity (EOGRT) study of titanium dioxide E 171-E in rats by oral administration via the diet carried out by LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG, Hamburg, Germany Report No. 36222. Summary report. 16 February 2022. Unpublished reported submitted to Health Canada by the Titanium Dioxide Manufacturers Association.

Tennant RW, Margolin BH, Shelby MD, Zeiger E, Haseman JK, Spalding J, Caspary W, Resnick M, Stasiewicz S, Anderson B and Minor R, 1987. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. *Science*. 236: 933–941.

Thoree, V., Skepper, J., Deere, H., Pele, L.C., Thompson, R.P.H. and Powell, J.J., 2008. Phenotype of exogenous microparticle-containing pigment cells of the human Peyer's patch in inflamed and normal ileum. *Inflammation Research*, *57*(8), pp.374-378.

Trouiller B, Reliene R, Westbrook A, Solaimani P and Schiestl RH. 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice. *Cancer Research*. 69: 8784–8789.

Tucker, L.B. and McCabe, J.T., 2017. Behavior of male and female C57BL/6J mice is more consistent with repeated trials in the elevated zero maze than in the elevated plus maze. *Frontiers in behavioral neuroscience*, *11*, p.13.

Uchida K, Kado S, Onoue M and Tohyama K. 1997. Relationship between the nature of mucus and crypt multiplicity in aberrant crypt foci in the rat colon. *Japanese Journal of Cancer Research.* 88: 807-814.

Ude VC, Brown DM, Viale L, Kanase N, Stone V, Johnston HJ. 2017. Impact of copper oxide nanomaterials on differentiated and undifferentiated Caco-2 intestinal epithelial cells; assessment of cytotoxicity, barrier integrity, cytokine production and nanomaterial penetration. *Particle and Fibre Toxicology.* 14: 31

Urrutia-Ortega, I.M., Garduño-Balderas, L.G., Delgado-Buenrostro, N.L., Freyre-Fonseca, V., Flores-Flores, J.O., González-Robles, A., Pedraza-Chaverri, J., Hernández-Pando, R., Rodríguez-Sosa, M., León-Cabrera, S. and Terrazas, L.I., 2016. Food-grade titanium dioxide exposure exacerbates tumor formation in colitis associated cancer model. *Food and Chemical Toxicology*, *93*, pp.20-31.

Verleysen E, Waegeneers N, De Vos S, Brassinne F, Ledecq M, Van Steen F, Andjelkovic M, Janssens R, Mathioudaki S, Delfosse L, Machiels R, Cheyns K and Mast J, 2021. Physicochemical characterization of nanoparticles in food additives in the context of risk identification. EFSA supporting publication 2021:EN-9992. 31 pp.

Verleysen E, Waegeneers N, Brassinne F, De Vos S, Jimenez IO, Mathioudaki S and Mast J, 2020. Physicochemical characterization of the pristine E171 food additive by standardized and validated methods. Nanomaterials (Basel), 10, 592.

Verma, A., Chhikara, I. and Dixit, D., 2014. Photocatalytic treatment of pharmaceutical industry wastewater over TiO_2 using immersion well reactor: synergistic effect coupling with ultrasound. *Desalination and Water Treatment*, *52*(34-36), pp.6591-6597.

Vila L, Garcia-Rodriguez A, Marcos R and Hernandez A, 2018. Titanium dioxide nanoparticles translocate through differentiated Caco-2 cell monolayers, without disrupting the barrier functionality or inducing genotoxic damage. *Applied Toxicology*. 1–11.

Vilanova, O., Mittag, J.J., Kelly, P.M., Milani, S., Dawson, K.A., Rädler, J.O. and Franzese, G., 2016. Understanding the kinetics of protein–nanoparticle corona formation. *ACS nano*, *10*(12), pp.10842-10850.

Vroman, L., 1962. Effect of adsorbed proteins on the wettability of hydrophilic and hydrophobic solids. *Nature*, *196*(4853), pp.476-477.

Wahn, U., Peters Jr, T. and Siraganian, R.P., 1981. Allergenic and antigenic properties of bovine serum albumin. *Molecular immunology*, *18*(1), pp.19-28.

Walczyk, D., Bombelli, F.B., Monopoli, M.P., Lynch, I. and Dawson, K.A., 2010. What the cell "sees" in bionanoscience. *Journal of the American Chemical Society*, *132*(16), pp.5761-5768.

Waller, T., Chen, C. and Walker, S.L., 2017. Food and industrial grade titanium dioxide impacts gut microbiota. *Environmental Engineering Science*, *34*(8), pp.537-550.

Walsh M, Clendenning M, Williamson E, Pearson S-A, Walters RJ, Nagler B, Packenas D, Win AK, Hopper JL, Jenkins MA, Haydon AM, Rosty C, English DR, Giles GG, McGuckin MA, Young MA, Young JP and Buchanan DD. 2013. Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype. *Modern Pathology.* 26:1642–1656

Wang, J., Wang, Y., Gao, J., Hu, P., Guan, H., Zhang, L., Xu, R., Chen, X. and Zhang, X., 2009. Investigation on damage of BSA molecules under irradiation of low frequency ultrasound in the presence of FeIII-tartrate complexes. *Ultrasonics sonochemistry*, *16*(1), pp.41-49.

Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J. and Li, Y., 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology letters*, *168*(2), pp.176-185.

Warheit, D.B., Brown, S.C. and Donner, E.M., 2015. Acute and subchronic oral toxicity studies in rats with nanoscale and pigment grade titanium dioxide particles. *Food and Chemical Toxicology*, *84*, pp.208-224.

Warheit, D.B., Hoke, R.A., Finlay, C., Donner, E.M., Reed, K.L. and Sayes, C.M., 2007. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. *Toxicology letters*, *171*(3), pp.99-110.

Warheit DB, Kreiling R, Levy LS. 2016. Relevance of the rat lung tumor response to particle overload for human risk assessment-Update and interpretation of new data since ILSI 2000. Toxicology. 374:42-59.

West, B. & Wyzan, H. (1963) Unpublished report by American Cyanamid Company [As cited in JECFA 1969].

WHO [World Health Organization] 1982. Titanium-Environmental Health Criteria 24.

Winkler, H.C., Notter, T., Meyer, U. and Naegeli, H., 2018. Critical review of the safety assessment of titanium dioxide additives in food. *Journal of nanobiotechnology*, *16*(1), pp.1-19.

Winter, M., Beer, H.D., Hornung, V., Krämer, U., Schins, R.P. and Förster, I., 2011. Activation of the inflammasome by amorphous silica and TiO_2 nanoparticles in murine dendritic cells. *Nanotoxicology*, *5*(3), pp.326-340.

Won HS, Maeng LS. Chae HS, Kim HK, Cho YS, Kang J-H, Jang HS, Ryu M-R. 2012. Sequential changes in aberrant crypt foci and lectin expression in the early and late stages of DMH-induced colon carcinogenesis in rats. *Gut Liver.* 6(2): 229-234.

Woodruff, R.S., Li, Y., Yan, J., Bishop, M., Jones, M.Y., Watanabe, F., Biris, A.S., Rice, P., Zhou, T. and Chen, T., 2012. Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay. *Journal of Applied Toxicology*, *32*(11), pp.934-943.

Wu Q-B and Sun G-P. 2015. Expression of COX-2 and HER-2 in colorectal cancer and their correlation. *World Joumal of Gastroenterology.* 21(20): 6206-6214.

Xu J, Shi H, Ruth M, Yu H, Lazar L, Zou B, Yang C, Wu A and Zhao J. 2013. Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. *PLoS ONE.* 8: e70618.

Yan J, Wang D, Li K, Chen Q, Lai W, Tian L, Lin B, Tan Y, Liu X and Xi Z, 2020. Toxic effects of the food additives titanium dioxide and silica on the murine intestinal tract: mechanisms related to intestinal barrier dysfunction involved by gut microbiota. Environmental Toxicology and Pharmacology, 80.

Yang, L., 2015. 2-Nanotechnology-enhanced metals and alloys for orthopedic implants. *Nanotechnology-Enhanced Orthopedic Materials. Oxford: Woodhead Publishing*, pp.27-47.

Yao, J.J., Lewallen, E.A., Trousdale, W.H., Xu, W., Thaler, R., Salib, C.G., Reina, N., Abdel, M.P., Lewallen, D.G. and van Wijnen, A.J., 2017. Local cellular responses to titanium dioxide from orthopedic implants. *BioResearch open access*, *6*(1), pp.94-103.

Yoshizaki, K., Asai, M. and Hara, T., 2020. High-fat diet enhances working memory in the Y-maze test in male C57BL/6J mice with less anxiety in the elevated plus maze test. *Nutrients*, *12*(7), p.2036.

Yu, X., Hong, F. and Zhang, Y.Q., 2016. Cardiac inflammation involving in PKCε or ERK1/2-activated NF-κB signalling pathway in mice following exposure to titanium dioxide nanoparticles. *Journal of hazardous materials*, *313*, pp.68-77.

Zhang, Z., Zhang, R., Xiao, H., Bhattacharya, K., Bitounis, D., Demokritou, P. and McClements, D.J., 2019. Development of a standardized food model for studying the impact of food matrix effects on the gastrointestinal fate and toxicity of ingested nanomaterials. *NanoImpact*, *13*, pp.13-25.

Zhang S, Jiang X, Cheng S, Fan J, Qin X, Wang T, Zhang Y, Zhang J, Qiu Y, Qiu J, Zou Z and Chen C, 2020. Titanium dioxide nanoparticles via oral exposure leads to adverse disturbance of gut microecology and locomotor activity in adult mice. Archives of Toxicology, 94, 1173–1190.

Zhang, T., Zhu, G., Lu, B., Qian, Z. and Peng, Q., 2021. Protein corona formed in the gastrointestinal tract and its impacts on oral delivery of nanoparticles. *Medicinal Research Reviews*, *41*(3), pp.1835-1850.

Zhou, D., Ji, Z., Jiang, X., Dunphy, D.R., Brinker, J. and Keller, A.A., 2013. Influence of material properties on TiO 2 nanoparticle agglomeration. *PloSone*, *8*(11), p.e81239.

Zirak RG, Lotfi A and Moghadam MS. 2016. Effects of the interaction of nanoanatase TiO_2 with bleomycin sulfate on chromosomal abnormalities in vivo. *International Journal of Advanced Biotechnology and Research.* 7: 1094–1108.

Appendix A – Literature Search Strategy

EMBASE

Database(s): **Embase** 1974 to 2021 June 29
Search Strategy:

#	Searches	Results
1	*titanium dioxide nanoparticle/ or *titanium dioxide/	16285
2	(Titanium dioxide or 13463-67-7 or E171 or E 171 or Pigment White 6 or Titanium oxide or 1385RN 59 or 1700 White or 234DA or 500HD or 63B1 White or "A 200 (pigment)" or "A 330 (pigment)" or A-Fil or A-Fil Cream or A-FN 3Aerolyst 7710 or Aerosil P 25 or Aerosil P 25S6 or Aerosil P 27 or Aerosil T 805 or AI3-01334 or "AK 15 (pigment)" or "Amperit 780.0" or AMT 100 or AMT 600 or Anatase or Atlas white titanium dioxide or AUF 0015S or Austiox R-CR 3 or "B 101 (pigment)" or Bayer R-FD 1 or Bayertitan A or Bayertitan AN 3 or Bayertitan R-FD 1 or Bayertitan R-FK 21 or Bayertitan R-FK-D or Bayertitan R-KB 2 or Bayertitan R-KB 3 or Bayertitan R-KB 4 or Bayertitan R-KB 5 or Bayertitan R-KB 6 or Bayertitan R-U 2 or Bayertitan R-U-F or Bayertitan R-V-SE 20 or Bayertitan T or Bistrater L-NSC 200C or Blend White 9202 or BR 29-7-2 or Brookite or "C 97 (oxide)" or C-Weiss 7 or "C.I. 77891" or "C.I. Pigment White" or Cab-O-Ti or Calcotone White T or CG-T or CI 77891 or CI Pigment white 6 or CL 310 or Cosmetic Hydrophobic TiO_2 9428 or Cosmetic Micro Blend TiO_2 9228 or Cosmetic White C47-5175 or Cosmetic White C47-9623 or Hombitan or Hombitan R 101D or Hombitan R 610K or Horse head a-410 or Horse Head A-410 or Horse head a-420 or Horse Head A-420 or Horse head r-710 or Horse Head R-710 or KH 360 or Kronos 2073 or Kronos cl 220 or Kronos RN 40P or Kronos RN 56 or Kronos titanium dioxide or Levanox White RKB or NCI-C04240 or NCI-C0424O or NSC 15204 or Orgasol 1002D White 10 Extra Cos or "P 25 (oxide)" or Pigment White 6 or Rayox or Rutile or Runa ARH 20 or Runa ARH 200 or Runa RH20 or Rutiox CR or Ti-Pure or Ti-pure R 900 or Ti-Pure R 901 or Tichlor or Tin dioxide dust or Tiofine or "Tiona T.D." or Tiona td or Tioxide or Tioxide A-DM or Tioxide AD-M or Tioxide R XL or Tioxide R-CR or Tioxide R-SM or "Tioxide R.XL" or Tioxide RHD or Tioxide RSM or Tipaque or Tipaque R 820 or Titafrance or Titan White or Titandioxid or Titania or Titanic anhydride or Titanic oxide or Titanium dioxide or Titanium oxide or Titanium peroxide or Titanium White or "Titanium(IV) oxide" or Titanox or Titanox 2010 or Titanox ranc or "Trioxide(s)" or Tronox or Unitane or Uniwhite AO or Uniwhite KO or Zopaque or Zopaque or TiO_2 13463-67-7 or 15FIX9V2JP).tw,kw.	18317
3	1 or 2 [Titanium dioxide]	25095
4	*Food dye/ or *food additive/ or exp *food/ or exp *beverage/ or *food intake/ or exp *diet/ or *dietary exposure/ or *food contamination/ or exp *infant feeding/	629558
5	(food or foods or feed or eat or eaten or diet or diets or dietary or drink* or consume or consumed or consuming).tw,kw.	1508666

6	(bakery or beer? or breast feed* or breastfeed* or cereal? or cider? or dairy or dietary or foodborne* or foodstuff* or grape? or ((infant or baby) adj2 formula*) or fruit? or juice* or meat? or milk or mushroom* or poultry or puree* or rice or spice? or vegetable* or wine?).tw,kw.	886601
7	(oral* adj4 (dos* or intak* or administ* or expos*)).tw,kw.	242759
8	((intravenous* or iv) adj4 (dos* or intak* or administ* or expos*)).tw,kw.	170415
9	*maternal exposure/ or *prenatal exposure/ or *meconium/ or (f?etal* or f?etus* or infant? or intrauterine or "in uter*" or maternal* or neonat* or "neo nat*" or newborn* or "newborn*" or "peri natal" or perinatal or pregnan* or "pre natal" or prenatal or placenta* or meconium?).tw,kw.	1769881
10	or/4-9	4046735
11	3 and 10	1911
12	exp *pharmacokinetics/ or exp *pharmacokinetic parameters/ or *pharmacodynamics/ or exp *drug mechanism/ or exp *drug response/ or exp *pharmacodynamic parameters/ or *toxicokinetics/	541702
13	((Titanium dioxide or 13463-67-7 or E171 or E 171 or Pigment White 6 or Titanium oxide or 1385RN 59 or 1700 White or 234DA or 500HD or 63B1 White or "A 200 (pigment)" or "A 330 (pigment)" or A-Fil or A-Fil Cream or A-FN 3Aerolyst 7710 or Aerosil P 25 or Aerosil P 25S6 or Aerosil P 27 or Aerosil T 805 or AI3-01334 or "AK 15 (pigment)" or "Amperit 780.0" or AMT 100 or AMT 600 or Anatase or Atlas white titanium dioxide or AUF 0015S or Austiox R-CR 3 or "B 101 (pigment)" or Bayer R-FD 1 or Bayertitan A or Bayertitan AN 3 or Bayertitan R-FD 1 or Bayertitan R-FK 21 or Bayertitan R-FK-D or Bayertitan R-KB 2 or Bayertitan R-KB 3 or Bayertitan R-KB 4 or Bayertitan R-KB 5 or Bayertitan R-KB 6 or Bayertitan R-U 2 or Bayertitan R-U-F or Bayertitan R-V-SE 20 or Bayertitan T or Bistrater L-NSC 200C or Blend White 9202 or BR 29-7-2 or Brookite or "C 97 (oxide)" or C-Weiss 7 or "C.I. 77891" or "C.I. Pigment White" or Cab-O-Ti or Calcotone White T or CG-T or CI 77891 or CI Pigment white 6 or CL 310 or Cosmetic Hydrophobic TiO_2 9428 or Cosmetic Micro Blend TiO_2 9228 or Cosmetic White C47-5175 or Cosmetic White C47-9623 or Hombitan or Hombitan R 101D or Hombitan R 610K or Horse head a-410 or Horse Head A-410 or Horse head a-420 or Horse Head A-420 or Horse head r-710 or Horse Head R-710 or KH 360 or Kronos 2073 or Kronos cl 220 or Kronos RN 40P or Kronos RN 56 or Kronos titanium dioxide or Levanox White RKB or NCI-C04240 or NCI-C0424O or NSC 15204 or Orgasol 1002D White 10 Extra Cos or "P 25 (oxide)" or Pigment White 6 or Rayox or Rutile or Runa ARH 20 or Runa ARH 200 or Runa RH20 or Rutiox CR or Ti-Pure or Ti-pure R 900 or Ti-Pure R 901 or Tichlor or Tin dioxide dust or Tiofine or "Tiona T.D." or Tiona td or Tioxide or Tioxide A-DM or Tioxide AD-M or Tioxide R XL or Tioxide R-CR or Tioxide R-SM or "Tioxide R.XL" or Tioxide RHD or Tioxide RSM or Tipaque or Tipaque R 820 or Titafrance or Titan White or Titandioxid or Titania or Titanic anhydride or Titanic oxide or Titanium dioxide or Titanium oxide or Titanium peroxide or Titanium White or "Titanium(IV) oxide" or Titanox or Titanox 2010 or Titanox ranc or "Trioxide(s)" or Tronox or Unitane or Uniwhite AO or Uniwhite KO or Zopaque or Zopaque or $TiO_2$13463-	817

	67-7 or 15FIX9V2JP) and (absorb* or absorp* or adsorp* or bioavailab* or biotransform* or eliminat* or half life or metabolis* or metaboliz* or plasma or accumulat* or activat* or clearance* or diffus* or distribut* or excret* or inactivat* or kinetic* or penetrat* or releas* or retention* or retain* or chronopharmacokinetic* or pharmacodynamic* or pharmacokinet* or neuropharmacol* or neuropharmacodynamic* or pharmacolo* or toxicokinet*)).ti.	
14	(absorb* or absorp* or adsorp* or bioavailab* or biotransform* or eliminat* or half life or metabolis* or metaboliz* or plasma or accumulat* or activat* or clearance* or diffus* or distribut* or excret* or inactivat* or kinetic* or penetrat* or releas* or retention* or retain* or chronopharmacokinetic* or pharmacodynamic* or pharmacokinet* or neuropharmacol* or neuropharmacodynamic* or pharmacolo* or toxicokinet*).ab. /freq=2	3903441
15	(((mode or modes or mechanism*) adj3 action*) or MoA).tw,kw.	225913
16	or/12-15 [pharmacokinetics pharmacodynamics]	4431039
17	11 and 16 [TiO_2 pharmacokinetics]	592
18	exp adverse drug reaction/ or exp death/ or drug safety/ or drug tolerance/ or drug interaction/ or food drug interaction/ or herb drug interaction/ or exp postmarketing surveillance/ or exp risk assessment/ or safety/ or exp side effect/ or case report/	5047290
19	exp toxicity/ or developmental toxicity/ or exp allergenicity/ or exp carcinogenicity/ or clastogen/ or exp genetic damage/ or exp hypersensitivity/ or exp mutagenicity/ or exp mutagenesis/	1609096
20	reproductive success/ or reproductive health/ or exp pregnancy complication/ or exp pregnancy disorder/	580014
21	(adverse* or allerg* or death? or fatal* or harm or harms or harmful or hypersensitiv* or interaction* or lethal* or risk or risks or safety or ((side* or acute or chronic) adj2 (effect* or event*)) or tolera* or toxic* or poison* or cardiotox* or cytotox* or dermatotox* or dermotox* or embryotox* or fetotox* or genotox* or hepatotox* or hepatox*or immunotox* or maternotox* or nephrotox* or neurotox* or ototoxic* or iatrogen* or teratogen* or mutagen* or cancer* or carcin* or malign* or tumor* or tumour* or case? report*).ti.	4909498
22	(adverse* or allerg* or death? or fatal* or harm or harms or harmful or hypersensitiv* or interaction* or lethal* or (risk? adj2 assess*) or safety or tolera* or toxic* or poison* or cardiotox* or cytotox* or dermatotox* or dermotox* or embryotox* or fetotox* or genotox* or hepatotox* or hepatox*or immunotox* or maternotox* or nephrotox* or neurotox* or ototoxic* or iatrogen* or teratogen* or mutagen* or cancer* or carcin* or malign* or tumor* or tumour* or case? report*).ab. /freq=2	5368138
23	(((gene* or genomic* or dna or chromosom*) adj3 (damag* or repair* or adduct? or aberrat* or break* or fragment*)) or clastogen* or micronucleus or mutation?).tw.	1163683
24	((reproduc* or pregnan* or gestat* or antenatal* or antepartum*) adj4 (tox* or fail* or disorder* or complicat* or health or aberrat* or success* or unsuccessful* or loss* or resorp*)).tw.	158261

25	((fetal or fetus or foetal or foetus or litter* or neonat* or offspring*) adj4 (complicat* or losing or loss* or lost or malform* or prematur* or resorp*)).tw.	43320
26	or/18-25	11888683
27	3 and (4 or 5 or 6 or 7) [TiO_2 food]	1684
28	26 and 27 [TiO_2 AE]	603
29	limit 28 to yr="2016-Current"	376

MEDALL

Database(s): **Ovid MEDLINE(R) ALL** 1946 to June 28, 2021

Search Strategy:

#	Searches	Results
1	(Titanium dioxide or 13463-67-7 or E171 or E 171 or Pigment White 6 or Titanium oxide or 1385RN 59 or 1700 White or 234DA or 500HD or 63B1 White or "A 200 (pigment)" or "A 330 (pigment)" or A-Fil or A-Fil Cream or A-FN 3Aerolyst 7710 or Aerosil P 25 or Aerosil P 25S6 or Aerosil P 27 or Aerosil T 805 or AI3-01334 or "AK 15 (pigment)" or "Amperit 780.0" or AMT 100 or AMT 600 or Anatase or Atlas white titanium dioxide or AUF 0015S or Austiox R-CR 3 or "B 101 (pigment)" or Bayer R-FD 1 or Bayertitan A or Bayertitan AN 3 or Bayertitan R-FD 1 or Bayertitan R-FK 21 or Bayertitan R-FK-D or Bayertitan R-KB 2 or Bayertitan R-KB 3 or Bayertitan R-KB 4 or Bayertitan R-KB 5 or Bayertitan R-KB 6 or Bayertitan R-U 2 or Bayertitan R-U-F or Bayertitan R-V-SE 20 or Bayertitan T or Bistrater L-NSC 200C or Blend White 9202 or BR 29-7-2 or Brookite or "C 97 (oxide)" or C-Weiss 7 or "C.I. 77891" or "C.I. Pigment White" or Cab-O-Ti or Calcotone White T or CG-T or CI 77891 or CI Pigment white 6 or CL 310 or Cosmetic Hydrophobic TiO_2 9428 or Cosmetic Micro Blend TiO_2 9228 or Cosmetic White C47-5175 or Cosmetic White C47-9623 or Hombitan or Hombitan R 101D or Hombitan R 610K or Horse head a-410 or Horse Head A-410 or Horse head a-420 or Horse Head A-420 or Horse head r-710 or Horse Head R-710 or KH 360 or Kronos 2073 or Kronos cl 220 or Kronos RN 40P or Kronos RN 56 or Kronos titanium dioxide or Levanox White RKB or NCI-C04240 or NCI-C0424O or NSC 15204 or Orgasol 1002D White 10 Extra Cos or "P 25 (oxide)" or Pigment White 6 or Rayox or Rutile or Runa ARH 20 or Runa ARH 200 or Runa RH20 or Rutiox CR or Ti-Pure or Ti-pure R 900 or Ti-Pure R 901 or Tichlor or Tin dioxide dust or Tiofine or "Tiona T.D." or Tiona td or Tioxide or Tioxide A-DM or Tioxide AD-M or Tioxide R XL or Tioxide R-CR or Tioxide R-SM or "Tioxide R.XL" or Tioxide RHD or Tioxide RSM or Tipaque or Tipaque R 820 or Titafrance or Titan White or Titandioxid or Titania or Titanic anhydride or Titanic oxide or Titanium dioxide or Titanium oxide or Titanium peroxide or Titanium White or "Titanium(IV) oxide" or Titanox or Titanox 2010 or Titanox ranc or "Trioxide(s)" or Tronox or Unitane or Uniwhite AO or Uniwhite KO or Zopaque or Zopaque or $TiO_2$13463-67-7 or 15FIX9V2JP).tw,kw,kf.	18857
2	exp *Food Additives/ or *Food Coloring Agents/ or exp *food/ or exp *eating/ or exp *diet/ or *Dietary Exposure/ or exp *food contamination/ or exp *Infant Nutritional Physiological Phenomena/	1014641

3	(food or foods or feed or eat or eaten or diet or diets or dietary or drink* or consume or consumed or consuming).tw,kw,kf.	1211170
4	(bakery or beer? or breast feed* or breastfeed* or cereal? or cider? or dairy or dietary or foodborne* or foodstuff* or grape? or ((infant or baby) adj2 formula*) or fruit? or juice* or meat? or milk or mushroom* or poultry or puree* or rice or spice? or vegetable* or wine?).tw,kf.	776704
5	(oral* adj4 (dos* or intak* or administ* or expos*)).tw,kf.	181258
6	((intravenous* or iv) adj4 (dos* or intak* or administ* or expos*)).tw,kf.	127203
7	*Maternal Exposure/ or *Maternal-Fetal Exchange/ or *Meconium/ or (f?etal* or f?etus* or infant? or intrauterine or "in uter*" or maternal* or neonat* or "neo nat*" or newborn* or "new born*" or "peri natal" or perinatal or pregnan* or "pre natal" or prenatal or placenta* or meconium?).tw,kw,kf.	1529769
8	or/2-7	3753899
9	1 and 8	1821
10	exp *Pharmacokinetics/ or *Toxicokinetics/	40318
11	((Titanium dioxide or 13463-67-7 or E171 or E 171 or Pigment White 6 or Titanium oxide or 1385RN 59 or 1700 White or 234DA or 500HD or 63B1 White or "A 200 (pigment)" or "A 330 (pigment)" or A-Fil or A-Fil Cream or A-FN 3Aerolyst 7710 or Aerosil P 25 or Aerosil P 25S6 or Aerosil P 27 or Aerosil T 805 or AI3-01334 or "AK 15 (pigment)" or "Amperit 780.0" or AMT 100 or AMT 600 or Anatase or Atlas white titanium dioxide or AUF 0015S or Austiox R-CR 3 or "B 101 (pigment)" or Bayer R-FD 1 or Bayertitan A or Bayertitan AN 3 or Bayertitan R-FD 1 or Bayertitan R-FK 21 or Bayertitan R-FK-D or Bayertitan R-KB 2 or Bayertitan R-KB 3 or Bayertitan R-KB 4 or Bayertitan R-KB 5 or Bayertitan R-KB 6 or Bayertitan R-U 2 or Bayertitan R-U-F or Bayertitan R-V-SE 20 or Bayertitan T or Bistrater L-NSC 200C or Blend White 9202 or BR 29-7-2 or Brookite or "C 97 (oxide)" or C-Weiss 7 or "C.I. 77891" or "C.I. Pigment White" or Cab-O-Ti or Calcotone White T or CG-T or CI 77891 or CI Pigment white 6 or CL 310 or Cosmetic Hydrophobic TiO_2 9428 or Cosmetic Micro Blend TiO_2 9228 or Cosmetic White C47-5175 or Cosmetic White C47-9623 or Hombitan or Hombitan R 101D or Hombitan R 610K or Horse head a-410 or Horse Head A-410 or Horse head a-420 or Horse Head A-420 or Horse head r-710 or Horse Head R-710 or KH 360 or Kronos 2073 or Kronos cl 220 or Kronos RN 40P or Kronos RN 56 or Kronos titanium dioxide or Levanox White RKB or NCI-C04240 or NCI-C04240 or NSC 15204 or Orgasol 1002D White 10 Extra Cos or "P 25 (oxide)" or Pigment White 6 or Rayox or Rutile or Runa ARH 20 or Runa ARH 200 or Runa RH20 or Rutiox CR or Ti-Pure or Ti-pure R 900 or Ti-Pure R 901 or Tichlor or Tin dioxide dust or Tiofine or "Tiona T.D." or Tiona td or Tioxide or Tioxide A-DM or Tioxide AD-M or Tioxide R XL or Tioxide R-CR or Tioxide R-SM or "Tioxide R.XL" or Tioxide RHD or Tioxide RSM or Tipaque or Tipaque R 820 or Titafrance or Titan White or Titandioxid or Titania or Titanic anhydride or Titanic oxide or Titanium dioxide or Titanium oxide or Titanium peroxide or Titanium White or "Titanium(IV) oxide" or Titanox or Titanox 2010 or Titanox ranc or "Trioxide(s)" or Tronox or Unitane or	882

	Uniwhite AO or Uniwhite KO or Zopaque or Zopaque or $TiO_2$13463-67-7 or 15FIX9V2JP) and (absorb* or absorp* or adsorp* or bioavailab* or biotransform* or eliminat* or half life or metabolis* or metaboliz* or plasma or accumulat* or activat* or clearance* or diffus* or distribut* or excret* or inactivat* or kinetic* or penetrat* or releas* or retention* or retain* or chronopharmacokinetic* or pharmacodynamic* or pharmacokinet* or neuropharmacol* or neuropharmacodynamic* or pharmacolo* or toxicokinet*)).ti.	
12	(absorb* or absorp* or adsorp* or bioavailab* or biotransform* or eliminat* or half life or metabolis* or metaboliz* or plasma or accumulat* or activat* or clearance* or diffus* or distribut* or excret* or inactivat* or kinetic* or penetrat* or releas* or retention* or retain* or chronopharmacokinetic* or pharmacodynamic* or pharmacokinet* or neuropharmacol* or neuropharmacodynamic* or pharmacolo* or toxicokinet*).ab. /freq=2	3141163
13	(((mode or modes or mechanism*) adj3 action*) or MoA).tw,kf.	182170
14	or/10-13 [pharmacokinetics pharmacodynamics]	3298198
15	9 and 14 [TiO_2 pharmacokinetics]	585
16	exp "Drug-Related Side Effects and Adverse Reactions"/ or exp Death/ or drug interactions/ or Food-Drug Interactions/ or herb-drug Interactions/ or Product Surveillance, Postmarketing/ or Risk Assessment/ or Safety/ or Case Reports/	2810050
17	exp Toxicity Tests/ or Abnormalities, Drug-induced/ or exp Chromosome Aberrations/ or exp DNA damage/ or Allergens/ or Carcinogens/ or exp Hypersensitivity/ or Mutagens/ or exp Mutagenicity Tests/ or exp Mutagenesis/	965367
18	Reproductive Health/ or exp Reproduction/ or exp Pregnancy complications/	1185868
19	(adverse* or allerg* or death? or fatal* or harm or harms or harmful or hypersensitiv* or interaction* or lethal* or risk or risks or safety or ((side* or acute or chronic) adj2 (effect* or event*)) or tolera* or toxic* or poison* or cardiotox* or cytotox* or dermatotox* or dermotox* or embryotox* or fetotox* or genotox* or hepatotox* or hepatox* or immunotox* or maternotox* or nephrotox* or neurotox* or ototoxic* or iatrogen* or teratogen* or mutagen* or cancer* or carcin* or malign* or tumor* or tumour* or case? report*).ti.	3940887
20	(adverse* or allerg* or death? or fatal* or harm or harms or harmful or hypersensitiv* or interaction* or lethal* or (risk? adj2 assess*) or safety or tolera* or toxic* or poison* or cardiotox* or cytotox* or dermatotox* or dermotox* or embryotox* or fetotox* or genotox* or hepatotox* or hepatox* or immunotox* or maternotox* or nephrotox* or neurotox* or ototoxic* or iatrogen* or teratogen* or mutagen* or cancer* or carcin* or malign* or tumor* or tumour* or case? report*).ab. /freq=2	3856054
21	(((gene* or genomic* or dna or chromosom*) adj3 (damag* or repair* or adduct? or aberrat* or break* or fragment*)) or clastogen* or micronucleus or mutation?).tw.	891588
22	((reproduc* or pregnan* or gestat* or antenatal* or antepartum*) adj4 (tox* or fail* or disorder* or complicat* or health or aberrat* or success* or unsuccessful* or loss* or resorp*)).tw.	121409

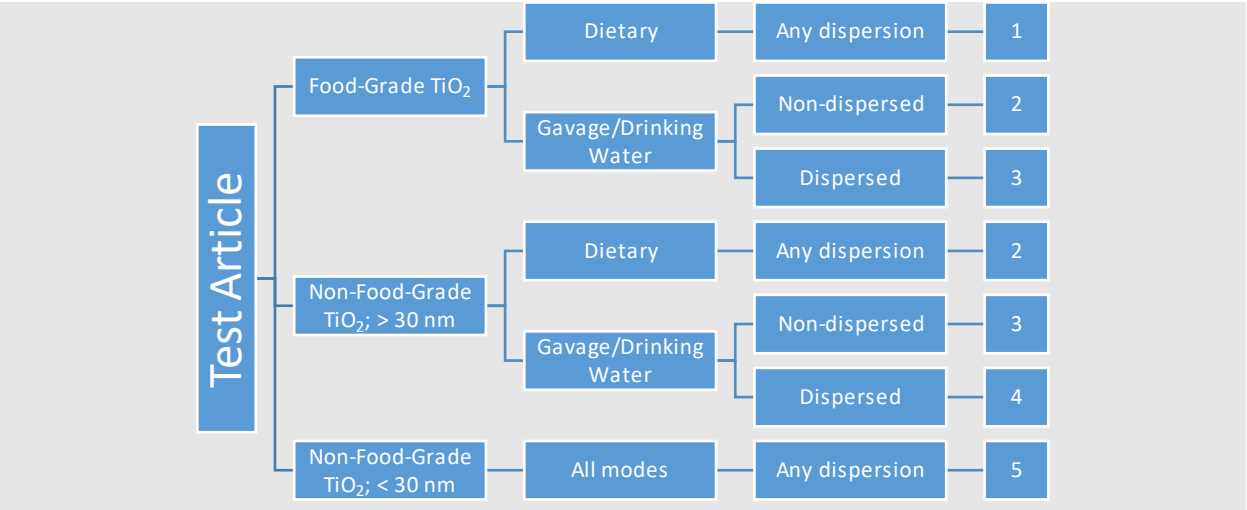
23	((fetal or fetus or foetal or foetus or litter* or neonat* or offspring*) adj4 (complicat* or losing or loss* or lost or malform* or prematur* or resorp*)).tw.	30871
24	or/16-23	9446176
25	1 and (2 or 3 or 4 or 5) [TiO_2 food]	1677
26	24 and 25 [TiO_2 AE]	534
27	limit 26 to yr="2016-Current"	324

Appendix B – Ranking Criteria

Study Ranking Criteria – *In Vivo*

In vivo studies were ranked according to A) relevance of test article and route of administration and B) reliability of results. Studies received an individual ranking for each of these criteria as well as an overall ranking. More details on each individual ranking and the overall ranking are provided below:

A) Relevance of Test Article and Route of Administration



B) Reliability of Results

The reliability of results was assigned a rank based on an assessment of following criteria:

Category	Criteria	Ranking
Characteristics of test article reported in study	Test article well characterized with information on particle size, crystalline form, surface treatments, and source provided	1
	Some information on the test article missing	2 or 3
Characteristics of test article verified	Test article verified using an appropriate analytical technique	1
	Test article used in study was not verified and instead authors relied on previously published information	2
	Test article was not verified and cannot be verified through other sources	3
Methodological Concerns	No major methodological concerns were identified	1
	Some methodological concerns were identified, but are unlikely to influence to outcome of the study	2
	Major methodological concerns have been identified and are likely to influence to outcome of the study	3
Statistical Concerns	No major statistical concerns were identified	1
	Some statistical concerns were identified, but are unlikely to influence to outcome of the study	2
	Major statistical concerns have been identified and are likely to influence to outcome of the study	3

Based on an assessment of the above criteria, the study was assigned an overall rank for reliability:

1) A reliability ranking of **'high'** was assigned if the majority of the criteria were classified as '1' and no criteria were classified as '3'.
2) A reliability ranking of **'moderate'** was assigned if the majority of the criteria were classified as '2' and no criteria were classified as '3'
3) A reliability ranking of **'low'** was assigned if at least one of the criteria was classified as '3'

C) Overall Study Ranking

The study was assigned an overall ranking based on the combined ranking of A) the relevance of the test article and route of administration and B) the reliability of the results.

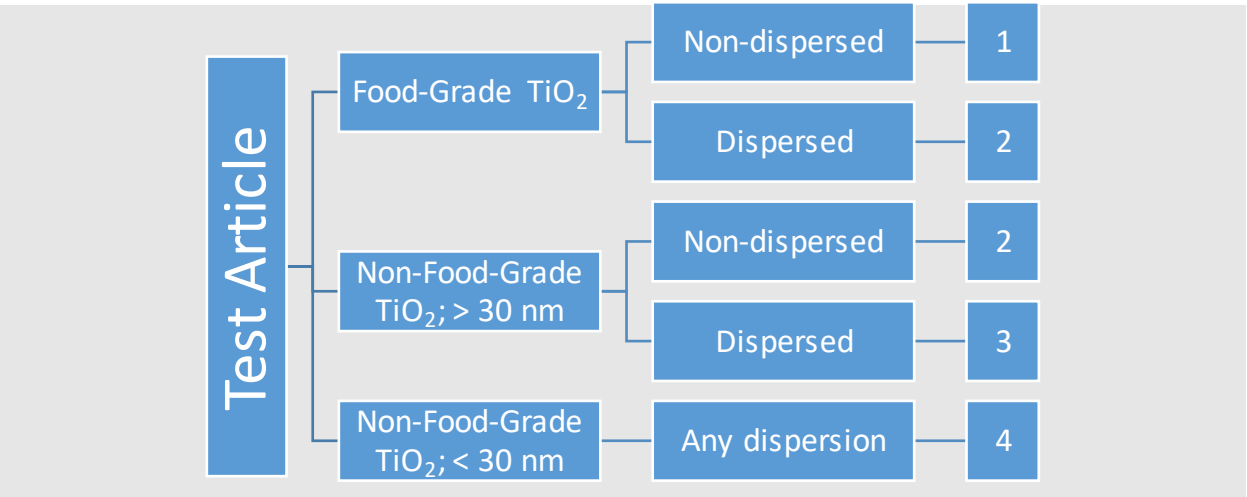
Overall Study Ranking	Relevance of Test Article and Route of Administration	Reliability of Results
Highly relevant	1	High
Relevant, but with limitations	1	Moderate
	2, 3 or 4	High or Moderate
Limited relevance	1, 2, 3 or 4	Low
	5	Any Rank

Only studies with an overall ranking of **'highly relevant'** and **'relevant, but with limitations'** were considered for inclusion of the review of TiO_2.

Study Ranking Criteria – *In Vitro*

In vitro studies will be ranked according to A) relevance of test article and B) reliability of results. Studies received an individual ranking for each of these criteria as well as an overall ranking. More details on each individual ranking and the overall ranking are provided below:

A) Relevance of Test Article



B) Reliability of Results

The reliability of results will be assigned a rank based on an assessment of following criteria:

Category	Criteria	Ranking
Characteristics of test article reported in study	Test article well characterized with information on particle size, crystalline form, surface treatments, and source provided	1
	Some information on the test article missing	2 or 3
Characteristics of test article verified	Test article verified using an appropriate analytical technique	1
	Test article used in study was not verified and instead authors relied on previously published information	2
	Test article was not verified and cannot be verified through other sources	3
Methodological Concerns	No major methodological concerns were identified	1
	Some methodological concerns were identified, but are unlikely to influence to outcome of the study	2
	Major methodological concerns have been identified and are likely to influence to outcome of the study	3
Statistical Concerns	No major statistical concerns were identified	1
	Some statistical concerns were identified, but are unlikely to influence to outcome of the study	2
	Major statistical concerns have been identified and are likely to influence to outcome of the study	3

Based on an assessment of the above criteria, the study was assigned an overall rank for reliability:

1) A reliability ranking of **'high'** was assigned if the majority of the criteria were classified as '1' and no criteria were classified as '3'.
2) A reliability ranking of **'moderate'** s assigned if the majority of the criteria were classified as '2' and no criteria were classified as '3'
3) A reliability ranking of **'low'** was assigned if at least one of the criteria was classified as '3'

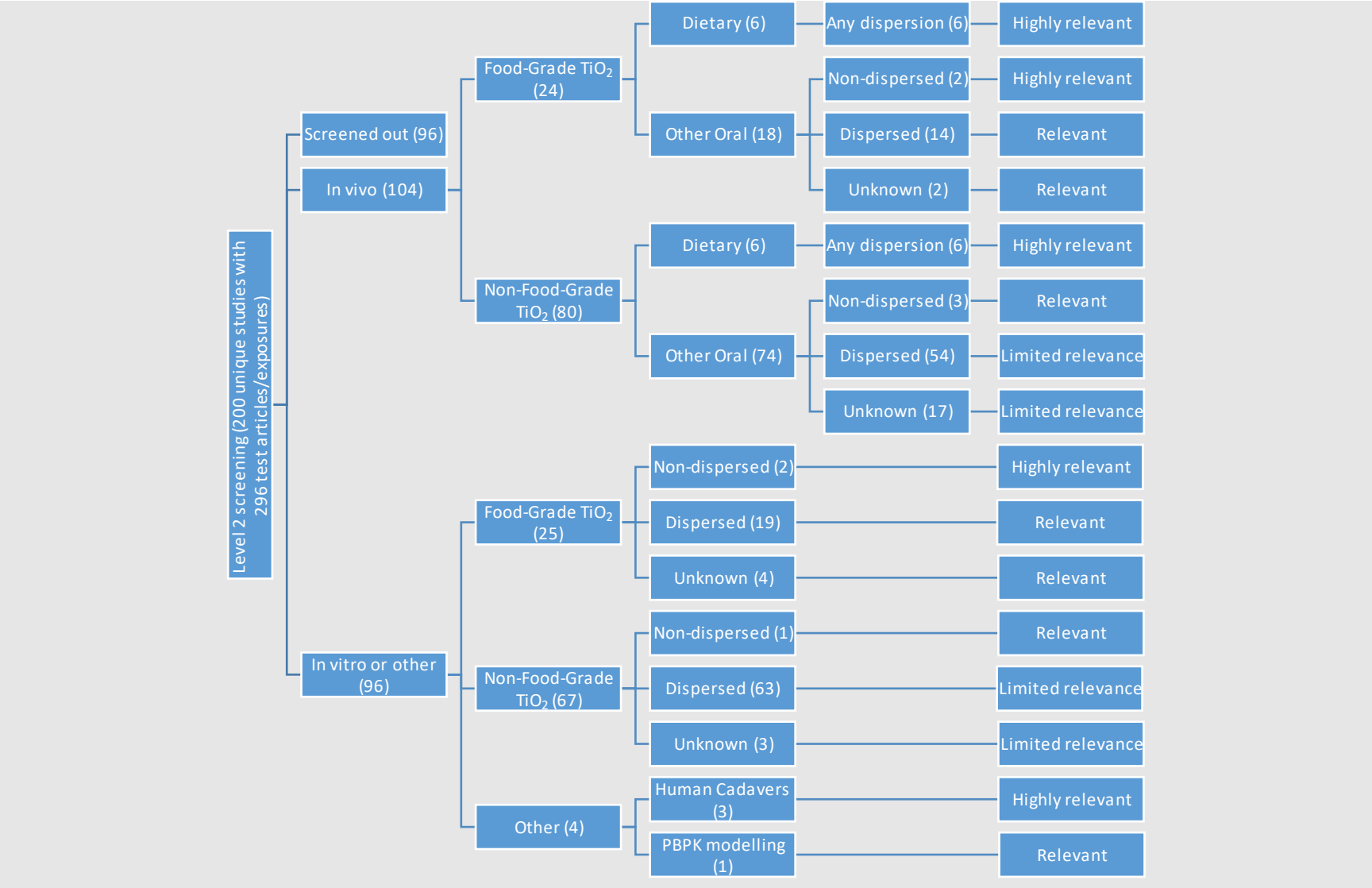
C) Overall Study Ranking

The study was assigned an overall ranking based on the combined ranking of A) the relevance of the test article and B) the reliability of the results.

Overall Study Ranking	Relevance of Test Article and Route of Administration	Reliability of Results
Highly relevant	1	High
Relevant, but with limitations	1	Moderate
	2 or 3	High or Moderate
Limited relevance	1, 2 or 3	Low
	4	Any Rank

Only studies with an overall ranking of **'highly relevant'** and **'relevant, but with limitations'** were considered for inclusion of the review of TiO_2.

Appendix C – Combined Results of Study Screening and Ranking from Both Library Searches



Appendix D – Physicochemical Properties of Some Common TiO_2 Forms

Table 4. Physicochemical properties of some TiO_2 forms commonly used as test articles in toxicity studies. This list is not exhaustive.

Form	D_{50} Primary Particle Size (nm)	Particle Size Distribution (nm)	% Nano (<100 nm)	Mass-Specific Surface Area (m^2/g)	Surface Coating & Composition	Purity & Elemental Composition	Crystalline Form	Form of Food-Grade TiO_2	Examples of Studies Cited in the State of the Science report that Used as Test Article
E171[a]	79-187 (TEM); 104-166 (SEM) Agglomerates (mode): 81-302 (TEM)	30-400 (TEM)	11.4-45.6% (by number; SEM); 5-74% (by number; TEM); 2-33% (by mass; TEM)	~8-10	Uncoated with superficial phosphate, K and C	>97.5% pure and may contain Si, Al, and P	> 99% anatase	Yes	Bettini *et al.* 2017; Blevins *et al.* 2019; Han *et al.* 2020; LPT 2020 as cited in EFSA 2021a; Farrell and Magnuson 2017
E171[b]	130-182 (TEM); 151 (SEM)	50-300 (SEM)	5.4% (by number; SEM) 4-20% (by number; TEM) 3% (by mass; TEM)	Not Reported	Not Reported	Not Reported	> 99% rutile	Yes	None cited in the State of the Science report
Unitane® 0-220[c]	109-124 (TEM); 113-135 (SEM)	Not Reported	26-44% (by number TEM); 20-40% (by number SEM)	8.4-9	Uncoated with Al, K, and phosphate	>98% pure and may contain Nb, Cl, Si, K, and P	> 99.5% anatase	Yes	NCI 1979
Micro-sized pigment-grade (pg-1)[d]	120 (TEM)	Not Reported	27% (by number; TEM)	8.1	Uncoated with C, K and P on surface	Purity not reported, but may contain K, P, and Nb	100% anatase	Yes	Donner *et al.* 2016
Micro-sized pigment-grade (pg-2)[d]	165 (TEM)	Not Reported	11% (by number; TEM)	7.1	Uncoated with C and Al on surface	Purity unknown, but may contain Al	100% rutile	Unclear	Donner *et al.* 2016
Micro-sized pigment-grade (pg-3)[d]	132 (TEM)	Not Reported	26% (by number; TEM)	17.1	Reported to be coated (no other details available) with C and Si on surface	Purity unknown, but may contain Al, Si, and Nb	100% rutile	Unclear	Donner *et al.* 2016

Form	D_{50} Primary Particle Size (nm)	Particle Size Distribution (nm)	% Nano (<100 nm)	Mass-Specific Surface Area (m^2/g)	Surface Coating & Composition	Purity & Elemental Composition	Crystalline Form	Form of Food-Grade TiO_2	Examples of Studies Cited in the State of the Science report that Used as Test Article
JRCNM-10200a[e]	115 (TEM)	Not Reported	Not Reported	Not Reported	Not Reported	Not Reported	Anatase	Unclear	Murugadoss *et al.* 2020
Micro-sized TiO_2[f]	Mean: 128 ± 33.4 (TEM)	~60-240	Not Reported	9.35	Not Reported	99.95% pure and may contain Se, Al, B, Pb, V, Sn, Mn, Cr, Mo, Sb, Hg, and Co	Rutile	Unclear	Duan *et al.* (2021)
Micro-sized cosmetic-grade[g]	Mean: 160 ± 59.4 (SEM)	Not Reported	Not Reported	Not Reported	Not Reported	Not Reported	Anatase	No	Sycheva *et al.* 2011
Aeroxide® TiO2 P25 (NM-105)[h]	10-45 (TEM)	<10-50	100% (by number; TEM)	~46-55	Uncoated with hydroxyl on surface	99.8% pure and may contain Si, Al, and Na	~81-88% anatase; ~12-19% rutile	No	Heo *et al.* 2020; Kampfer *et al.* 2021; Sofranko *et al.* 2021; Waller *et al.* 2017
Engineered TiO_2-NPs[i]	7-10 (TEM); Agglomerates ~50 (TEM)	Polydisperse with narrow size distribution	Not Reported	Not Reported	Not Reported	Not Reported	Anatase	No	Kreyling *et al.* 2017a,b
Engineered TiO_2-NPs[j]	< 100 (based on specific surface area likely 12 nm or less)	Not Reported	Not reported, but based on specific surface area likely to be 100% nano	>150	Not Reported	Not Reported	Anatase	No	Ebrahimzadeh Bideskan *et al.* 2017
Engineered TiO_2-NPs[k]	Mean: 90 (TEM)	40-140	Not Reported	Not Reported	Not Reported	Not Reported	Not Reported	No	Kandeil *et al.* 2020

* P = phosphorus; Si = silicon; Al = aluminum; K = potassium; Nb = niobium; Se = selenium; B = boron; Pb = lead; V = vanadium; Sn = tin; Mn = manganese; Cr = chromium; Mo = molybdenum; Sb = antimony; Hg = mercury; Co = cobalt.

a = Description of physicochemical properties from Verleysen *et al.* (2020), Dudefoi *et al.* (2017a), EFSA (2019b; 2021a), LNE (2020), and TDMA (2019).

b = Description of physicochemical properties from Verleysen *et al.* (2020) and EFSA (2019b; 2021a).

c = Description of physicochemical properties from NCI (1979), TDMA (2022), and MRI (1974; 1975a,b).

d = Description of physicochemical properties from Donner *et al.* (2016).

e = Description of physicochemical properties from JRC (2016).

f = Description of physicochemical properties from Duan *et al.* (2021).

g = Description of physicochemical properties from Sycheva *et al.* (2011).

h = Description of physicochemical properties from JRC Nanomaterials Repository (2016), Rasmussen *et al.* (2014), Dudefoi *et al.* (2017a), and Geiss *et al.* (2020).

i = Description of physicochemical properties from Kreyling *et al.* (2017a,b).
j = Description of physicochemical properties from Ebrahimzadeh Bideskan *et al.* (2017).
k = Description of physicochemical properties from Kandeil *et al.* (2020).
l = Description of physicochemical properties from Verleysen *et al.* (2020).

Appendix E – Summary of Studies Investigating Toxicokinetics

Table 5. Summary table of test results of *in vivo* toxicokinetic studies via the oral route.

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
Ammendolia *et al.* (2017)	Adult male and female SD rats (10 per group per sex); Treated by **oral gavage** for 5 d; TiO_2-NPs suspended in ultrapure water by sonication for 15 minutes (dispersions prepared daily); Rats divided into 3 groups of 20 (*n* = 10 males; *n* = 10 females): 0, 1 or 2 mg/kg bw/d; small intestine 24 h after last treatment.	**TiO_2-NPs**, anatase, spherical shape (20-60 nm); irregular shape (40 X 60 nm); Source Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK.	Ti concentrations in the small intestine were 0.08 ± 0.02 µg/g (control), 0.09 ± 0.02 µg/g (1 mg/kg bw/d, and 0.13 ± 0.03 µg/g (2 mg/kg bw/d).
Chen *et al.* (2020)	Three-week old male SD rats (6 per group); Treated by **oral gavage** for 90 days; TiO_2-NPs suspended in distilled water by sonication (dispersions prepared fresh every day); Rats divided into 4 groups: 0, 2, 10, or 50 mg/kg bw/d; Blood, organs, and tissues taken on day 91.	**TiO_2-NPs**, anatase, 29 ± 9 nm; Source: Shanghai Macklin Reagent Co. Ltd, China	Ti concentrations in blood, liver, intestine, lung, kidney, and testicles were *not* statistically significant different from control; Ti concentrations in the spleen and heart were below LOD; In the colon, Ti concentration were higher in the colon in the 50 mg/kg bw/d group compared to other treatment groups and control; Concluded the higher concentration in the colon was related to TiO_2. NPs were observed attaching to the surface of colonic mucosa tissue (not in mucosa cells).
Cho *et al.* (2013)	Six-week-old male and female spf Sprague Dawley rats (*n* = 11 per sex per group). A 14-d range finding study was performed prior to the 13-week subchronic study in which animals were exposed to TiO_2-NPs suspended in distilled water via **oral gavage** at doses of 0, 260.4, 520.8 or 1041.5 mg/kg bw/d. No treatment-related findings were observed and the same doses were used for the 13-week study. The concentrations of Ti in the basal diet and water were not known. To evaluate tissue distribution, tissue samples from the liver, spleen, kidney, and brain were obtained and weighed following terminal sacrifice. Five animals from each group were randomly assigned to a metabolic cage immediately after gavage, and urine and feces samples were collected for 24 h.	Powder-form **TiO_2-NPs** (80:20 anatase to rutile) were obtained from ABC Nanotech Co., Ltd. (Daejeon, Korea). Particles had a primary size of 21 nm, surface area of 50 ± 15 m^2/g and a hydrodynamic diameter of 37.8 ± 0.4 nm.	After 13-weeks of repeated oral dosing, a small but dose-dependent increase in the Ti content was observed that reached statistical significance from untreated controls only in males at the two highest doses. No significant increase in Ti concentration was observed in liver, spleen, kidney or brain in any treated group relative to controls. Urinary Ti showed no significant increase in treated groups whereas a clear, dose-dependent increase in fecal Ti was observed, suggesting most particles were not absorbed from the GI tract. Overall, absorption of Ti following subchronic repeated oral administration of TiO_2-NPs was found to be minimal in the rat.

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
Coméra *et al*. (2020)	Adult C57BL/6 mice (12 to 18 weeks; sex not reported) (4 per group) **Experiment 1** Treated by **oral gavage** (single dose); E171 suspended in water; Mice divided into 2 groups of 4; 0 or 40 mg/kg bw; measured particle content 2, 4, 8, 24 h after treatment (assuming that 1 mouse was examined at each time point). **Experiment 2** A **closed mid-jejunal loop** of 10 cm filled with sonicated E171 (300 µg/L) in buffer or buffer control for 30 minutes.	**E171**, > 95% anatase, 20-340 nm (118 nm); 44.7% nano; Suspended in milliQ water using the NANOGENOTOX dispersion protocol; Source: French commercial supplier of food colouring agents High agglomeration of test substance in exposure medium was reported.	**Experiment 1** Presence of particles detected by laser reflective confocal microscopy; Increase in particle content observed in ileal and jejunal villi and colon crypts; In ileal and jejunal villi, max particle content at 4 h with return to basal levels at 8 h; In colon, non-significant increase in particle content 4 h with return to basal levels at 8 h; Increase in particles in Peyer's patches at only 8 h; Increase in particle content at 4 and 8 h by 3.5 and 4.1-fold, respectively; concentration below LOD in tissues at all time points; Concluded 0.007% of Ti present in intestine at 4 h; TiO_2 primarily absorbed in ileum, partly in jejunum, and too a lesser degree in colon; absorption primarily occurs via villi and too a lesser degree via Peyer's patches. **Experiment 2** Inhibiting paracellular pathway reduced the absorption of TiO_2 but did not completely block it suggesting that endocytosis is also involved in the transport of TiO_2; Inhibiting the transepithelial passage did not affect absorption.
Farrell and Magnuson (2017)	Male and female SD rats (10 per group/sex); Exposed via the **diet** daily for 7 days (test article incorporated into feed); Rats divided into 2 groups 20 (*n* = 10 males and n-10 females): control or ~200 mg/kg diet ww (equivalent to ~30 mg/kg bw/d in male rats); control diet contained basal concentration of 7-9 mg/kg diet ww of Ti; After 7 days TiO_2 containing diet replaced with untreated diet; Rats sacrificed 1h, 24h, 72h after the removal of the TiO_2 containing diet	<u>Four test articles:</u> 1. **Rutile TiO_2** (thick platelet – pearlescent green) (Engelhard, Peekskill, N.Y.; Experimental Product EP96037) 2. **Rutile TiO_2** (thin platelet – pearlescent gold) (Engelhard, Peekskill, N.Y.; Experimental Product EP96032) 3. **Rutile TiO_2** (standard commercial grade; product number 2558 meeting USP & 21 CFR 73.575 specifications) (Brenntag, South Plainfield, N.J., U.S.A. 4. **Anatase TiO_2**, D_{50} = 133 to 146 nm (standard commercial grade; product number 3328 meeting FCC/E171/USP/EP/JP specifications) (Brenntag, South Plainfield, N.J., U.S.A.).	Ti levels in liver, kidneys, muscle, whole blood, urine, and feces; Ti levels were generally below the LOQ/LOD at most time points; Observations of Ti levels above the LOD were reported, but this was sporadic and occurred at similar levels and frequency when compared to the control group; Authors concluded that **E171 administered via the diet was not appreciably absorbed and distributed in mammalian tissues and there was no evidence of accumulation following repeated exposure for 7 days**

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
Geraets *et al.* (2014)	Male and female Wistar rats. **Oral study:** Animals were dosed by **oral gavage** either once (3 males per group, 4 TiO_2-NPs and controls) or during five consecutive days (3 males per group, 4 TiO_2-NPs and controls; in addition 3 females per group for NM-101 and controls). The dose was 2.3 mg, corresponding to 6.8 – 8.6 mg/kg bw. Rats were sacrificed and tissue sampling was done 24 h after the last exposure (Day 2 or Day 6). **IV study:** Rats received a single or repeated dose (on 5 consecutive days) of 2.3 mg TiO_2 via **injection in the tail vein**. Blood and tissue samples were collected at Day 2 and Day 90 for the single IV administration (3 male and 3 female animals per group), and at Day 6 (i.e. the first day after the last repeated exposure), 14, 30 and 90 after the repeated IV administration (3 male and 3 female animals per group, except for Day 14 and Day 30 which included 3 male animals only). Additional blood samples were collected via orbita puncture at Day 1 (single dose) and Day 5 (repeated doses) at 5, 10, 20 and 30 minutes, and 1, 2, 4, 8, and 24 hours after dosing in order to evaluate the elimination of Ti from the blood after the IV administration. Control rats (vehicle treated) were included (2 male and 1 female animals per group).	Five different TiO_2 particles from the JRC nanomaterials repository were used in this study: 1. **NM-100** (anatase; 200 – 220 nm primary particle size, studied in IV route only); 2. **NM-101** (anatase; 6 nm primary particle size, studied in oral route only); 3. **NM-102** (anatase; 20 nm primary particle size); 4. **NM-103** (rutile, 20 nm primary particle size, coated with Al_2O_3 and a polysiloxane polymer layer); 5. **NM-104** (rutile, 20 nm primary particle size, coated with Al_2O_3)	**Oral study:** Of the 30 liver/spleen samples of exposed animals, one liver sample (NM-102 group) was at the LOD (0.03 µg Ti/g tissue)and 1 liver sample (NM-103 group) was above the LOD but below the LOQ (0.09 µg Ti/g tissue). No Ti levels above the LOD were detected in spleen of any exposed animals, although one of the three untreated controls had a Ti concentration at the LOD in both the spleen and liver. All MLN samples contained Ti concentrations above the LOD although only in the NM-104 group were levels in exposed animals greater than unexposed controls. In this group, the total MLN Ti content was estimated to represent approximately 0.003% of the administered dose. **IV study:** After single administration, Ti could be detected at the first tissue measurement point (24 h after administration) in all investigated tissues, at levels above those as measured in controls. Repeated exposure to all TiO_2-NPs was found to result in up to 5 times higher tissue Ti levels compared to single doses, indicating no significant elimination occurred in this timeframe. In descending order, the highest Ti tissues for all NPs were observed in liver, spleen and lung.
Guillard *et al.* (2020)	Human (placentas: *n* = 22; meconium: *n* = 18) **Experiment 1** **Placentas** collected from women who gave birth to term babies with no complications; **meconium samples** of infants collected in the first few days after birth; placentas and meconium samples were not related. **Experiment 2**	**Experiment 1** n/a **Experiment 2** **E171**, anatase, 20 to 440 nm (mean 104.9 nm); 55 % nano; Particles prepared following the NANOGENOTOX dispersion protocol; Source: French commercial supplier of food colouring agents	**Experiment 1** Ti concentrations in placentas ranged from 0.01 to 0.48 mg/kg (median 0.05 mg/kg); Only 9 of 18 meconium samples were above LOQ, with a median concentration of 0.25 mg/kg; the presence of particles were also confirmed in 2 placentae and 2 meconium samples. **Experiment 2** Model confirmed TiO_2 is capable of crossing placental membranes, with the number of

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
	Ex vivo human **placenta perfusion model** to quantify transplacental transfer across of TiO_2; perfusion medium contained 0 (n = 2) or 15 µg/mL (n = 13).		particles increasing in the fetal exudate in the first 40 minutes of a 60 minute perfusion model; 47% of particles were <100 nm.
Hendrickson *et al.* (2016)	Adult male SD Rats (6 per group); Treated by **oral gavage** for 28 d; Dispersed in 1% aqueous starch solution containing 0.1% Tween-80; Rats administered TiO_2 dispersions immediately after sonication Rats divided into 3 groups of 6: 0, 250 mg/kg bw day (NM-101) or 250 mg/kg bw/d (NP-25); blood samples and organs taken on day 28.	Two test articles: 1. **TiO_2-NPs**, anatase, spherical, uncoated, 5-10 nm; Source: JRC Nanomaterials Repository, Italy (NM-101). 2. **TiO_2-NPs**, anatase, spherical 20-25 nm, uncoated; Source: Sigma-Aldrich, USA (NP-25).	Ti concentrations were the highest in liver > spleen > small intestine > kidney; Ti concentrations were below the LOD in lungs, brain, testicles, heart, and blood.
Hendrickson *et al.* (2020)	Male Wistar rats (n = 12); Rats treated by **isolated intestinal loop technique** with 50 mg/kg bw; Three hours post-dosing intestine, liver, and spleen examined; TiO_2-NPs suspended in saline solution by sonication for 5 minutes (dispersions administered immediately after preparation).	**TiO_2-NPs**, rutile, primary particle size was needle or rod-like shape 5x30 nm; formed loose agglomerates ~100 nm or larger; Source: Sigma-Aldrich (USA)	NPs detected on the surface and between microvilli of mucosal cells of the small intestine and in mucosal tissue; particles detected in Peyer's patches as both single NPs and as agglomerates ranging from 20-60 nm; In liver parenchymal tissue, aggregates (150-200 nm) observed up to 300 nm; In spleen red pulp single NPs (20-30 nm), agglomerates (up to 100 nm) and conglomerates (up to 800 nm) were observed.
Heringa *et al.* (2018)	Male and female humans (n = 9 women aged 77 to 104; n = 6 men aged 56 to 87); Measured Ti concentration in liver and spleen of **deceased subjects.**	n/a	8 of 15 liver samples and 1 of 15 spleen samples were below the LOD; When above LOD, Ti concentrations were 40 ng/g and 80 ng/g in the liver and spleen, respectively; Average particle size of 86-426 nm in liver and 88-445 nm in spleen (lowest size was LOD); almost all Ti was present as particles.
Janer *et al.* (2014)	Male Sprague Dawley rats were allocated to a vehicle control or TiO_2 group (6 animals per group). A single dose of 100 mg/kg TiO_2-NPs was administered by **oral gavage**. On the day following administration, animals were sacrificed and spleen, liver, small and large intestines, and mesenteric lymph nodes were removed.	**TiO_2-NPs**, synthesized using flame spray pyrolisys, were obtained from L'Urederra (Spain) under the framework of the EU project Nanopolytox. Reported to be "mainly anatase" with a D_{50} of 18 ± 8 nm. Particles were dispersed by sonication in MQ water containing 2 mM sodium citrate as a dispersant.	There was no statistically significant increase in Ti levels in any of the tissues evaluated 24 h after the administration of 100 mg/kg TiO_2-NPs. No TiO_2-NPs were located in the small intestine when examined by SEM. In Peyer's patches, TiO_2-NP agglomerates were observed in the cytoplasm of at least one cell but not inside the mitochondria or nucleus.
Jones *et al.* (2015)	Adult humans aged 30-56 years, weight range 63-124 kg (n = 5 women, n = 4 men); Exposed to single **oral** dose of 5 mg/kg bw TiO_2 dispersed in water; Three test articles used and administered at least 4 weeks apart (most volunteers that	Three test articles: 1. **TiO_2-NPs**, 10 nm (peak), ~100% < 50 nm by number, anatase, Source: NanoAmor	No significant differences between pre-and post-dose biomarkers were observed for any particles size.

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
	participated were exposed to all three test articles); Blood collected at baseline, 2h, 4h, 24h, and 48h; Urine samples collected in time collections over 4 days (24h pre dosing and 72h post dosing).	2. **TiO_2-NPs**, 70nm (peak), 95% of particles ranged from 48 to 154 nm by number, rutile, Source: Sigma-Aldrich 3. **TiO_2**, 1800 nm (peak), 100% > 105 nm by number, rutile, Source: Sigma-Aldrich	
Kreyling *et al.* (2017b)	8-10 week old female Wistar-Kyoto rats (4 rats per time point); Treated by **oral gavage** (single dose;) and sacrificed 1 h, 4h, 24h or 7 days after treatment; Aqueous [^{48}V]TiO_2-NPs suspension (administered immediately after dispersion to non-fasted rats); Rats sacrificed at 1, 4, and 24 h were dosed with 10 µg each (equivalent to ~49.82, 30.8 and 44.44 µg/kg bw) and rats sacrificed after 7 days were dosed with 30 µg each (equivalent to ~78 µg/kg bw). **Experiment 1 (MAIN-1):** To determine biodistribution of TiO_2 rats were divided into 4 groups of 4 and sacrificed 1 h, 4h, 24h or 7 days after treatment; **Experiment 2 (MAIN-2):** To determine retention in GIT walls rats were divided into 3 groups of 4 and sacrificed 1h, 4h, and 24h after treatment (all doses with 10 µg each).	**TiO_2-NPs**, anatase, Approximately spherical aggregates/agglomerates roughly 50 nm in diameter; primary particle size approximately 7 to 10 nm; Source: commercially available ST-01 (Ishihara Sangyo Ltd., Japan)	Approximately 0.6% of the dose absorbed during the first hour after treatment; After 7 days approximately 0.5% of the dose remained; The distribution patterns of TiO_2 varied between rats; Measurable levels were observed after 4 h in spleen, kidneys, heart, and uterus; Maximum retention reached in spleen, kidney, and heart at 24 h. In liver, lung, and blood, retention declined from 4h to 7 days; In brain uterus, kidneys, highest concentrations observed on day 7; Peak concentration in liver was 12.5% of absorbed dose at 4 h and 2.6% in the spleen at 24 h; Concluded that the slow excretion kinetics likely results in the accumulation of systemically circulating particles in some organs with chronic exposure.
MacNicoll *et al.* (2015)	8-wk old male Sprague Dawley rats were used in this experiment. TiO_2 particles (5 mg/kg bw) were administered to rats (n = 6 per group) by **oral gavage** as a suspension either in deionised water or 5 % ovalbumin solution. Samples of blood, feces and urine were collected at intervals over 4 days following dosing. After 96 h post-administration of TiO_2, rats were killed and tissue samples of liver, brain, heart, kidney, spleen, and GIT were collected.	<u>Six test articles:</u> 1. 99.7 % **TiO_2-NPs**, anatase, 15 nm (Sigma cat # 637254); 2. 99.5 % **TiO_2-NPs,** 80 % anatase/20 % rutile, 25 nm (Nanocomposix); 3. 99.7 % **TiO_2-NPs**, anatase, 40 nm (NanoAmor 5430 MR); 4. 99.5 % **TiO_2-NPs**, rutile, 40-50 nm (Sigma cat # 637252); 5. 99.7 % **TiO_2**, anatase, 120 nm (NanoAmor 5430 MR); 6. 99.5 % **TiO_2**, rutile, up to 5000 nm (Sigma cat # 224227)	In general, the mean concentrations of Ti in organs from treated rats were not significantly higher than vehicle controls. In the GIT samples, however, the mean concentration of Ti taken from rats treated with '<100 nm' particles was significantly higher than the mean concentration of Ti in the samples taken from rats in the control group. The results show that oral administration of TiO_2 particles did not lead to translocation of Ti to blood, urine, or distribution to various organs in rats at any of the time intervals studied during the 96 h post-treatment. With the possible exception of one particle size, the mean concentration of Ti in

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
		Particles were dispersed by sonication either in deionised water or a 5 % ovalbumin solution. All exposure experiments were performed in the presence of FCS (concentration not stated).	the GIT was not significantly affected by treatment.
Pele *et al.* (2015)	Human (age and sex unknown); *n* = 8 (results from only 5 participants); Ingested two gelatine **capsules** each containing 50 mg E171 with 250 mL of water; Blood samples taken 0, 30 minutes and 1, 1.5, 2, 3, 6, 8, and 10 h after ingestion	**E171**, anatase, D_{50} = 260 nm; Source : Kronos® 1171, Fagron UK	Semi-quantitative measure of particles using reflectance as well as measurements of Ti concentrations using ICP-MS; Significant increases in signals at 2 h onwards, with a peak at 6 h; Highest blood concentration at 6 hrs was 11 ng/mL, which declined to 5 ng/mL at 10 h. Size of absorbed particles was not determined.
Peters *et al.* (2020)	Male and female humans aged 83 to 97 (women) and aged 64 to 98 (men) (*n* = 8 women; *n* = 7 men for liver, spleen, kidney and *n* = 7 women; *n* = 5 men for jejunum and ileum); Measured Ti concentration in liver, spleen, kidney, jejunum, and ileum of **deceased subjects.**	n/a	In 4/15 liver, 2/15 spleen, 1/15 kidney and 1/12 jejunum, Ti concentrations were below LOD; Average concentration in the liver (0.03 µg/g), spleen (0.06 µg/g), kidney (0.08 µg/g), jejunum (0.34 µg/g), and ileum (0.43 µg/g); particles sizes ranged from 50 to 500 nm (lowest size LOD); Majority of Ti in organs consisted of particulate matter ranging in size from 50 to 500 nm (mode ranges from 100 to 160 nm).
Riedle *et al.* (2020)	6 week old male and female C57BL/6 mice (18 per group); Exposed via **diet** daily for 6, 12, and 18 wks (test article was formulated into diet); Mice divided into 4 groups of 18: 0, 6.25, 62.5, or 625 mg/kg diet (equivalent to approximately 0, 1, 10, or 100 mg/kg bw); *n* = 6 mice/group euthanized at 6, 12, and 18 weeks	**E171**, anatase, 119 nm; Source: Sensient Colors (St. Louis, MO, USA)	Presence of 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; Evidence of dose-response.
Talamini *et al.* (2019)	8 week-old male NFR mice (4 per group); Treated **orally** 3 days/week for 3 weeks (i.e., total of 9 treatments in 21 d); E171 freshly dispersed in water; Test article slowly dripped into mouth via pipette with each drop swallowed; Mice divided into 2 groups of 4: 0 or 5 mg/kg bw/d (equivalent to daily dose of ~2 mg/kg bw/d); Mice euthanized on day 21 (3 days after last dose)	**E171**, anatase, Mean diameter of 201.2 ± 8.5 nm; **Freshly dispersed in water (no sonication or de-agglomeration to simulate realistic conditions)**; 35% nano (determined by TEM); Source: Pretiox AVo1PhG (Giusto Faravelli S.p.A. Milan Italy)	Ti concentrations in the suspension were determined before and after the passage through the pipette tip; Liver, stomach, and whole intestine were cleaned with ultrapure water to remove any adhering blood or residue in the GI tract; Ti concentrations in tissues were determined by single particle ICP-MS analysis; 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;

Study	Species-strain, doses and dosing regimen	Test Article (source)	Result
			Ti concentrations in lungs, spleen, stomach, small intestine ***not*** statistically significant between treated and control mice; Ti concentrations in the brain, kidney, and testis below LOQ
Tassinari *et al*. (2014)	Young sexually mature male and female SD rats (7 per dose per sex); Treated by **oral gavage** for 5 d; Suspended in distilled water by sonication for 15 minutes (dispersions prepared daily); Rats divided into 3 groups of 14 (*n* = 7 males and *n* = 7 females): 0, 1 or 2 mg/kg bw/d; blood samples and organs taken on day 6.	**TiO_2-NPs**, anatase, Based on TEM analysis, primary particles were spherical in shape (20-60 nm) and irregular in shape (40-60 nm), but large agglomerates of particles were also observed (size not reported); Based on SEM analysis, the mean diameter was 284 ± 43 nm with 13% of particles < 100 nm, 87% of particles ranging from 30 to 900 nm and 48% of particles ranging from 100 to 300 nm, but agglomerates up to 1.6 µm were also observed; Source: Sigma-Aldrich Company Ltd. (Gillingham, Dorset, UK).	Ti concentrations in organs and tissues were not significantly different from controls with the exception of the spleen and ovaries in the 2 mg/kg bw group; agglomerates of Ti particles (200-400 nm) were identified in the spleen of the 2 mg/kg bw group.
West and Wyzan (1963) as cited in JECFA 1969	Male humans (*n* =5); **Ingested** 5g of E171 suspended in milk for 3 consecutive days; Urine samples taken for 5 days	**E171** (no other details available)	No increase in urinary Ti levels suggesting that no significant absorption occurred.

Appendix F – Summary of Studies Investigating Acute Toxicity

Table 6. Summary table of acute toxicity studies of TiO_2 particles via the oral route.

Study	Study type, species, doses and dosing regimen	Test Article (source)	Result
Wang *et al.* (2007)	Acute toxicity study in mice according to OECD Guideline 420 (Acute Oral Toxicity - Fixed Dose Method) CD-1 (ICR) mice (10 per sex per group) were fasted overnight before being dosed with a vehicle control (0.5% hydroxypropyl methylcellulose; HPMC) or one of three sizes of suspended TiO_2 particles by **oral gavage** at 5000 mg/kg bw.	Three test articles: 1. **TiO_2-NPs**, (25 nm), crystalline structure not specified; 2. **TiO_2-NPs**, (80 nm), crystalline structure not specified; 3. **TiO_2**, (155 nm), crystalline structure not specified.	Animals were sacrificed 14 days after dosing. The LD_{50} was determined to be > 5000 mg/kg bw. Histopathologic findings were reported in brain, liver and kidney with the 80 and 155 nm particles but not the 25 nm particles.
Warheit *et al.* (2007) Note that a similar study is also described in Warheit *et al.* 2015.	Acute toxicity study in rats according to OECD Guideline 425 (Acute Oral Toxicity: Up-and-Down Procedure) Three fasted female Crl:CD(SD) rats were administered TiO_2 particles suspended in water by **oral gavage** at a dose of 5000 mg/kg bw.	**TiO_2** particles were 79% rutile and 21% anatase. Composition was ~90 wt% TiO_2, 7% alumina, and 1% amorphous silica. Median particle size was 140 nm in water and surface area was 38.5 m^2/g. Particles underwent a neutralization process during production.	No deaths occurred and no gross lesions were present at necropsy. The LD_{50} was determined to be > 5000 mg/kg bw.
Ranjan *et al.* 2020	Acute toxicity study in female Wistar rats according to OECD Guideline 420 (Acute Oral Toxicity - Fixed Dose Method). The experimental design included four groups (*n* = 5 per group) with three different doses (500 mg/kg bw, 1000 mg/kg bw and 2000 mg/kg bw) and a control group. Test article was administered via **oral gavage**.	**TiO_2-NPs** (average size 28.3 ± 3.1 nm) were synthesized in-house using a microwave-irradiation-assisted hybrid chemical approach.	No mortality was observed in either the vehicle control or the NP-treated rats. Thus, the acute oral LD_{50} is estimated to be greater than 2000 mg/kg bw. At the end of the study, serum was extracted from the sacrificed animals and various biochemical parameters were analyzed. It was observed that except for ALT, all the other enzymes and protein estimation were not significantly altered. A significant dose-dependent increase in ALT levels was reported.

Appendix G – Biomarkers of Colorectal Cancer

The development of colorectal cancer is a multistep process that typically begins with the appearance of preneoplastic lesions (i.e., ACF/ABCs) that progress to pre-malignant lesions (i.e., polyps and adenomas) and then to malignant lesions (i.e., carcinomas; Conteduca *et al.* 2013; Dekker *et al.* 2019; Kuipers *et al.* 2015). The progression of colorectal cancer can be affected by a wide variety of factors, including genetics/epigenetics, growth factors, obesity, and chronic inflammation in the intestinal tract, among others (Conteduca *et al.* 2013; Carvalho *et al.* 2012; Hermsen *et al.* 2002).

Colonic crypts are tubular structures located in the lining of the colon and rectum that originate from a single mesenchymal stem cell located at the base of the crypt, which produce epithelial cells that renew the crypt approximately every five days (Alrawi *et al.* 2006a; Humphries and Wright 2008). ABCs are typically larger than healthy colonic crypts and have a thicker epithelium as well as a visible, irregular luminal opening, whereas ACF are areas of colonic mucosa than contain one or more ABCs and can be classified as non-hyperplastic, hyperplastic, dysplastic or mixed (Alrawi *et al.* 2006a; Pretlow *et al.* 1991; Quintanilla *et al.* 2019). Many consider ACF/ABCs to be the earliest identifiable preneoplastic abnormality in the development of colorectal cancer (Alrawi *et al.* 2006a; Clapper *et al.* 2020; Magnuson *et al.* 1993; Uchida *et al.* 1997).

In 1987, ACF/ABCs were first identified as possible a precursor of colorectal cancer (Bird 1987) and since then, multiple studies have demonstrated their role in predicting tumour formation in the colon of laboratory animals (Kristiansen 1996; Magnuson *et al.* 1993; Pretlow *et al.* 1992; Uchida *et al.* 1997). Crypt multiplicity (i.e., number of ABCs per focus) is a consistent predictor of tumour incidence in carcinogen-treated laboratory animals; generally ACF with high crypt multiplicity (i.e., four or more ABCs) were correlated with colonic tumour incidence, whereas ACF consisting of three or less crypts were not predictive (Alrawi *et al.* 2006a; Magnuson *et al.* 1993; Pretlow *et al.* 1992; Kristiansen 1996). The importance of crypt multiplicity in colon cancer is further supported by Uchida *et al.* (1997) who demonstrated that the mucinous profile of ACF with four or more crypts in DMH-treated rats resembled the mucinous profile observed in human colorectal cancer (i.e., increase in ACF with sialomucin predominance relative to ACF with sulfomucin predominance). Caderni *et al.* (1995) also noted a positive correlation between the number of sialomucin-producing ACF and crypt multiplicity as well as a significant association between sialomucin-producing ACF and tumour incidence in DMH-treated rats; however, only ACF consisting of 14 or more crypts were associated with tumour incidence. The number of ACF per colon is another common parameter often measured, although multiple studies have noted that it is not a good predictor of tumour incidence in carcinogen-treated laboratory animals (Caderni *et al.* 1995; Magnuson *et al.* 1993). Other studies have noted the considerable heterogeneity in ACF histology as well as the impact differences in study methodology or procedures (such as animal strain, mode of exposure, duration of experimental procedure, etc.) may have on the number of ACF in the colon of carcinogen-treated laboratory animals (Suzui *et al.* 2013). Pre-treatment with a colon carcinogenesis initiator itself can also result in inter-individual variability of approximately 20-40% in ACF formation in rodents (see Blevins *et al.* 2019; Rodrigues *et al.* 2002; Won *et al.* 2012 for examples of inter-individual variability within DMH treatment groups). While some ACF do progress to adenomas and cancer, many ACF in carcinogen-treated rodents spontaneously regress over time (Choi *et al.* 2015; Shpitz *et al.* 1996), which may limit its usefulness as a biomarker of colonic tumorigenesis.

The spontaneous formation of ACF is also not an uncommon observation in control mice and rats in the absence of a colon carcinogenesis initiator despite colon cancer in these species being exceedingly rare. For example, Furukawa *et al.* (2002) reported that ~42.5% of untreated, male and female F344 rats spontaneously developed ACF in the colon over a 16-week period (~112 days), of which ~77% of ACF had 1-3 ABCs per focus and ~23% of ACF had ≥4 ABCs per focus, but the spontaneous development of colorectal tumours was found to be ≤ 0.34%. In another study, approximately 35 ACF spontaneously developed per colon in Wistar rats receiving saline by gavage for 90 days, with the majority of ACF comprised of ≤3 ABCs and only one or two ACF with ≥4 ABCs per focus; no tumours were reported (Singh *et al.* 2022). However, neither of these studies were likely long enough to fully assess whether any of the ACF would progress to neoplasia.

In 1991, ACF/ABCs were first identified in the colon mucosa of human surgical resections and cadavers and were found to be similar to those previously identified in rodent colons following treatment with carcinogens (Pretlow *et al.* 1991). The authors noted that the number of ACF per cm^2 of colonic mucosa were significantly higher in individuals with colon cancer compared to those without colon cancer or other predisposing conditions. However, it should be noted that ACF have also been detected in the colons of ~15 to 77% of healthy patients (Adler *et al.* 2002; Hurlstone *et al.* 2005; Takayama *et al.* 1998). Since then, many studies have suggested ACF may be a risk factor for human colorectal cancer and therefore, may represent a useful, early biomarker for the development of colorectal cancer in humans (Anderson *et al.,* 2012; Di Gregorio *et al.* 1997; Drew et al., 2018; Kowalczyk et al., 2020; Takayama *et al.* 1998). In particular, several studies reported that the risk for colorectal cancer in humans was strongly associated with the presence of dysplastic ACF in the proximal colon and rectum as well as the number of crypts per focus (≥20 crypts per focus) and the number of ACF per colon (>6 ACF per colon; Anderson *et al.,* 2012; Clapper *et al.* 2020; Drew *et al.* 2018; Kowalczyk *et al.* 2020; Takayama *et al.* 1998). This has led some to recommend stratifying ACF based on the presence of certain features, particularly dysplasia, when determining risk for colorectal cancer (Clapper *et al.* 2020; Di Gregorio *et al.* 1997). Although the difficultly in identifying dysplastic ACF *in situ* (Clapper *et al*. 2020) as well as the dynamic nature of ACF over time in humans (i.e., less than half of ACF re-identified after one-year; Schoen *et al.* 2008) may limit the utility of this feature as a biomarker of human colorectal neoplasia.

The stepwise progression of ACF to colorectal cancer in humans is complex and likely follows multiple routes, depending on ACF histology and many other factors. The classic adenoma-carcinoma pathway, in which normal colonic mucosa evolve into ACF/ABCs that give rise to tubular and tubulovillous adenomatous polyps (adenomas) and subsequently progress to cancer, is the most common pathway reported and accounts for ~70-90% of human colorectal cancers (Kuipers *et al.* 2015; Dekker *et al.* 2019; Siskova *et al.* 2020). As mentioned, the presence of dysplasia may increase the likelihood of ACF becoming adenomas (Di Gregorio *et al.* 1997); although, the vast majority of adenomas (~90-95%) do not progress to cancer despite most sporadic colorectal cancers beginning as adenomas (Hermsen *et al.* 2002; Conteduca *et al.* 2013). In the last decade, it was discovered that ~10-20% of human colorectal cancers arise from serrated polyps (i.e., hyperplastic polyps, sessile serrated polyps/adenomas, and traditional serrated adenomas) via the serrated neoplasia pathway (Kuipers *et al.* 2015; Dekker *et al.* 2019). Hyperplastic ACF with a serrated histology has been suggested as a potential precursor to the serrated neoplasia pathway (Rosenberg *et al.* 2007). Conversely, others have argued that hyperplastic ACF may be more likely to progress to hyperplastic polyps, which are largely considered non precancerous lesions (Di

Gregorio *et al.* 1997), and still others reported no association between hyperplastic ACF and hyperplastic polyps or other types of serrated polyps (Lance and Hamilton 2008; Cho *et al.* 2008). The percentage of ACF that progress to colorectal cancer via either pathway is unclear, although some have estimated that approximately 25% of ACF exhibit "some degree of genomic instability" similar to most adenomas and carcinomas, suggesting only a small portion may develop into cancer over time (Alrawi *et al.* 2006a,b).

Overall, the use of ACF as a biomarker of colorectal cancer risk in laboratory animals and humans is controversial. Very few of these lesions progress to neoplasia, with most spontaneously regressing over time in rodents (Choi *et al.* 2015; Shpitz *et al.* 1996) and humans (Schoen *et al.* 2008). In carcinogen-treated rodents, Inter-individual variability in ACF formation can be high (~20-40%; see Blevins *et al.* 2019; Rodrigues *et al.* 2002; Won *et al.* 2012 for examples). In humans, some have strongly argued against the use of ACF as a biomarker due to the lack of a clear association between certain ACF features (such as the number of ACF per colon, presence of dysplasia, lumen morphology, and vascular pattern intensity) and the risk of colorectal cancer (Quintanilla *et al.* 2019; Cho *et al.* 2008; Lance and Hamilton 2008). In a recent case-control study, only the number of large ACF (>40 crypts per focus) was found to be associated with increased risk of colorectal cancer, although Quintanilla *et al.* (2019) concluded that even this endoscopic feature has "dubious clinical utility" since it did not accurately predict ACF histopathology or abnormal molecular features of ACF indicative of colorectal cancer. The authors also noted inconsistencies in histological classifications of ACF among pathologists, with considerable overlap between cases and controls (Quintanilla *et al.* 2019). The difficulty in the histological characterization of ACF is likely attributable to the considerable heterogeneity in ACF morphology, the lack of standardization in the classification of ACF among pathologists as well as differences in methodology and tissue sampling procedures across studies (Alrawi *et al.* 2006a; Quintanilla *et al.* 2019; Suzui *et al.* 2013).

Other commonly used biomarkers to determine the risk and/or progression of colorectal cancer include measurements of various proteins in colorectal cancer tissue, serum protein levels, as well as various genetic and epigenetic changes. For example, in human colorectal cancer tissue samples, decreased expression of Muc2 (key structural component of colonic mucus) and increased expression of COX2 (inflammatory marker) and HER2 (growth factor) were associated with increased malignancy and poor prognosis (Chao *et al.* 2018; Wu and Sun 2015). Decreased serum levels of L1CAM (a structural protein found on neuronal cell membranes) are found in patients with colorectal cancer and may be a promising biomarker for the early detection of colorectal cancer (Chu *et al.* 2020). A vast number of genomic mutations involving the activation of oncogenes (e.g., KRAS, BRAF, etc.) and the inactivation of tumour suppressor genes (e.g., APC, TP53, etc.), epigenetic instability related to excessive CpG island DNA methylation as well as microsatellite and chromosomal instability have also been reported in both major colorectal cancer pathways (Alrawi *et al.* 2006a; Conteduca *et al.* 2013; Kuipers *et al.* 2015; Dekker *et al.* 2019; Siskova *et al.* 2020). Many other biomarkers of colorectal cancer have also been proposed, which have been summarized elsewhere (e.g., see Alrawi *et al.* 2006a; Conteduca *et al.* 2013; Siskova *et al.* 2020).

Appendix H – Summary of Studies Investigating Genotoxicity

Table 7. Summary table of test results of *in vitro* genotoxicity studies with food-grade TiO_2.

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Mutagenicity assays			
BioReliance 2021a	*Salmonella typhimurium* TA98, TA100,, TA1535, TA1537 and the tryptophan locus of *Escherichia coli* WP2 *uvr*A; Exposed to E171 at concentrations of 33.3, 100, 333, 1000, 3333, or 5000 µg/plate in the presence and absence of Aroclor 1254-induced rat liver S9 for 48 to 72 hours; Water was used as vehicle control; the positive control varied depending on experimental condition: 2-aminoanthracene (all strains, +S9), 2-nitrofluorene (TA98, -S9), sodium azide (TA100 & TA1535, -S9), 9-aminoacridine (TA1537, -S9), MMS (WP2 *uvr*A, -S9); Conducted in accordance with OECD guideline 471 Bacterial reverse mutation assay	**E171-E** (anatase, D_{50}: 99.9 ± 2.0 nm; 50-51% < 100 nm) The test article was suspended in water by vortex mixing (i.e., the test article was "stirred using a stir plate and sterile stir bar during dose formulation preparation…and dosing to maintain a homogenous mixture"). The test article was reported to form workable suspensions in water at concentrations of approximately 5 to 50 mg/ml.	Negative (+/- S9) for frameshift or basepair substitution mutations at concentrations up to 5000 µg/plate. However, the bacterial reverse mutation test may not be suitable for the assessment of nanomaterials (or insoluble particles, in general) since they may not be readily taken up by the bacterial cells used in this assay (Doak, *et al.* 2012; OECD 2014; Kumari *et al.* 2010). TiO_2-NPs are also known to have antibacterial/bacteriostatic properties (see Khashan *et al.* 2021; Lopez de Dicastillo *et al.* 2020), which further limits the suitability of the bacterial reverse mutation test for the assessment of these materials. Cellular uptake of particles was not confirmed; No evidence of cytotoxicity, but a reduction in revertant count was observed at ≥3333 µg/plate in TA1537 with metabolic activation.
BioReliance 2021b	Hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus (*hprt*) of Chinese hamster ovary (CHO) cells; CHO cells were exposed to E171 at concentrations of 0.156, 0.313, 0.625, 1.25, 2.50, 7.5, 15, or 30 µg/ml in the presence and 0.0781, 0.313, 0.625, 1.25, 2.50, 7.5, 15, or 30 µg/ml absence of Aroclor 1254-induced rat liver S9 (5±0.5 hours); Serum free medium was used as the vehicle control; the positive control varied depending on experimental condition: EMS (-S9) and benzo(a)pyrene (+S9); Conducted in accordance with OECD guideline 476 Mammalian cell forward gene mutation assay	**E171-E** (anatase, D_{50}: 99.9 ± 2.0 nm; 50-51% < 100 nm) The test article was suspended in serum free medium by vortex mixing.	Negative (+/- S9) for forward mutations at concentrations up to 30 µg/ml. However, there was no evidence of cellular uptake of the test article in CHO cells in a subsequent TEM imaging analysis and therefore, the lack of genotoxic effects observed could be related to the lack of exposure of the test article to the DNA. No evidence of cytotoxicity.

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Dunkel et al. (1985)	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538, *E. coli* WP2 *uvr*A; Exposed to concentrations of TiO_2 up to 10 mg/plate for an unknown duration with and without S9; S9 was prepared from the livers of male Fischer 344 rats, B6C3F1 mice, and Syrian hamsters with or without Aroclor 1254 pre-treatment; Positive and negative controls were included (no other details provided); Bacterial reverse mutation assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	Negative (+/- S9), but the bacterial reverse mutation test may not be suitable for the assessment of nanomaterials (or insoluble particles, in general) since they may not be readily taken up by the bacterial cells used in this assay (Doak, *et al.* 2012; OECD 2014; ; Kumari *et al*. 2010). TiO_2-NPs are also known to have antibacterial/bacteriostatic properties (see Khashan *et al.* 2021; Lopez de Dicastillo *et al*. 2020), which further limits the suitability of the bacterial reverse mutation test for the assessment of these materials. Particle uptake not evaluated and no information on cytotoxicity was reported.
Myhr and Capary (1991)	Thymidine kinase locus in L5178Y mouse lymphoma cells; Exposed to TiO_2 at concentrations of 1.56, 3.13, 6.25, 12.5, 25, 50, or 100 µg/ml for 2 days with or without S9; S9 was prepared from the livers of male Fischer 344 rats with Aroclor 1254 pre-treatment; Culture medium was used as negative control and 5 nl/ml MMS or 250 nl/ml EMS was used as the positive control; Mammalian cell gene mutation assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. Suspension of TiO_2 prepared in water (no other details available)	Negative (+/- S9), but particle uptake not evaluated and no information on cytotoxicity reported; TiO_2 co-pelleted with cells after 4-hour treatment period and therefore, the actual exposure continued through the 2-day expression period
Tennant *et al.* (1987)	*S. typhimurium*/microsome	**TiO_2 (Unitane® 0-220)**	Negative (+/- S9), but the bacterial reverse mutation test may not be suitable for the

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Exposed to concentrations of TiO_2 up to 10 mg/plate for an unknown duration with and without S9; No information about positive and negative controls provided; Bacterial reverse mutation assay	Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	assessment of nanomaterials (or insoluble particles, in general) since they may not be readily taken up by the bacterial cells used in this assay (Doak, *et al.* 2012; OECD 2014; Kumari *et al.* 2010). TiO_2-NPs are also known to have antibacterial/bacteriostatic properties (see Khashan *et al.* 2021; Lopez de Dicastillo *et al.* 2020), which further limits the suitability of the bacterial reverse mutation test for the assessment of these materials. Particle uptake not evaluated and no information on cytotoxicity reported.
	L5178Y mouse lymphoma cells; Exposed to concentrations of TiO_2 up to 1.6 µg/plate for an unknown duration without S9; No information about positive and negative controls provided; Mammalian cell gene mutation assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	Negative, but particle uptake not evaluated and no information on cytotoxicity reported.
Micronucleus and chromosomal aberration assays			
BioReliance 2021c	Human peripheral blood lymphocytes (HPBLs) obtained from a healthy, non-smoking female;	**E171-E** (anatase, D_{50}: 99.9 ± 2.0 nm; 50-51% < 100 nm)	Negative for the induction of micronuclei at concentrations up to 30 µg/ml at 4 hours (+/- S9) and 24 hours (-S9). However, cellular uptake of the test article was not confirmed

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Exposed to E171 at concentrations of 0.3, 1, 10, or 30 µg/ml for 4 hours with or without Aroclor 1254-induced rat liver S9, and 24 hours without Aroclor 1254-induced rat liver S9; HPBLs were treated for 4 hours (+/- S9) and 24 hours (-S9); Water was used as the vehicle control; Mitomycin C and vinblastine were used as the positive controls without S9 and cyclophosphamide was used as the positive control with S9; Conducted in accordance with OECD guideline 487 Mammalian cell micronucleus assay	The test article was reported to form workable suspensions in water via vortex mixing at concentrations of approximately 5 to 50 mg/ml.	and therefore, the negative results could also be related to the lack of exposure; No evidence of cytotoxicity at any treatment concentrations for any exposure condition.
Franz *et al.* (2020)	HT29-MTX-E12 cells; Exposed to E171 at concentrations of 0.5, 5 or 50 µg/ml for 48 hours without S9; Negative controls were untreated cells; etoposide (0.25-1 µM, 48 hours) and vinblastine (2.5-10 nM, 48 hours) were used as the clastogenic and aneugenic positive controls, respectively; Mammalian cell micronucleus assay	**Food-grade TiO_2 (E171)**; 170 nm; anatase (Hombitan AFDC, Venator). Stable suspensions generated by bath sonicating test article in double distilled water for 10 minutes. The suspensions were intentionally prepared to generate bigger agglomerates that more closely resembled the particle size distribution of E171 in the food matrix.	Negative for micronuclei and hypodiploid nuclei, but the presence of agglomerates interfered with the detection of micronuclei in the flow cytometry-based scoring method used in this study; Agglomeration status of the particles in the exposure media was not confirmed and cellular uptake of particles was not evaluated; No evidence of cytotoxicity after 24 and 48 hours.
Ivett *et al.* (1989)	Chinese hamster ovary (CHO) cells; Exposed to TiO_2 at concentrations of 15, 20 or 25 µg/ml for 2 hours with S9 and 8 hours without S9; S9 was prepared from the livers of male Fischer 344 rats with Aroclor 1254 pre-treatment; Solvent was used as negative control, mitomycin C was used as positive control without S9, and cyclophosphamide was used as positive control with S9;	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-	Negative (+/- S9), but particle uptake not evaluated and no information on cytotoxicity reported; Highest dose limited by solubility; positive result observed at 20 µg/ml in first trial (+S9), but response was not reported in subsequent trial and chemical judged to be negative.

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Chromosomal aberration assay	specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	
Proquin *et al.* (2017)	Human colonic epithelial HCT116 cells; Exposed to E171 at concentrations of 5, 10, 50, or 100 µg/cm^2 (equivalent to 50, 100, 500, 1000 µg/ml) for 24 hours without S9; Negative controls were untreated cells Mammalian cell micronucleus assay	**Food-grade TiO_2 (E171)**; 39% < 100 nm; anatase (Sensient Technologies Company). Stable suspensions generated by bath sonicating test article in 30 minutes (no other details provided).	Positive concentration-dependent increase in the incidence of micronucleated binucleated cells per 1000 binucleated cells at 5, 10, and 50 µg/cm^2. The cells exposed to the highest concentration (100 µg/cm^2) could not be assessed due to the presence of agglomerated particles, which interfered with the identification of micronuclei. No evidence of cytotoxicity was reported under test conditions; The authors also reported "E171 [seemed] to interact with the centromere region of kinetochore poles during mitosis"; however, based on the published photomicrographs, it is difficult to determine whether the particles were located inside the cells or on the surface of the cells. No positive control.
Tennant *et al.* (1987)	Chinese hamster ovary (CHO) cells; Exposed to concentrations of TiO_2 up to 25 µg/ml for unknown duration without S9; No information about positive and negative controls provided; Chromosomal aberration assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of	Negative, but particle uptake not evaluated and no information on cytotoxicity reported.

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
		particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	
Comet assays			
Brown *et al.* (2019)	Caco-2 cells (undifferentiated); Exposed to concentrations of 3.9, 7.8 or 15.6 µg/cm^2, equivalent to 12.5, 25, and 50 µg/ml for 4 hours; Untreated cells were used a negative control; H_2O_2 and $KBrO_3$ used as positive controls; Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; Three size groups of particles: 135±6, 305±61, 900±247 nm; 99.8% anatase and 0.2% rutile (Bolsjehuset – a supplier of candy ingredients). Stable suspensions generated by sonicating test article for 10 minutes without pause (no other details available).	Negative for DNA strand breaks and increase in Fpg-sensitive sites; No evidence of cytotoxicity; Visual scoring method used to assess the nuclei for DNA damage (unclear if conducted in a blinded manner); Cellular uptake of particles not confirmed.
	Human HepG2 liver cells; Exposed to concentrations of 3.9, 7.8 or 15.6 µg/cm^2, equivalent to 12.5, 25, and 50 µg/ml for 4 hours; Untreated cells were used a negative control; H_2O_2 and $KBrO_3$ used as positive controls; Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; Three size groups of particles: 135±6, 305±61, 900±247 nm; 99.8% anatase and 0.2% rutile (Bolsjehuset – a supplier of candy ingredients). Stable suspensions generated by sonicating test article for 10 minutes without pause (no other details available).	Negative for DNA strand breaks and increase in Fpg-sensitive sites; Evidence of cytotoxicity reported at the highest dose tested (15.6 µg/cm^2); Visual scoring method used to assess the nuclei for DNA damage (unclear if conducted in a blinded manner); Cellular uptake of particles not confirmed.
	Mouse embryonic cells; Exposed to concentrations of 0.98, 1.95, 3.9, 7.8 or 15.6 µg/cm^2, equivalent to 3.13, 6.25, 12.5, 25, and 50 µg/ml for 4 hours; Untreated cells were used a negative control; H_2O_2 and $KBrO_3$ used as positive controls; Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; Three size groups of particles: 135±6, 305±61, 900±247 nm; 99.8% anatase and 0.2% rutile (Bolsjehuset – a supplier of candy ingredients). Stable suspensions generated by sonicating test article for 10 minutes without pause (no other details available).	Positive for increase in DNA strand breaks at the highest dose tested (15.6 µg/cm^2), but not Fpg-sensitive sites; No information on cytotoxicity reported; Cellular uptake of particles not confirmed.
	Mouse embryonic cells; Exposed to concentrations of 0.98, 1.95, 3.9, 7.8 or 15.6 µg/cm^2, equivalent to 3.13, 6.25, 12.5, 25, and 50 µg/ml for 24 hours;	**Food-grade TiO_2 (E171)**; Three size groups of particles: 135±6, 305±61, 900±247 nm; 99.8% anatase and 0.2% rutile (Bolsjehuset – a supplier of candy ingredients).	Negative for changes in DNA damage, oxidative stress, p53-mediated cellular stress, or unfolded protein response;

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Untreated cells were used a negative control; cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response- endoplasmic reticulum stress), and $KBrO_3$ used as positive controls; ToxTracker reporter cell assay	Stable suspensions generated by sonicating test article for 10 minutes without pause (no other details available).	Excessive levels of cytotoxicity observed (approximately 25 to 75% of control) making the results difficult to intepret; Cellular uptake of particles not confirmed.
Dorier *et al.* (2017)	Mono-culture of Caco-2 cells (undifferentiated) and co-culture of Caco-2 cells (undifferentiated) and HT29-MTX mucus secreting cells; Acute: exposed to E171 at concentrations of 10 or 50 µg/ml for 6, 24 or 48 hours; Chronic: exposed to E171 at concentrations of 10 or 50 µg/ml twice a week for three weeks; Untreated cells were used a negative control; 50 nM H_2O_2 used as positive control; Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; mean 118±53 nm, range 20-340 nm, 44.7% < 100 nm; anatase with <1% rutile (French commercial supplier of food colouring). Stable suspensions generated using an indirect cup type sonicator; test article sonicated in water for 30 minutes.	Negative for DNA strand breaks and increase in Fpg-sensitive sites in acute exposure experiment and for DNA strand breaks in chronic exposure experiment in both the mono- and co-cultures, Positive, slight increase in Fpg-sensitive sites at both doses in the mono-culture, but only the 50 µg/ml in the co-culture in chronic exposure experiment; DNA damage was reported as fold-change from negative control (no absolute values for the treatment groups or negative or positive controls were presented) Exposure concentrations used "exceeded the estimated human daily exposure to TiO_2 [by] approximately 10,000-fold", Authors reported that E171 accumulated in the cells, but method used (ICP-MS of lysed cells) could not distinguish between Ti accumulation in cells and Ti bound to the cell membrane and the TEM images were two dimensional making it difficult to determine if the particles were inside the cell or on the cell surface. No evidence of cytotoxicity in acute or chronic conditions; Undifferentiated Caco-2 cells do not resemble mature enterocyte-like cells and are less representative of *in vivo* conditions;

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
			The authors reported that E171 accumulated in the cells; however, it is not possible to surmise from the two dimensional TEM images if the particles were inside of the cells or on the surface of the cells; Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis.
Dorier *et al.* (2019)	Co-culture of Caco-2 cells (undifferentiated) and HT29-MTX mucus secreting cells; Exposed to E171 at a concentration of 50 µg/ml for 24 hours; Untreated cells were used a negative control; 30 µg/ml MMS as positive control for alkaline comet assay and A549 cells exposed to 1 µM riboflavin and then irradiated with UVA as positive control for Fpg-comet assay; Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; mean 118±53 nm, range 20-340 nm, 44.7% < 100 nm; anatase with <1% rutile (French commercial supplier of food colouring). Stable suspensions generated using an indirect cup type sonicator; test article sonicated in water for 30 minutes.	Negative (+/- S9) for DNA double strand breaks (as measured by 53BP1 immunostaining and 8-oxo-dGuo levels in cells); no evidence that DNA repair mechanisms were impacted; No evidence of cytotoxicity after 6 or 48 hours; Cellular uptake of particles not confirmed; only single concentrations evaluated; Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis; Undifferentiated Caco-2 cells do not resemble mature enterocyte-like cells and are less representative of *in vivo* conditions.
Franz *et al.* (2020)	HT29-MTX-E12 cells; Exposure to E171 at concentrations of 0.5, 5 or 50 µg/ml 48 hours; Untreated cells were used a negative control; MMS (25 mM) and EMS (1 nM) used as positive controls; Mammalian alkaline comet assay	**Food-grade TiO_2 (E171)**; 170 nm; anatase (Hombitan AFDC, Venator). Stable suspensions generated by bath sonicating test article in double distilled water for 10 minutes. The suspensions were intentionally prepared to generate bigger agglomerates that more closely resembled the particle size distribution of E171 in the food matrix.	Negative for DNA strand breaks; No evidence of cytotoxicity; Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage; Agglomeration status of the particles in the exposure media was not confirmed and cellular uptake of particles was not evaluated.
Gea *et al.* (2019)	Human BEAS-2B bronchial epithelial cells;	**Food-grade TiO_2 (E171)**; 150 nm; anatase (Faravelli Group).	Positive; dose-dependent increase in DNA strand breaks, Fpg-sensitive sites, and oxidative damage starting at 50 µg/ml in light;

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Exposure to E171 at concentrations of 20, 50, 80, 120 or 160 µg/ml (equivalent to 5.2, 13.0, 20.7, 31.2 or 41.6 µg/cm^2) for 24 hours with light or in darkness; Unexposed cells and cells treated with 1% DMSO were used as negative controls; Mammalian alkaline comet assay +/- Fpg	Stable suspensions generated by bath sonicating test article in 100 minutes (no other details provided).	dose-dependent increase in DNA strand breaks and Fpg-sensitive sites starting at 80 µg/ml in darkness; oxidative damage only at highest dose tested (160 µg/ml) in darkness; The presence of DMSO may have affected the oxidative response and the 24 hour duration may have allowed for DNA repair to occur; No evidence of cytotoxicity after 24 hours at concentrations up to 80 µg/ml, but did not test the two highest concentrations used (i.e., 120 and 160 µg/ml); Presence of particles observed in lung cells; Relevance of E171 exposure to lung cells *in vitro* to human dietary exposure to food-grade TiO_2 is uncertain; No positive control.
Proquin *et al*. (2017)	Undifferentiated human Caco-2 intestinal epithelial cells; Exposed to E171 at a concentration of 0.143 µg/cm^2 (equivalent to 1 µg/ml) for 24 hours with or with co-exposure to 20 µg/ml AOM; Negative controls were untreated cells; H_2O_2 and AOM were used as positive controls; Mammalian alkaline comet assay	**Food-grade TiO_2 (E171)**; 39% < 100 nm; anatase (Sensient Technologies Company). Stable suspensions generated by bath sonicating test article in 30 minutes (no other details provided).	Positive, with similar levels of DNA damage in E171 and E171+AOM conditions; No evidence of cytotoxicity at concentration of E171 and AOM tested; Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage; Only a single concentration tested and the cellular uptake of particles at the E171 concentration tested was not confirmed. Undifferentiated Caco-2 cells do not resemble mature enterocyte-like cells and are less representative of *in vivo* conditions.

Study	Cell line, Exposure Concentrations and Duration, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Other genotoxicity assays			
Ivett *et al.* (1989)	Chinese hamster ovary (CHO) cells; Exposed to TiO_2 at concentrations of 2.5, 8.5 or 25 µg/ml for 2 hours with S9 and 25 hours without S9; S9 was prepared from the livers of male Fischer 344 rats with Aroclor 1254 pre-treatment; Solvent was used as negative control, mitomycin C was used as positive control without S9, and cyclophosphamide was used as positive control with S9; Sister chromatid exchange assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	Negative (+/- S9), but the *in vitro* sister chromatid exchange assay in mammalian cells was deleted by the OECD in 2014 due to "a lack of understanding of the mechanism(s) of action of the effect detected by the test" (OECD 2017); Highest dose limited by solubility; particle uptake not evaluated and no information on cytotoxicity was reported.
Tennant *et al.* (1987)	Chinese hamster ovary (CHO) cells; Exposed to concentrations of TiO_2 up to 25 µg/ml for unknown duration without S9; No information about positive and negative controls provided; Sister chromatid exchange assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation	Negative, but the *in vitro* sister chromatid exchange assay in mammalian cells was deleted by the OECD in 2014 due to "a lack of understanding of the mechanism(s) of action of the effect detected by the test" (OECD 2017); Particle uptake not evaluated and no information on cytotoxicity was reported.

Table 8. Summary table of test results of *in vivo* genotoxicity studies with food-grade TiO_2 and test articles highly comparable to food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Mutagenicity assays			
None identified			
Micronucleus and chromosomal aberration assays			
Shelby *et al.* (1993)	9 to 14-week-old male B6C3F1 mice (*n* = 5 per group); Treated by **intraperitoneal injection** daily for 3 days with vehicle control (corn oil) or doses of 250, 500, or 1000 mg/kg bw/d TiO_2 in corn oil in the first experiment and vehicle control (corn oil) or doses of 500, 1000, or 1500 mg/kg bw/d TiO_2 in corn oil in the second experiment. Positive control: dimethylbenzanthracene (12.5 mg/kg in corn oil, presumably by intraperitoneal injection). Mice euthanized 24 hours after last treatment and bone marrow (femur) and peripheral blood samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. Doses were prepared using corn oil as the test vehicle and then suspended in solution with a Tek-Mar Tissumizer® homogenizer.	Negative in both experiments (bone marrow erythrocytes and peripheral blood reticulocytes) <u>Experiment 1:</u> A statistical significant positive trend observed for bone marrow erythrocytes, with a statistically significant increase in micronucleated cells per 1000 PCEs at the highest dose tested compared to controls, but no clear dose-response and elevated test results were within the range of control data for the same sex and strain reported by the same authors. No evidence of micronuclei formation in peripheral blood erythrocytes. <u>Experiment 2:</u> A statistically significant increase in micronucleated cells per 1000 PCEs observed at the intermediate dose compared to control, but not at the lowest or highest doses tested. Elevated test results were within the range of control data for the same sex and strain reported by the same authors. Unclear what impact the sample preparation method had on agglomeration status of TiO_2 particles.
Shelby and Witt (1995)	9 to 14-week-old male B6C3F1 mice (*n* = 8 per group); Single **intraperitoneal injection** of vehicle control (corn oil) or doses of 625, 1250, or 2500 mg/kg bw TiO_2 in corn oil. Positive control: presumably dimethylbenzanthracene (12.5 mg/kg in corn oil,	**TiO_2 (Unitane® 0-220)** Test article sourced from NTP repository of all chemicals tested in carcinogenicity bioassays (Radian Corporation) and therefore, it is likely that it was the same material used in the NCI two-year TiO_2 cancer bioassay (**Unitane® 0-220**; anatase; 113-135 nm and 109-124 nm by SEM	Negative at both time points (bone marrow cells)

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	presumably by intraperitoneal injection), but data not presented. Mice euthanized 17 and 36 hours after last treatment and bone marrow (femur) samples were collected. Mammalian bone marrow chromosomal aberration assay	and TEM, respectively; up to 44% of particles < 100 nm by number). Based on similarities in both elemental composition, median particle diameter, mass-specific surface area, and percentage of particles < 100 nm by number, Unitane® 0-220 can be considered highly comparable to the current form of TiO_2 added to food. No information on sample preparation was available	
Comet assays			
Bettini *et al.* (2017)	Adult male Wistar rats (10 per group); Treated by **gavage** daily for 7 days with either vehicle control (water) or 10 mg/kg bw/d of E171 dispersed in water. Rats were euthanized (timing not reported) and Peyer's patch cells were collected. Mammalian alkaline comet assay +/- Fpg	**Food-grade TiO_2 (E171)**; mean 118±53 nm, range 20-340 nm, 44.7% < 100 nm; anatase (French commercial supplier of food colouring). Stable dispersions generated using the NANOGENOTOX dispersion protocol (particles ultrasonicated for 16 minutes at 20 kHz in 0.05% w/v BSA).	Negative (Peyer's patch cells). E171 particles were detected in Peyer's patch cells of treated rats confirming exposure. Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. No positive control and only a single dose group tested.
Jensen *et al.* (2019)	8-13 week old female lean Zucker (Crl:ZUC-Lepr^{fa}) rats (10 per group); Treated by **gavage** once a week for 10 weeks with vehicle control (sterile water with 2% FBS) or doses of 50, or 500 mg/kg bw/d E171 dispersed in filtered sterile water with 2% FBS. Rats were euthanized 24 hours after the last treatment and liver and lung tissue were collected. Positive control: $KBrO_3$ (5 mM) exposed THP-1 cells. Mammalian alkaline comet assay +/- Fpg or hOGG1	**Food-grade TiO_2 (E171)**; Three size groups of particles: 135±6, 305±61, 900±247 nm; 99.8% anatase and 0.2% rutile (Bolsjehuset – a supplier of candy ingredients). Stable dispersions generated using the ENPRA dispersion protocol (particles ultrasonicated at 20 kHz for 16 minutes in filtered sterile water with 2% FBS). Doses were prepared daily and administered immediately to rats.	Equivocal (liver and lung cells) No evidence of DNA strand breaks and Fpg- or hOGG1-sensitive sites in liver and lung cells and E171 treatment did not affect repair of $KBrO_3$-induced oxidative damage in lung tissue. However, limitations described below raise some questions as to the reliability of the study findings and make the negative results difficult to interpret. Systemic exposure to the test article in the liver and lung was not confirmed and DNA damage was not evaluated in an appropriate target tissue (i.e., GIT);

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
			Only two dose groups were evaluated; an outof date dosing regime was used; a visual scoring method was used to determine the level of DNA damage The analysis of DNA damage occurred 24 hours after last exposure instead of 2-6 hours after last dose, which may have allowed DNA repair to occur; Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis.

Table 9. Summary table of test results of *in vivo* genotoxicity studies with TiO_2 particles with a mean diameter of > 100 nm. The test articles used in these studies were insufficiently characterized to allow for comparison with the food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Mutagenicity assays			
None identified			
Micronucleus and chromosomal aberration assays			
Donner *et al.* (2016)	7 to 8-week-old male and female Crl:CD(SD) or Wistar Crl:WI(Han) rats (5 per sex per group; 7 per sex at highest dose level); Single **gavage** administration of vehicle control (sterile water) or doses of 500, 1000, or 2000 mg/kg bw TiO_2 dispersed in sterile water. Positive control: 10 mg/kg bw cyclophosphamide (intraperitoneal injection). Peripheral blood samples collected 48 and 72 hours after last treatment. Conducted in accordance with OECD guideline 474 Mammalian erythrocyte micronucleus assay	<u>Three test articles:</u> 1. **TiO_2 (pigment grade; pg-1);** median particle diameter of 120 nm; 27% of particles < 100 nm; anatase; mass-specific surface area of 8.1 m^2/g; isoelectric point around pH 4; whole particle and surface elemental composition including K, P, and Nb (source unknown) 2) **TiO_2 (pigment grade; pg-2);** median particle diameter of 165 nm; 11% of particles < 100 nm; rutile; mass-specific surface area 7.1 m^2/g; isoelectric point around pH 6; whole particle and surface elemental composition including Al (source unknown) 3) **TiO_2 (pigment grade; pg-3);** median particle diameter of 132 nm; 26% of particles < 100 nm; rutile; mass-specific surface area of 17.1 m^2/g; isoelectric point around pH 4; whole particle and	Equivocal at both time points for all three test articles (peripheral blood reticulocytes). However, Ti concentrations measured in the blood and liver were consistently low (≤1.14 µg/g blood and ≤0.316 µg/g liver), indicating low oral bioavailability of the pg-1 material regardless of dose administered. There was also no evidence of a dose-response, with similar Ti concentrations measured across the pg-1 dose groups and negative controls. Therefore, the lack of genotoxic effects observed for the three pigment-grades of TiO_2 were considered to be related to the lack of exposure to the test articles on account of their low oral bioavailability.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
		surface elemental composition including Al, Si, and Nb; particles reported to be coated, but no other details provided (source unknown) Stable dispersions generated by ultrasonicating the test articles in water for 3 hours at 50W.	The physicochemical properties of anatase pg-1 material were consistent with food grade TiO_2, however, not enough data was available on the current rutile forms of TiO_2 added to food to determine if the pg-2 and pg-3 materials were comparable to food-grade TiO_2.
Sycheva *et al.* (2011)	Male CBAxB6 mice (6 per group); Treated by **gavage** daily for 7 days with vehicle control (distilled water) or doses of 40, 200 or 1000 mg/kg bw/d TiO_2 dispersed in distilled water. Mice were euthanized 24 hours after the last treatment and bone marrow (femur), forestomach, colon, and testis samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2 (cosmetic grade)**; mean 160 ± 59.4 nm; anatase (marketed as a cosmetic ingredient in Russia). TiO_2 particles dispersed in distilled water (no other details provided).	Equivocal (bone marrow erythrocytes) Negative (forestomach and colon epithelia and testis spermatids) A statistically significant increase in micronuclei formation in bone marrow PCEs at the highest dose tested; however, this increase was also small in magnitude and of uncertain biological significance. The analysis of micronuclei incidence in bone marrow PCEs deviated from OECD test guidelines (474; 1997), with 1000 PCEs scored instead of the recommended 2000 or more PCEs. There was also no evidence of cytotoxicity observed in bone marrow PCEs and systemic exposure to the bone marrow was not confirmed. No evidence of micronuclei, nuclear protrusions, or typical nuclei in epithelial cells of the forestomach or colon or induced micronuclei in spermatids, but there was evidence of cytotoxicity. No positive control. In addition, the relevance of this study for the assessment of TiO_2 as a food additive was questionable since test article administered was not sufficiently characterized to allow for comparison with the form of TiO_2 added to food.
Comet assays			
Murugadoss *et al.* (2020)	8-week-old female C57BL/6JRj mice (4-5 per group);	**TiO_2 (JRC NM10200a)**; mean 117 nm; anatase (JRC nanomaterial); median equivalent circle	Equivocal (blood cells)

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Single **gavage** administration of vehicle control (suspension medium) or doses of 10, 50 or 250 µg TiO_2 per mouse (equivalent to ~0.6, 2.9 or 14.7 mg/kg bw). Mice were euthanized 3 days post-dosing and blood samples were collected. Positive control: Hydrogen peroxide (100 µM for 15 minutes) exposed blood cells from untreated mice. Mammalian alkaline comet assay	diameter of small and large agglomerates were 122 and 352 nm, respectively. Stable dispersions generated by ultrasonicating the test article in suspension media designed to produce small or large agglomerates using a probe sonicator (7056J) by using different pH conditions (pH 7.5 and pH 2, respectively). Particle suspensions were immediately stabilized with 0.25% BSA and the suspension dispersed at pH 2 was readjusted to pH 7-7.5. The median equivalent circle diameter was 122 nm for small agglomerates and 352 nm for large agglomerates.	A significant positive result was observed starting at the lowest dose tested for large agglomerates, and at the intermediate dose with small agglomerates, but no evidence of dose-response. Similar levels of DNA damage observed at all doses for both sizes of agglomerates and therefore, the biological significance of the dose-response relationship was considered uncertain. In addition, the TiO_2 particles would have had to be taken up by the cell nucleus to interact with the DNA to produce positive results and there is no evidence this occurs for particles of this size. There was also no reported change in blood Ti levels in response to TiO_2 treatment suggesting low bioavailability. Only a single sampling time 72 hours post-dosing was evaluated and positive control data was not reported Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. The study was designed to evaluate the properties/behaviour of nanomaterials under specific experimental conditions to generate small and large agglomerates of which the relevance to human exposure to food-grade TiO_2 through the diet is uncertain. In addition, the relevance of this study for the assessment of TiO_2 as a food additive was questionable since test article administered was not sufficiently characterized to allow for comparison with the form of TiO_2 added to food.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Sycheva *et al.* (2011)	Male CBAxB6 mice (5 per group); Treated by **gavage** daily for 7 days with vehicle control (distilled water) or doses of 40 or 200mg/kg bw/d TiO_2 dispersed in distilled water. Mice were euthanized 24 hours after the last treatment and bone marrow (femur), brain, and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2 (cosmetic grade)**; mean 160 ± 59.4 nm; anatase (marketed as a cosmetic ingredient in Russia). TiO_2 particles dispersed in distilled water (no other details provided).	Equivocal (bone marrow cells) Negative (brain and liver cells) For bone marrow cells, a statistically significant increase in % tail DNA was observed at both doses tested, but this increase was of uncertain biological significance since it was small in magnitude and there was no evidence of a dose response. In addition, the TiO_2 particles would have had to be taken up by the cell nucleus to interact with the DNA to produce positive results and there is no evidence this occurs for particles of this size. No information on organ toxicity was provided and systemic exposure to the test article in the bone marrow, liver, and brain was not confirmed. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Only two dose groups were tested, the analysis of DNA damage occurred 24 hours after the last exposure (which may have allowed DNA repair to occur) and there was no positive control. In addition, the relevance of this study for the assessment of TiO_2 as a food additive was questionable since test article administered was not sufficiently characterized to allow for comparison with the form of TiO_2 added to food.

Table 10. Summary table of test results of *in vivo* genotoxicity studies with non-food-grade TiO_2-NPs with a mean diameter of < 100 nm.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Mutagenicity assays			
Louro *et al.* (2014)	3-month-old C57B1/6-Tg (*LacZ*) mice (sex unknown), 5-6 per group; Treated by **intravenous injection** via caudal vein for 2 days with vehicle control (dispersion medium) or doses of 10 or 15 mg/kg bw/d TiO_2-NPs in dispersion medium. Positive control: *N*-ethyl-*N*-nitrosourea (120 mg/kg/bw by intraperitoneal injection). Mice were euthanized 28 days after last treatment and liver and spleen tissue samples were collected. Transgenic rodent gene mutation assay (*LacZ*)	**TiO_2-NPs**; mean 22nm; anatase (NM-102; JRC nanomaterial) Stable dispersions generated using the NANOGENOTOX dispersion protocol (particles ultrasonicated for 16 minutes at 400 W in 0.05% w/v BSA) and then diluted with PBS.	Negative (liver and spleen cells). Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Sadiq *et al.* (2012)	6-7-week-old male B6C3F1 mice (5 per group); Treated by **intraperitoneal injection** daily for 3 days with vehicle control (PBS) or doses of 0.5, 5, 50 mg/kg bw/d TiO_2-NPs in PBS. Positive control: *N*-ethyl-*N*-nitrosourea (140 mg/kg/bw by intraperitoneal injection) Peripheral blood samples collected 1 day pre-dosing and 1, 2, 4, and 6 weeks after the first dose. Erythrocyte *Pig-α* gene mutation assay	**TiO_2-NPs**; mean 12.1 nm; anatase (synthesized by sol-gel method) Stable dispersions generated with vigorous mixing and sonication test article in PBS (no other details provided).	Negative (peripheral blood reticulocytes). Unclear if dosing occurred by intravenous or intraperitoneal injection, but the EFSA FAF Panel (2021a) confirmed with authors that dosing occurred via intraperitoneal injection. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Suzuki *et al.* (2016)	8-week-old male *gpt* Delta C57BL/6J mice (5 per group); Treated by **intravenous injection** via caudal vein once a week for 4 weeks with vehicle control (disodium phosphate) or doses of 2, 10, 50 mg/kg bw/d TiO_2-NPs in disodium phosphate.	**TiO_2-NPs**; mean 21 nm; 80% anatase, 20% rutile (Sigma) Stable dispersion generated by ultrasonicating test article in 2 mg/mL disodium phosphate in water bath.	Negative for *Pig-α* gene mutations (peripheral blood erythrocytes) and negative for *gpt* and Spi^- gene mutations (liver cells). Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Positive control: *N*-ethyl-*N*-nitrosourea (70 mg/kg) or diethylnitrosamine (160 mg/kg) by intraperitoneal injection. Mice were euthanized 9 days after last treatment and peripheral blood and liver tissue samples were collected. Erythrocyte *Pig-α* gene mutation assay and transgenic rodent gene mutation assay (*gpt,* Spi$^-$)	Vehicle control was also ultrasonicated in water bath.	
Suzuki *et al.* (2020)	8-week-old male *gpt* Delta C57BL/6J mice (6 per group); Treated by **intravenous injection** via caudal vein once a week for 4 weeks with vehicle control (disodium phosphate) or doses of 2, 10, 50 mg/kg bw/d TiO_2-NPs in disodium phosphate. Mice were euthanized 90 days after last treatment and liver tissue samples were collected. Transgenic rodent gene mutation assay (*gpt,* Spi$^-$)	**TiO_2-NPs**; mean 21 nm; 80% anatase, 20% rutile (Sigma) Stable dispersion generated by ultrasonicating test article in 2 mg/mL disodium phosphate in water bath. Vehicle control was also ultrasonicated in water bath.	Negative (liver cells). Reported that mutation assays conducted as previously described (Suzuki *et al.* 2016), which likely includes positive control: *N*-ethyl-*N*-nitrosourea (70 mg/kg) or diethylnitrosamine (160 mg/kg) by intraperitoneal injection. However, no positive control data reported. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Trouiller *et al.* (2009)	4-5-month-old pregnant C57Bl/6Jp^{un}/p^{un} mice (5 per group) Exposed to TiO_2-NPs in **drinking water** at a concentration of 300 µg/mL (equivalent to 50 mg/kg bw/d based on average weight of 30 grams per mouse and average daily water intake of 5 mL) for 10 days (from 8.5 to 18.5 post-coitum). Vehicle control was water. Offspring (fetuses) were euthanized at 20 days and their eyes (retinal pigment epithelium) were collected. DNA deletion assay (p^{un} locus)	**TiO_2-NPs**; mean 21 nm; 75% anatase, 25% rutile (Degussa-Evonik) Stable dispersion generated by ultrasonicating test article in drinking water for 15 minutes.	Positive for an increase in the frequency of DNA deletions in fetuses (number of eyespots per retinal pigment epithelium) No positive control. The equivalent dose was reported as 500 mg/kg bw/d, but based on information reported by the authors the equivalent dose was actually 50 mg/kg bw/d. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Micronucleus and chromosomal aberration assays			
Ali *et al.* (2019)	Male Swiss Albino mice (15 per group);	<u>Two test articles:</u> 1. **TiO_2-NPs**; mean 21 nm; crystalline form unknown (Sigma)	Positive (bone marrow cells).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Treated by **gavage** daily for 5 days with vehicle control (physiological saline) or doses of 50, 250, 500 mg/kg bw/d TiO_2-NPs in physiological saline. Mice were euthanized after last treatment and bone marrow was collected. Mammalian bone marrow chromosomal aberration assay	2. **TiO_2-NPs**; mean 80 nm; crystalline form unknown (Sigma) Sample preparation unclear, but presumably followed method described in Trouiller *et al.* (2009) – ultrasonicated for 15 minutes (no other information available).	Dose-dependent increase in mean percentage of metaphases with chromosomal aberrations starting at lowest dose tested for both test articles. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Chakrabarti *et al.* (2019)	7-8-week-old male and female Swiss albino mice (5 per sex per group); Treated by **gavage** for 90 days with vehicle control (water) or doses of 200, 500 mg/kg bw/d TiO_2-NPs in water. Positive control: cyclophosphamide (40 mg/kg bw) via intraperitoneal injection on day 88. Mice were euthanized after last treatment and bone marrow (femur) samples were collected Mammalian erythrocyte micronucleus and mammalian bone marrow chromosomal aberration assays	**TiO_2-NPs**; mean 58 nm; crystalline form unknown (Sigma) Suspended test article in water (no other details provided).	Positive (bone marrow cells) Statistically significant increase in percentage of micronucleated polychromatic erythrocyte at highest dose tested, but evidence of dose response. Statistically significant increase in total number of chromosomal aberrations at highest dose tested but evidence of dose-response. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Chen *et al.* (2014)	8-week-old male Sprague Dawley rats (7 per group); Treated by **gavage** daily for 30 days with vehicle control (ultrapure water) or doses of 10, 50, or 200 mg/kg bw/d TiO_2-NPs in ultrapure water. Rats were euthanized after last treatment and bone marrow (femur) samples were collected Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 75 ± 15 nm; anatase (Shanghai Aladdin Reagent Co. Ltd, China) Stable dispersions generated by ultrasonicating test article in ultrapure water for 15 minutes and stirred on vortex agitator before each use.	Negative (bone marrow cells). No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Dobrzynska *et al.* (2014)	14-week-old male Wistar rats (7 per group per time point);	**TiO_2-NPs**; mean 21 nm; 85% anatase, 15% rutile (NM-105; JRC nanomaterial)	Positive (bone marrow cells). increase in number of micronuclei per 1000 PCE at 24 hours, but not 1 or 4 weeks; No effect on PCE%. Negative for chromosome damage at all time points.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Single **intravenous injection** via caudal vein of vehicle control (0.9% NaCl solution) or 5 mg/kg bw TiO_2 in BSA/PBS solution. Rats were euthanized 24 hours, 1 week and 4 weeks after treatment and bone marrow (femur) samples were collected. Mammalian erythrocyte micronucleus assay	Stable dispersions generated by ultrasonicating (output 20) test article in PBS; bovine serum albumin added after sonication.	No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Donner *et al.* (2016)	7 to 8-week-old male and female Crl:CD(SD) or Wistar Crl:WI(Han) rats (5 per sex per group; 7 per sex at highest dose level); Single **gavage** administration of vehicle control (sterile water) or doses of 500, 1000, or 2000 mg/kg bw TiO_2 dispersed in sterile water. Positive control: 10 mg/kg bw cyclophosphamide (intraperitoneal injection). Peripheral blood samples collected 48 and 72 hours after last treatment. Mammalian erythrocyte micronucleus assay	Three test articles: 1. **TiO_2-NPs**; 42 nm; anatase (source unknown) 2. **TiO_2-NPs**; 43 nm; 83.5% anatase, 16.5% rutile (source unknown) 3. **TiO_2-NPs**; 47 nm; rutile (source unknown) Stable dispersions generated by ultrasonicating the test articles in water for 3 hours at 50W.	Negative (peripheral blood reticulocytes). Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
El-Bassyouni *et al.* (2017)	Adult male Albino mice (5 or 10 per group); Treated by **intraperitoneal injection** once a week for 4 weeks with vehicle control (saline water) or doses of 100, 200, or 400 mg/kg bw/d TiO_2-NPs in saline water. Positive control: 40 mg/kg bw cyclophosphamide (intraperitoneal injection). Mice were euthanized (timing not reported) and bone marrow (femur) samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; particle size unknown; anatase (BDH) No information about sample preparation was provided.	Positive (bone marrow cells). Increased incidence of micronucleated PCEs at highest dose tested, but not at lower or intermediate doses. Evidence of dose-response. Unclear if the authors tested 5 or 10 mice per treatment group. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
El-Ghor *et al.* (2014)	Male Swiss Webster mice (5 per group); Treated by **intraperitoneal injection** daily for 5 days with vehicle control (deionized water) or doses of	**TiO_2-NPs**; mean 44 nm; anatase/rutile (Sigma) No information about sample preparation was provided.	Positive (bone marrow cells).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	500, 1000, or 2000 mg/kg bw/d TiO_2-NPs in deionized water. Positive control: 25 mg/kg bw cyclophosphamide (intraperitoneal injection). Mice were euthanized 24 hours after final treatment and bone marrow (femur) samples were collected. Mammalian erythrocyte micronucleus assay		Increased incidence of micronucleated PCEs and decrease in PCEs/NCEs ratio starting at lowest dose with evidence of dose-response. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Fadoju *et al.* (2019)	6-8-week-old male Swiss mice (5 per group per time point); Treated by **intraperitoneal injection** daily for 5 days with vehicle control (MilliQ water) or doses of 9.38, 18.75, 37.5, 75, or 150 mg/kg bw/d TiO_2-NPs in MilliQ water. Positive control: 25 mg/kg bw cyclophosphamide (intraperitoneal injection). Mice were euthanized 6 hours after final treatment and bone marrow samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; < 25 nm; anatase (Sigma) Stable dispersions generated by ultrasonicating test article in MilliQ water in a sonication bath three times for 3 minutes with a 30-second pause in between and then vortexed for 5 minutes.	Positive (bone marrow erythrocytes). Increase in micronucleated PCEs and decrease in PCEs/NCEs ratio at all doses at 5 days; Increase in micronucleated PCEs at 10 days only in highest dose tested and decrease in PCEs/NCEs ratio at 10 days starting at doses ≥ 37.5 mg/kg bw/d. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Grissa *et al.* (2015)	4-month-old male Wistar rats (6 per group); Treated by **gavage** daily for 60 days with vehicle control (distilled water) or doses of 50, 100, or 200 mg/kg bw/d TiO_2-NPs in distilled water. Rats were euthanized 24 hours after the final treatment and bone marrow (femur) were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; 5-12 nm, anatase (AZ Tech - paint ingredient for the ceramic sector) Stable dispersions generated by ultrasonicating test article in distilled water for 30 minutes and then mechanically vibrating the solution for 5 minutes before each use.	Positive (bone marrow erythrocytes). Increased incidence of micronucleated PCEs starting at 100 mg/kg bw/d. Decrease in %PCE at highest dose tested. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Kazimirova *et al.* (2019)	8-week-old female Wistar rats (6-8 per group per time point); Single **intravenous injection** of vehicle control (physiological solution with 10% v/v rat serum) or	**TiO_2-NPs**; mean 21 nm; anatase/rutile (70:30 or 80:20), (Evonik) Stable dispersions generated by ultrasonicating test article in physiological solution with 10% v/v rat serum for 15 minutes at 150W.	Negative at all time points (bone marrow erythrocytes). No positive control.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	0.59 mg/kg bw TiO_2-NPs in physiological solution with 10% v/v rat serum. Rats were euthanized 1 day, 1, 2, or 4 weeks after treatment and bone marrow (femur) samples were collected Mammalian erythrocyte micronucleus assay		Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Kumar *et al.* (2016)	8-week-old male Wistar rats (6 per group); Treated by **intravenous injection** via caudal vein once a week for 30 days with vehicle control (saline) or doses of 5, 25, or 50 mg/kg bw/d TiO_2-NPs in saline. Rats were euthanized and peripheral blood samples were collected (timing not reported). Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; 10-20 nm, crystalline form unknown, surface chemical composition: 56% titanium, 26% oxygen; 18% copper; (Sigma) Stable dispersions generated by ultrasonicating test article in saline for 15 minutes.	Positive (peripheral blood cells), Increase in micronuclei formation starting at 25 mg/kg bw/d. Details of study method/results not well described. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Lotfi *et al.* (2016)	8-week-old male Balb/c mice (4 per group); Single **intraperitoneal injection** of vehicle control (sterile water) or doses of 100, 1000, or 3000 mg/kg bw/d TiO_2 in sterile water in the first experiment and vehicle control (sterile water) or 1000 mg/kg bw/d TiO_2 in sterile water in the second experiment. Untreated control group also included in both experiments. Mice were euthanized 24 hours after treatment (experiment 1) and 24, 48, 72, and 96 hours after treatment (experiment 2) and bone marrow (femur) were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 28.88 nm; rutile (synthesized by sol-gel method) Stable dispersions generated by ultrasonicating test article in saline for 15 minutes.	Positive in both experiments (bone marrow cells). Experiment 1: Increase in micronuclei frequency in polychromatic erythrocytes starting at 1000 mg/kg bw/d at 24 h. Experiment 2: Increase in micronuclei frequency in polychromatic erythrocytes at 24, 48, and 72 h, but not 96 hours No positive control. Authors report positive control as treatment with sterile water (not a true positive control) and negative control as no treatment. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Louro *et al.* (2014)	3-month-old C57B1/6-Tg (*LacZ*) mice (sex unknown), 5-6 per group; Treated by **intravenous injection** via caudal vein for 2 days with vehicle control (dispersion medium) or doses of 10, or 15 mg/kg bw/d TiO_2-NPs in dispersion medium. Positive control: *N*-ethyl-*N*-nitrosourea (120 mg/kg/bw by intraperitoneal injection). Peripheral blood samples were collected 42 hours after last treatment. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 22nm; anatase (NM-102; JRC nanomaterial) Stable dispersions generated using the NANOGENOTOX dispersion protocol (particles ultrasonicated for 16 minutes at 400 W in 0.05% w/v BSA) and then diluted with PBS.	Negative (peripheral blood reticulocytes). Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Manivannan *et al.* (2020)	Male Swiss albino mice (5 per group); Treated by **gavage** daily for 28 days with vehicle control (double distilled water) or doses of 0.2, 0.4 or 0.8 mg/kg bw of TiO_2-NPs dispersed in double distilled water. Positive control: single intraperitoneal injection of 2.5 mg/kg bw mitomycin C Mice were euthanized 18 hours after final treatment and bone marrow samples were collected. Mammalian bone marrow chromosomal aberration assay	**TiO_2-NPs**; mean 25 nm, range 21-31 nm; rutile (Sigma) Stable dispersions generated by ultrasonicating test article in double distilled water for 30 minutes at 100 W and 30 kHz.	Positive (bone marrow cells) A statistically significant increase in the percentage of aberrant cells and number of chromosome aberration per cell starting at ≥ 0.4 mg/kg bw/d with evidence of dose response. Chromatid and iso-chromosome breaks were most common aberrations. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Rizk *et al.* (2017)	Male Swiss Albino mice (15 per group per time point) Treated by **intraperitoneal injection** daily for 7, 14 or 45 days with vehicle control (hydroxylpropyl methylcellulose) or doses of 50, 250 or 500 mg/kg bw of TiO_2-NPs dispersed in hydroxylpropyl methylcellulose. Mice were euthanized and bone marrow (femur) were collected (timing not reported).	**TiO_2-NPs**; 21 nm; crystalline form unknown (Sigma) No information about sample preparation was provided.	Positive at 45 days (bone marrow cells) A statistically significant increase in % aberrations at 45 days starting at lowest dose tested with evidence of dose response. Negative at 7 and 14 days. Only 300 erythrocytes were analyzed per animal (should be at least 500 erythrocytes for bone marrow according to OECD guidelines).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mammalian bone marrow chromosomal aberration assay		No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Rizk *et al.* (2020)	Male Swiss Albino mice (25 per group per time point) Treated by **intraperitoneal injection** daily for 7, 14 or 45 days with vehicle control (1% tween 80) or doses of 50, 250 or 500 mg/kg bw of TiO_2-NPs dispersed in 1% tween 80. Methods indicate mammalian erythrocyte micronucleus assay will be conducted, but results reported are for a mammalian bone marrow chromosomal aberration assay.	**TiO_2-NPs**; 83.4 nm; crystalline form unknown (Sigma) Stable dispersions generated by ultrasonicating test article in 1% tween 80 for 15 minutes.	Difficult to interpret (discrepancy between method and results presented) Unclear if lowest dose is 50 or 150 mg/kg bw/d (probably 50 mg/kg bw/d) No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Sadiq *et al.* (2012)	6-7-week-old male B6C3F1 mice (5 per group); Treated by **intraperitoneal injection** daily for 3 days with vehicle control (PBS) or doses of 0.5, 5, 50 mg/kg bw/d TiO_2-NPs in PBS. Positive control: *N*-ethyl-*N*-nitrosourea (140 mg/kg by intraperitoneal injection) Peripheral blood samples collected 1 day after final treatment. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 12.1 nm; anatase (synthesized by sol-gel method) Stable dispersions generated with vigorous mixing and sonication test article in PBS (no other details provided).	Negative (peripheral blood reticulocytes). Unclear if dosing occurred by intravenous or intraperitoneal injection, but the EFSA FAF Panel (2021a) confirmed with authors that dosing occurred via intraperitoneal injection. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Shukla *et al.* (2014)	6-week-old male Swiss albino mice (5 per group); Treated by **gavage** daily for 14 days with vehicle control (MilliQ water) or doses of 10, 50 or 100 mg/kg bw/d TiO_2-NPs in MilliQ water. Positive control: ethylmethanesulfonate (to control for DNA damage). Mice were euthanized 24 hours after final treatment and bone marrow (femur) samples were collected.	**TiO_2-NPs**; 20-50 nm, 99.7% anatase (Sigma) Stable dispersions generated by sonicated test article in Milli-Q water for 20 minutes at 30W using a probe sonicator (2.5 minutes pulse on, 1.5 minutes pulse off).	Positive (bone marrow cells). Increased micronuclei formation at highest dose tested with evidence of dose response. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mammalian erythrocyte micronucleus assay		
Suzuki *et al.* (2016)	8-week-old male *gpt* Delta C57BL/6J mice (5 per group); Treated by **intravenous injection** via caudal vein once a week for 4 weeks with vehicle control (disodium phosphate) or doses of 2, 10, 50 mg/kg bw/d TiO_2-NPs in disodium phosphate. Mice were euthanized 9 days after last treatment and peripheral blood samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 21 nm; 80% anatase, 20% rutile (Sigma) Stable dispersion generated by ultrasonicating test article in 2 mg/mL disodium phosphate in water bath. Vehicle control was also ultrasonicated in water bath.	Negative (peripheral blood reticulocytes) No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Sycheva *et al.* (2011)	Male CBAxB6 mice (6 per group); Treated by **gavage** daily for 7 days with vehicle control (distilled water) or doses of 40, 200 or 1000 mg/kg bw/d TiO_2 dispersed in distilled water. Mice were euthanized 24 hours after the last treatment and bone marrow (femur) samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; 33 ± 16.7 nm; anatase (sourced from a Russian cosmetic supplier). TiO_2 particles dispersed in distilled water (no other details provided).	Negative (bone marrow cells). The analysis of micronuclei incidence in PCEs deviated from OECD test guidelines (474; 1997), with 1000 PCEs scored instead of the recommended 2000 or more PCEs. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Trouiller *et al.* (2009)	4-5-month-old male C57Bl/6Jp^{un}/p^{un} mice (5 per group) Exposed to TiO_2-NPs in **drinking water** at a concentration of 60, 120, 300 or 600 µg/mL (equivalent to 10, 20, 50 or 100 mg/kg bw/d based on average weight of 30 grams per mouse and average daily water intake of 5 mL) for 5 days. Vehicle control was water. Peripheral blood samples were collected (timing not reported). Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; mean 21 nm; 75% anatase, 25% rutile (Degussa-Evonik) Stable dispersion generated by ultrasonicating test article in drinking water for 15 minutes just before use.	Positive (peripheral blood erythrocytes). Increase in the frequency of micronuclei per polychromatic erythrocytes at highest dose tested, with no evidence of dose response. No positive control. The equivalent doses were reported as 50, 100, 250, and 500 mg/kg bw/d, but based on information reported by the authors the equivalent dose was actually 10, 20, 50 and 100 mg/kg bw/d.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
			Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Xu *et al.* (2013)	Male and female ICR mice (4 per sex per group); Single **intravenous injection** of vehicle control (saline) or doses of 140, 300 or 645 mg/kg bw TiO_2-NPs in saline. Positive control: 20 mg/kg bw cyclophosphamide via intraperitoneal injection 24 and 48 hours before the mice were euthanized. Mice were euthanized 14 days after treatment and bone marrow samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; 42.50 ± 60 nm, anatase (Hangzhou Wanjing materials Co, Ltd) Stable dispersion generated by ultrasonicating test article in saline for 3 minutes at 400W.	Negative (bone marrow cells). Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Zirak *et al.* (2016)	Male Balb/c mice (4 per group); Single **intraperitoneal injection** of vehicle control (sterile water) or doses of 10, 100 or 500 mg/kg bw TiO_2-NPs in sterile water. Untreated control group also included. Mice were euthanized 24 hours after treatment and bone marrow samples were collected. Mammalian erythrocyte micronucleus assay	**TiO_2-NPs**; 20.17 nm; anatase (synthesized by sol-gel method) Stable dispersion generated by ultrasonicating test article in sterile water for 15 minutes (no other details provided).	Positive (bone marrow cells). A statistically significant increase in the frequency of micronuclei formation in polychromatic erythrocytes with no evidence of dose response. No positive control. Authors report positive control as treatment with sterile water (not a true positive control) and negative control as no treatment. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Comet assays			
Asare *et al.* (2016)	8-12-week-old male $Ogg1^{-/-}$ knockout (KO) and $Ogg1^{+/+}$ wild type (WT) mice (5-6 per group per time point); Single **intravenous injection** via caudal vein of vehicle control (dH20/10X BSA/10X PBS) or 5 mg/kg bw TiO_2-NPs in dH20/10X BSA/10X PBS.	**TiO_2-NPs**; 21 nm; crystalline form unknown (Degussa-Evonik) Stable dispersion generated by ultrasonicating test article in in dH20/10X BSA/10X PBS (4.2 x 105 kJ/m3). No other details provided.	Positive on Day 7 for increase in Fpg sensitive sites in WT mice, but no evidence of DNA strand breaks (testis cells) Negative on Day 1 in KO and WT mice (lung, testis, liver cells) and on Day 7 in KO mice (lung, testis, liver cells) and WT mice (lung, liver cells).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Positive control: mice exposed to 0-5 Gy of x-rays. Mice were euthanized 1 or 7 days after treatment and liver, lung and testis tissue samples were collected. Mammalian alkaline comet assay +/- Fpg		Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Azim *et al.* (2015)	Male Albino mice (10-14 per group); Treated by **gavage** daily for 14 days with either vehicle control (1% tween 80) 150 mg/kg bw/d of TiO_2-NPs dispersed in 1% tween 80. Mice were euthanized (timing not reported) and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 21 nm; anatase (Sigma) Stable dispersion generated by ultrasonicating test article in 1% tween 80 for 15 minutes (no other details provided).	Positive for DNA damage (tail moment); (liver cells) No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Bettini *et al.* (2017)	Adult male Wistar rats (10 per group); Treated by **gavage** daily for 7 days with either vehicle control (water) or 10 mg/kg bw/d of TiO_2-NPs dispersed in water. Rats were euthanized (timing not reported) and Peyer's patch cells were collected. Mammalian alkaline comet assay +/- Fpg	**TiO_2-NPs**; mean 22 nm, range 15-24 nm, 85% anatase, 15% rutile (NM-105, JRC nanomaterial) Stable dispersions generated using the NANOGENOTOX dispersion protocol (particles ultrasonicated for 16 minutes at 400 W in 0.05% w/v BSA).	Negative (Peyer's patch cells) Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Chakrabarti *et al.* (2019)	7-8-week-old male and female Swiss albino mice (5 per sex per group); Treated by **gavage** for 90 days with vehicle control (water) or doses of 200, 500 mg/kg bw/d TiO_2-NPs in water. Positive control: cyclophosphamide (40 mg/kg bw) via intraperitoneal injection on day 88.	**TiO_2-NPs**; mean 58 nm; crystalline form unknown (Sigma) Suspended test article in water (no other details provided).	Positive (liver and kidney cells). Statistically significant increase in DNA damage (tail length, % tail DNA, tail moment, Olive moment) at highest dose tested. Preparation of the slides, lysis and electrophoresis was conducted under

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mice were euthanized 24 hours after last treatment and liver and kidney tissue samples were collected Mammalian alkaline comet assay		dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Dekanski *et al.* (2018)	7-week-old female NMRI/Han mice (5 per group); Single dose administered over 4 treatments separated by 30 minutes. Treated by **gavage** with 1000 or 2000 mg/kg bw TiO_2-NPs in a solution of 0.01M HCl in water (pH 2). Unclear if control animals received vehicle. Whole blood samples were collected 2 and 24 hours after the final treatment. Mammalian alkaline comet assay	**TiO_2-NPs**; mean diameter 45 Å (4.5 nm); crystalline form unknown (synthesized by acidic hydrolysis of titanium(IV) chloride) No information about sample preparation was provided.	Negative for DNA damage at both time points (blood cells). Mice were fasted for 4 hours prior to experiment; food and water returned 2 hours after the last dose. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Dobrzynska *et al.* (2014)	14-week-old male Wistar rats (7 per group per time point); Single **intravenous injection** via caudal vein of vehicle control (0.9% NaCl solution) or 5 mg/kg bw TiO_2 in BSA/PBS solution. Rats were euthanized 24 hours, 1 week and 4 weeks after treatment and bone marrow (femur) samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; mean 21 nm; 85% anatase, 15% rutile (NM-105; JRC nanomaterial) Stable dispersions generated by ultrasonicating (output 20) test article in PBS; bovine serum albumin added after sonication.	Negative for DNA damage (bone marrow leukocytes) Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
El-Bassyouni *et al.* (2017)	Adult male Albino mice (10 per group); Treated by **intraperitoneal injection** once a week for 4 weeks with vehicle control (saline water) or doses of 100, 200, or 400 mg/kg bw/d TiO_2-NPs in saline water.	**TiO_2-NPs**; particle size unknown; anatase (BDH) No information about sample preparation was provided.	Positive (liver). A statistically significant increased in DNA damage at the highest dose tested

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Positive control: 40 mg/kg bw cyclophosphamide (intraperitoneal injection). Mice were euthanized (timing not reported) and liver tissue samples were collected. Mammalian alkaline comet assay		Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
El-Din *et al.* (2019)	Adult male Albino rats (8 per group); Treated by **gavage** daily for 90 days with vehicle control (0.9% saline) or doses of 1200 mg/kg bw/d TiO_2-NPs in 0.9% NaCl. Rats were euthanized after final treatment and cardiac tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; < 100 nm; mixture of anatase and rutile (Sigma) Stable dispersions generated by ultrasonicating test article for 10 minutes (no other details provided).	Equivocal (cardiac myocytes). Evidence of DNA damage, but only alongside obvious tissue damage. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
El-Ghor *et al.* (2014)	Male Swiss Webster mice (5 per group); Treated by **intraperitoneal injection** daily for 5 days with vehicle control (deionized water) or doses of 500, 1000, or 2000 mg/kg bw/d TiO_2-NPs in deionized water. Positive control: 25 mg/kg bw cyclophosphamide (intraperitoneal injection). Mice were euthanized 24 hours after final treatment and bone marrow (femur), brain, and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; mean 44 nm; anatase/rutile (Sigma) No information about sample preparation was provided.	Positive for DNA damage (tail length, % DNA in tail mean, tail moment) at all doses in all three cell types (bone marrow, brain, and liver cells) Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Elnagar *et al.* (2018)	Male Albino rats (10 per group);	**TiO_2-NPs**; 21 nm; crystalline form unknown (Titanos)	Equivocal (testes cells).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Treated by **gavage** daily for 12 weeks with vehicle control (5% gum acacia solution) or doses of 1200 mg/kg bw/d TiO_2-NPs in 5% gum acacia solution. An untreated control group was also included. Rats were euthanized (timing not reported) and the testicular tissue samples were collected. Mammalian alkaline comet assay	No information about sample preparation was provided.	Authors report positive increase in DNA damage in TiO_2 treated rats compared to treated and untreated controls, but no quantitative data was provided to confirm this statement. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Fadda *et al.* (2018)	Male Wistar rats (10 per group); Treated by **gavage** daily for 21 days with vehicle control (1% carboxylmethyl cellulose) or doses of 1000 mg/kg bw/d TiO_2-NPs in 1% carboxylmethyl cellulose. Rats were euthanized after the final treatment and liver tissues samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 60 nm; anatase (Sigma) Stable dispersions generated by ultrasonicating test article for 20 minutes (no other details provided).	Equivocal (liver cells). Evidence of DNA damage, but only alongside obvious tissue damage and inflammation. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Fadda *et al.* (2019)	Male Wistar rats (10 per group); Treated by **gavage** daily for 5 days with doses of 600 mg/kg bw/d TiO_2-NPs in 1% tween. Control rats were untreated. Rats were euthanized after the final treatment and liver tissues samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 60 nm; anatase (Sigma) Stable dispersions generated by ultrasonicating test article for 20 minutes (no other details provided).	Equivocal (liver cells). Evidence of DNA damage, but only alongside obvious tissue damage and inflammation. No positive control or vehicle control group. Preparation of the slides, lysis and electrophoresis was conducted under

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
			dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Grissa *et al.* (2015)	4-month-old male Wistar rats (6 per group); Treated by **gavage** daily for 60 days with vehicle control (distilled water) or doses of 50, 100, or 200 mg/kg bw/d TiO_2-NPs in distilled water. Rats were euthanized 24 hours after the final treatment and blood samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 5-12 nm, anatase (AZ Tech - paint ingredient for the ceramic sector) Stable dispersions generated by ultrasonicating test article in distilled water for 30 minutes and then mechanically vibrating the solution for 5 minutes before each use.	Positive (peripheral blood leukocytes). Dose dependent increase was observed that reached statistical significance at 100 mg/kg bw/d. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Grissa *et al.* (2017)	4-month-old male Wistar rats (6 per group); Treated by **gavage** daily for 60 days with vehicle control (distilled water) or doses of 100 mg/kg bw/d TiO_2-NPs in distilled water. Rats were euthanized 24 hours after the final treatment and blood samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 5-10 nm, anatase (AZ Tech - paint ingredient for the ceramic sector) Stable dispersions generated by ultrasonicating test article in distilled water for 30 minutes and then mechanically vibrating the solution for 5 minutes before each use.	Positive (peripheral blood leukocytes). Evidence of DNA damage in treated rats compared to controls, but unconventional DNA damage scores reported. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Hashem *et al.* (2020)	Adult male Wistar rats (10 per group); Treated by **gavage** daily for 90 days with vehicle control (0.5% hydroxypropyl methylcellulose) or	**TiO_2-NPs**; particle size and crystalline form unknown (Sigma)	Equivocal (spleen cells) Evidence of DNA damage, but only alongside tissue damage and inflammation.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	doses of 20 or 40 mg/kg bw/d TiO_2-NPs in 0.5% hydroxypropyl methylcellulose. Rats were euthanized 24 hours after the final treatment and spleen tissue samples were collected. Mammalian alkaline comet assay	Authors report test article to be E171, but Sigma does not sell food-grade TiO_2 particles and no particle size or size distribution data was presented. Particles were suspended by unknown method (no other details were provided).	No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Hassanein *et al.* (2017)	Adult male Sprague Dawley rats (10 per group) Treated by **gavage** daily for 6 weeks with vehicle control (1% tween 80) or 150 mg/kg bw/d TiO_2-NPs in 1% tween 80. Rats were euthanized 24 hours after the final treatment and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 21 nm; crystalline form unknown (Sigma) No information about sample preparation was provided.	Equivocal (liver cells). Evidence of DNA damage, but only alongside obvious tissue damage and inflammation. No positive control. Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Kazimirova *et al.* (2019)	8-week-old female Wistar rats (6-8 per group per time point); Single **intravenous injection** of vehicle control (physiological solution with 10% v/v rat serum) or 0.59 mg/kg bw TiO_2-NPs in physiological solution with 10% v/v rat serum. Rats were euthanized 1 day, 1, 2, or 4 weeks after treatment and blood samples were collected. Positive control: hydrogen peroxide exposed blood cells. Mammalian alkaline comet assay +/- Fpg	**TiO_2-NPs**; mean 21 nm; anatase/rutile (70:30 or 80:20), (Evonik) Stable dispersions generated by ultrasonicating test article in physiological solution with 10% v/v rat serum for 15 minutes at 150W.	Positive (peripheral blood mononuclear cells). Increased % DNA in tail one day after the final treatment in the absence of Fpg, but not with Fpg or at other time points. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Kumar *et al.* (2016)	8-week-old male Wistar rats (6 per group); Treated by **intravenous injection** via caudal vein once a week for 30 days with vehicle control (saline) or doses of 5, 25, or 50 mg/kg bw/d TiO_2-NPs in saline. Rats were euthanized and samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 10-20 nm, crystalline form unknown, surface chemical composition: 56% titanium, 26% oxygen; 18% copper; (Sigma) Stable dispersions generated by ultrasonicating test article in saline for 15 minutes.	Equivocal (splenocytes). Evidence of DNA damage (tail length, tail moment, tail migration) starting at 25 mg/kg bw/d, but only alongside tissue damage and inflammation. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Li *et al.* (2017b)	6 to 7-week-old male B6C3F1 mice (5 per group) Treated by **intraperitoneal injection** daily for 3 days with vehicle control (PBS) or doses of 50 mg/kg bw/d TiO_2-NP in PBS. Positive control: 100 mg/kg bw methyl methanesulphonate via intraperitoneal injection. Mice were euthanized 4 hours after the final dose and liver and lung tissue samples were collected. Mammalian alkaline comet assay +/- hOGG1 or EndoIII	**TiO_2-NPs**; 8.9 to 15.3 nm; anatase (synthesized by sol-gel method) Stable dispersions generated by ultrasonicating test article in PBS for 1 minute (output 100W, frequency 40 kHz).	Positive for DNA damage (increase in % DNA in tail) in liver but not lung tissue (standard alkaline comet assay); Positive for DNA damage (increase in % DNA in tail) in liver and lung tissue (hOGG1 and EndoIII modified alkaline comet assays) Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Louro *et al.* (2014)	3-month-old C57B1/6-Tg (*LacZ*) mice (sex unknown), 5-6 per group; Treated by **intravenous injection** via caudal vein for 2 days with vehicle control (dispersion medium) or doses of 10, or 15 mg/kg bw/d TiO_2-NPs in dispersion medium. Positive control: *N*-ethyl-*N*-nitrosourea (120 mg/kg/bw by intraperitoneal injection).	**TiO_2-NPs**; mean 22nm; anatase (NM-102; JRC nanomaterial) Stable dispersions generated using the NANOGENOTOX dispersion protocol (particles ultrasonicated for 16 minutes at 400 W in 0.05% w/v BSA) and then diluted with PBS.	Negative at both doses (liver and spleen cells). Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. OECD guidelines recommend collecting samples 2-6 hours post-dosing.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mice were euthanized 28 days after the final treatment and liver and spleen tissue samples were collected. Mammalian alkaline comet assay		Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Manivannan *et al.* (2020)	Male Swiss albino mice (5 per group); Treated by **gavage** daily for 28 days with vehicle control (double distilled water) or doses of 0.2, 0.4 or 0.8 mg/kg bw of TiO_2-NPs dispersed in double distilled water. Mice were euthanized after final treatment and liver, spleen, thymus, bone marrow, and lymph node tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; mean 25 nm, range 21-31 nm; rutile (Sigma) Stable dispersions generated by ultrasonicating test article in double distilled water for 30 minutes at 100 W and 30 kHz.	Positive (liver, bone marrow, spleen, thymus, lymph nodes). A statistically significant increase in % tail DNA was observed starting at the lowest dose tested in the spleen, at the intermediate dose in the liver, lymph node and thymus, and at the highest dose tested in the bone marrow. Evidence of dose response in most tissues. No information on tissue/organ toxicity was provided. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Martins Jr *et al.* (2017)	Male Wistar rats (6 per group); Treated by **oral gavage** daily for 45 days, with either vehicle control (sodium citrate buffer, pH 4.5) or 0.5 mg/kg TiO_2-NPs in sodium citrate buffer, pH 4.5. Rats euthanized after final treatment and blood and liver tissue samples were collected Mammalian alkaline comet assay	**TiO_2-NPs**; 42 nm; crystalline form unknown (Sigma) No information about sample preparation was provided.	Negative (blood and liver). No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Meena *et al.* (2015a)	8-week-old male Wistar rats (6 per group); Treated by **intravenous injection** via caudal vein once a week for 30 days with vehicle control (1M PBS) or doses of 5, 25, or 50 mg/kg bw/d TiO_2-NPs in 1M PBS. Rats were euthanized after final treatment (timing not reported) and sperm cells were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 10-20 nm; anatase (Sigma) That authors report that the test article was 'dissolved' in PBS. Unclear if solution was sonicated for the experiment, but the authors reported sonicating the test article for 10 minutes in distilled water for characterization.	Positive (sperm cells). A significant increase in DNA damage (tail length, tail movement, tail migration) observed starting at intermediate dose with evidence of dose response. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Meena *et al.* (2015b)	8-week-old male Wistar rats (6 per group); Treated by **intravenous injection** via caudal vein once a week for 4 weeks with vehicle control (1M PBS) or doses of 5, 25, or 50 mg/kg bw/d TiO_2-NPs in 1M PBS. Rats were euthanized after final treatment (timing not reported) and sperm cells were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 10-20 nm; anatase; surface chemical composition: 56% titanium, 26% oxygen; 18% copper; (Sigma) Stable dispersions generated by ultrasonicating test article in 1M PBS for 15 minutes at 35 kHz.	Positive (brain cells) A significant increase in DNA damage (tail length, tail movement, tail migration) observed starting at the intermediate dose with evidence of dose response. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Modrzynska *et al.* (2018)	6-week-old female C57BL/6 (B6JBOM-F) mice (9 per group); Single **gavage** or **intravenous injection** administration of vehicle control or 162 µg of TiO_2 in a volume of 50 µL per mouse (equivalent to dose of ~7.4 mg/kg bw, assuming an average body weight of	**TiO_2-NPs**; 10.5 nm; crystalline form unknown (NanoAmor) Stable dispersions generated by ultrasonicating test article in 2% v/v mouse serum from C57BL/6 mice in nanopure water for 15 minutes (no other details provided).	Negative (liver). No positive control. Method did not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	22 grams). Test vehicle was 2% v/v mouse serum from C57BL/6 mice in nanopure water. Control mice receive the test vehicle. Mice were euthanized 1, 28, and 180 days after treatment and liver tissue samples were collected. Mammalian alkaline comet assay	Test vehicle also sonicated prior to exposure to control mice.	OECD guidelines recommend collecting samples 2-6 hours post-dosing. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Mohamed and Hussien *et al.* (2016)	10 to 12-week-old male Swiss Webster mice (3 per group); Treated by **gavage** daily for 5 days with 500 mg/kg bw/d TiO_2-NPs. Unclear was the test vehicle was, but the particles were characterized in water. The control group was untreated. Mice were euthanized 24 hours, 7 days and 14 days after treatment and brain tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 46.23 ± 3.45 nm; 22% anatase, 77% rutile (Sigma) Stable dispersions generated by ultrasonicating test article (no other details provided).	Equivocal (brain cells). Evidence of DNA damage, but only alongside obvious tissue damage. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Mohamed (2015)	10 to 12-week-old male Swiss Webster mice (5 per group per time point); Treated by **gavage** daily for 5 days with vehicle control (distilled water) or doses of 5, 50, or 500 mg/kg bw/d TiO_2-NPs in distilled water. Mice were euthanized 24 hours, 7 days and 14 days after the final treatment and gastric tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 46.23 ± 3.45 nm; 22% anatase, 77% rutile (Sigma) Stable dispersions generated by ultrasonicating test article (no other details provided).	Equivocal (gastric cells). Evidence of DNA damage in all treatment groups, but only alongside obvious cytotoxicity (as measured by various oxidative stress markers); Evidence of dose response as well as evidence of increased DNA damage over time. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
Moran-Martinez *et al.* (2018)	10 to 12-week-old male Long Evans rats (3 per group) Treated by **intraperitoneal injection** daily for 3 days with vehicle control (xylocaine: 2% injectable solution of lidocaine without epinephrine) or doses of 5 mg/kg bw TiO_2-NPs in xylocaine: 2% injectable solution of lidocaine without epinephrine. Rats were euthanized 48 and 72 hours after final treatment and blood samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 21 nm; crystalline form unknown (Sigma) Dispersed with mixture of water and ethanol (1:4); stability of suspension obtained by magnetic stirring (no other details provided).	Negative (lymphocytes). No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Murugadoss *et al.* (2020)	8-week-old female C57BL/6JRj mice (4-5 per group); Single **gavage** administration of vehicle control (suspension medium), 10, 50 or 250 µg TiO_2 in suspension medium per mouse (equivalent to ~0.6, 2.9 or 14.7 mg/kg bw). Mice were euthanized 3 days post-dosing and blood samples were collected. Positive control: Hydrogen peroxide (100 µM for 15 minutes) exposed blood cells from untreated mice. Mammalian alkaline comet assay	**TiO_2-NPs**; 17 nm; anatase (JRC nanomaterial) Stable dispersions generated by ultrasonicating the test article in two different suspension media designed to produce small or large agglomerates using a probe sonicator (7056J). Particle suspensions were immediately stabilized with 0.25% BSA. The median equivalent circle diameter was 122 nm for small agglomerates and 352 nm for large agglomerates.	Positive (leukocytes). For small agglomerates, a statistically significant increase in % tail DNA was observed in all treatment groups, but with no evidence of dose response. For large agglomerates, no statistically significant differences in % tail DNA were observed in any treatment group. Methods do not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Niu *et al.* (2017)	6 to 8-week-old male and female laboratory bred Kun Ming mice (4 per sex per group); Treated by **gavage** daily for 7 days with vehicle control (PBS) or doses of 2000 mg/kg bw/d TiO_2-NPs in PBS. Mice were euthanized 7 days after the final treatment and heart, liver, and kidney tissue samples were collected.	**TiO_2-NPs**; 10-25 nm; anatase (Sigma) Stable dispersions generated by suspending the test article in PBS and vigorously stirring and then sonicating for 20 minutes at 60W.	Equivocal (liver, kidney, heart). A statistically significant increase in tail DNA % and olive tail moment in all three organs compared to control, but only in the presence of organ toxicity. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mammalian alkaline comet assay		dim/yellow light to prevent additional DNA damage. Mice were fasted overnight before treatment. Food and water were provided 2 hours post-dosing. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Shi *et al.* (2015)	6-8 week old wild type ICR mice and $Nrf2^{(-/-)}$ ICR mice (4 per sex per group); Wild type mice were treated by **gavage** daily for 7 days with vehicle control (PBS) or doses of 500, 1000 or 2000 mg/kg bw/d TiO_2-NPs in PBS. $Nrf2^{(-/-)}$ mice received vehicle control (PBS) or 1000 mg/kg bw/d TiO_2-NPs in PBS. Mice were euthanized 1 day after the final treatment and liver and kidney tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 10–25 nm; anatase (Sigma) Stable dispersions generated by ultrasonicating the test article in PBS for 20 minutes at 60W before use.	Positive (liver and kidney). Dose-dependent increase in % tail DNA observed from the lowest dose. The response was greater in $Nrf2^{(-/-)}$ mice. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Mice were fasted overnight before treatment. Food and water were provided 2 hours post-dosing. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Shukla *et al.* (2014)	6-week-old male Swiss albino mice (5 per group); Treated by **gavage** daily for 14 days with vehicle control (MilliQ water) or doses of 10, 50 or 100 mg/kg bw/d TiO_2-NPs in MilliQ water. Positive control: ethylmethanesulfonate (to control for DNA damage) and potassium dichromate (to control for oxidative stress).	**TiO_2-NPs**; 20-50 nm, 99.7% anatase (Sigma) Stable dispersions generated by sonicated test article in Milli-Q water for 20 minutes at 30W using a probe sonicator (2.5 minutes pulse on, 1.5 minutes pulse off).	Positive (liver cells). A statistically significant dose-dependent increase in % tail DNA was observed at concentrations of 50 mg/kg bw and higher, with evidence of dose-response (with and without Fpg). A statistically significant dose-dependent increase in Olive tail moment in all dose groups, with evidence of dose-response (with and without Fpg).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Mice were euthanized 24 hours after final treatment and liver tissue samples were collected. Mammalian alkaline comet assay +/- Fpg		Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Suzuki *et al.* (2016)	8-week-old male *gpt* Delta C57BL/6J mice (5 per group); Treated by **intravenous injection** via caudal vein once a week for 4 weeks with vehicle control (disodium phosphate) or doses of 2, 10, 50 mg/kg bw/d TiO_2-NPs in disodium phosphate. Mice were euthanized 3 days after last treatment and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; mean 21 nm; 80% anatase, 20% rutile (Sigma) Stable dispersion generated by ultrasonicating test article in 2 mg/mL disodium phosphate in water bath. Vehicle control was also ultrasonicated in water bath.	Negative (liver). No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Sycheva *et al.* (2011)	Male CBAxB6 mice (5 per group); Treated by **gavage** daily for 7 days with vehicle control (distilled water) or doses of 40, 200 or 1000 mg/kg bw/d TiO_2 dispersed in distilled water. Mice were euthanized 24 hours after the last treatment and bone marrow (femur), brain, and liver tissue samples were collected. Mammalian alkaline comet assay	**TiO_2-NPs**; 33 ± 16.7 nm; anatase (sourced from a Russian cosmetic supplier). TiO_2 particles dispersed in distilled water (no other details provided).	Positive (bone marrow, liver). Negative (brain). Positive results (% tail DNA) observed starting at the lowest dose tested in bone marrow and at the highest dose tested in liver. No positive control. Preparation of the slides, lysis and electrophoresis was conducted under dim/yellow light to prevent additional DNA damage. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Trouiller *et al.* (2009)	4-5-month-old male C57Bl/6Jp^{un}/p^{un} mice (5 per group)	**TiO_2-NPs**; mean 21 nm; 75% anatase, 25% rutile (Degussa-Evonik)	Equivocal (peripheral white blood cells) Increase in tail moment, but cytotoxicity not evaluated making it difficult to interpret results

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Exposed to 500 mg/kg bw/d TiO_2-NPs in **drinking water** (concentration TiO_2-NPs unclear). Other dose calculations for other genotoxicity assays were incorrect and therefore, it is unclear what concentration of TiO_2-NPs the mice were exposed to. Vehicle control was water. Peripheral blood samples were collected (timing not reported). Mammalian alkaline comet assay	Stable dispersion generated by ultrasonicating test article in drinking water for 15 minutes just before use.	No positive control. Methods do not indicate if controlled for ambient light during slide preparation, lysis or electrophoresis. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Other genotoxicity assays			
Asare *et al.* (2016)	8-12-week-old male $Ogg1^{-/-}$ knockout (KO) and $Ogg1^{+/+}$ wild type (WT) mice (6 per group per time point); Single **intravenous injection** via caudal vein of vehicle control (dH20/10X BSA/10X PBS) or 5 mg/kg bw TiO_2-NPs in dH20/10X BSA/10X PBS. Positive control: mice exposed to 0-5 Gy of x-rays. Mice were euthanized 1 or 7 days after treatment and sperm cells were collected. Sperm chromatin structure assay	**TiO_2-NPs**; 21 nm; crystalline form unknown (Degussa-Evonik) Stable dispersion generated by ultrasonicating test article in in dH20/10X BSA/10X PBS (4.2 x 105 kJ/m3). No other details provided.	Negative on Day 1 and 7 for sperm DNA fragmentation in KO and WT mice (sperm cells) Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Chen *et al.* (2014)	8-week-old male Sprague Dawley rats (7 per group); Treated by **gavage** daily for 30 days with vehicle control (ultrapure water) or doses of 10, 50, or 200 mg/kg bw/d TiO_2-NPs in ultrapure water. Rats were euthanized after last treatment and bone marrow (femur) samples were collected γ- H_2AX bone marrow assay	**TiO_2-NPs**; mean 75 ± 15 nm; anatase (Shanghai Aladdin Reagent Co. Ltd, China) Stable dispersions generated by ultrasonicating test article in ultrapure water for 15 minutes and stirred on vortex agitator before each use.	Positive (brain cells) Increase in DNA fragmentation compared to control. No positive control. Unstandardized genotoxicity assay. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Kandeil *et al.* (2020)	Adult male Albino rats (20 per group)	**TiO_2-NPs**; 90 nm; crystalline form unknown (Sigma) TiO_2 particles prepared by high-energy ball mill.	Positive (brain cells) Increase in DNA fragmentation compared to control.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	Treated by **gavage** daily for 14 days with vehicle control (distilled water) of doses of 500 mg/kg bw/d TiO_2-NPs in distilled water. Rats were euthanized (timing not reported) and brain tissue samples were collected. Brain DNA fragmentation assay	Stable dispersions generated by ultrasonicating test article in distilled water for 15 minutes (no other details provided).	No positive control. Unstandardized method used for detection of DNA damage. Lack of details provided with respect to both the methods and results. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Li *et al.* (2010)	Female CD-1 (ICR) mice (10 per group); Treated by **intraperitoneal injection** daily for 14 days with vehicle control (0.5% hydroxypropyl methylcellulose) or doses for 5, 10, 50, 100, or 150 mg/kg bw/d TiO_2-NPs in 0.5% hydroxypropyl methylcellulose. Mice were euthanized after the final treatment and liver tissue samples were collected. DNA binding assay	**TiO_2-NPs**; 5 nm; anatase (synthesized by controlled hydrolysis of titanium tetrabutoxide) Stable dispersions generated by ultrasonicating for 30 minutes and then mechanically vibrating for 5 minutes.	Positive for DNA binding (liver cells). Accumulated in liver DNA by inserting itself into DNA base pairs or binding to DNA nucleotides that were bound with 3 oxygen or nitrogen atoms and 2 phosphorous atoms of DNA. Change in DNA conformation at doses ≥ 50 mg/kg bw/d. No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Minigalieva *et al.* (2018)	Male outbred white rats (minimum of 12 per group); Treated by **intraperitoneal injection** three times per week for 6 weeks (up to 18 injections) with vehicle control (deionized water) or doses of 0.5 mg/mL TiO_2-NPs in deionized water (equivalent to ~2.5 mg/kg bw/d). Rats were euthanized after final treatment and blood samples were collected. Random amplification of polymorphic DNA assay	**TiO_2-NPs**; 27 nm; crystalline form unknown; source unknown Particles dispersed into suspensions (no other details provided).	Positive (blood nucleated cells) A small but statistically increase in DNA fragmentation coefficient in treated rats compared to controls. No positive control. Unstandardized genotoxicity assay. Used a fragmentation coefficient to characterize the degree of DNA damage (ratio of total radioactivity of all tail fractions to that of the head).

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
			Lack of details provided with respect to the methods. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Mohamed and Hussein (2016)	10 to 12-week-old male Swiss Webster mice (3 per group); Treated by **gavage** daily for 5 days with 500 mg/kg bw/d TiO_2-NPs. Unclear was the test vehicle was, but the particles were characterized in water. The control group was untreated. Mice were euthanized 24 hours, 7 days and 14 days after treatment and brain tissue samples were collected. Single strand conformation polymorphism analysis of presenilin 1 gene	**TiO_2-NPs**; 46.23 ± 3.45 nm; 22% anatase, 77% rutile (Sigma) Stable dispersions generated by ultrasonicating test article (no other details provided).	Equivocal Unstandardized genotoxicity assay and test method has low sensitivity No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Mohamed (2015)	10 to 12-week-old male Swiss Webster mice (5 per group per time point); Treated by **gavage** daily for 5 days with vehicle control (distilled water) or doses of 5, 50, or 500 mg/kg bw/d TiO_2-NPs in distilled water. Mice were euthanized 24 hours, 7 days and 14 days after the final treatment and gastric tissue samples were collected. Single strand conformation polymorphism analysis of presenilin 1 gene	**TiO_2-NPs**; 46.23 ± 3.45 nm; 22% anatase, 77% rutile (Sigma) Stable dispersions generated by ultrasonicating test article (no other details provided).	Equivocal Unstandardized genotoxicity assay and test method has low sensitivity No positive control. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Trouiller *et al.* (2009)	4-5-month-old male C57Bl/6Jp^{un}/p^{un} mice (5 per group) Exposed to TiO_2-NPs in **drinking water** at a concentration of 60, 120, 300 or 600 µg/mL (equivalent to 10, 20, 50 or 100 mg/kg bw/d based on average weight of 30 grams per mouse and	**TiO_2-NPs**; mean 21 nm; 75% anatase, 25% rutile (Degussa-Evonik) Stable dispersion generated by ultrasonicating test article in drinking water for 15 minutes just before use.	Positive (bone marrow). A significant increase in % of γ-H2AX positive cells; evidence of dose response. Unstandardized genotoxicity assay.

Study	Species-strain, Doses and Dosing Regimen, Assay	Test Article (source) and Sample Preparation	Result/Remarks
	average daily water intake of 5 mL) for 5 days. Vehicle control was water. Mice were euthanized after final treatment and bone marrow (femur) samples were collected. γ- H_2AX bone marrow assay		Lack of details provided with respect to the methods. No positive control. The equivalent doses were reported as 50, 100, 250, and 500 mg/kg bw/d, but based on information reported by the authors the equivalent dose was actually 10, 20, 50 and 100 mg/kg bw/d. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Appendix I – Summary of Studies Investigating Inflammation and Immunotoxicity

Table 11. Summary table of studies providing evidence of immune dysregulation or inflammation following exposure to TiO_2 via the oral route.

Study	Species-strain, dose and dosing regimen	Test Article (source)	Result
Riedle *et al.* 2020	C57BL/6 mice (6 per sex per group) were exposed to 0, 1, 10, or 100 mg/kg bw/d food-grade TiO_2 via the **diet** for 18 weeks.	**Food-grade TiO_2** (anatase, 119 nm) from Sensient Colors (St. Louis, MO, USA). Test article was formulated into diet.	No evidence of gross perturbation of immune-cell physiology or inflammation at doses up to 100 mg/kg bw/d via the diet. Authors demonstrate uptake of TiO_2 by Peyer's patches, validating the delivery model.
Blevins *et al.* 2019	Male Wistar Han IGS rats (15 per group) were exposed to E171 in a standard **diet** at doses equal to 1.81 (basal diet), 4.76, 31.43 or 373.86 mg/kg bw/d for 7 days and 1.1-1.5 (basal diet), 3.0-4.1, 19.0-25.7, or 236-300 mg/kd bw/d for 100 days (doses based on food consumption, measured Ti concentration in diet and body weight data; control reflects background in basal diet). In the 100 day study, E171 was administered alone or following pretreatment with 1,2-dimethylhydrazine (DMH) to initiate colon carcinogenesis.	**Food-grade E171-E** was supplied by the Titanium Dioxide Manufacturers Association. E171 was formulated in pelleted diet at a concentration of 0, 40, 400 or 5,000 mg/kg diet.	Dietary E171 produced no signs of overt toxicity up to the highest dose tested. No effects were observed on T helper cells in Peyer's patches or systemically. E171 had no effects on pro-inflammatory cytokine production.
LPT 2020 as cited in EFSA 2021a	GLP- and OECD TG 443-compliant extended one-generation reproductive toxicity (EOGRT) study in CD/Crl:CD(SD) rats. The material tested was a commercial formulation of E171 that was administered in the **diet**. The F0 generation was exposed to E171 via the diet at doses of 0, 100, 300 or 1000 mg/kg bw/d for 10 weeks prior to mating until the F1 generation was weaned. The F1 generation received the same diets until PND 4 or 8 of the F2 generation. The background level of TiO_2 in the diet ranged from 11-31 mg Ti/kg diet.	**Food-grade E171-E** (anatase, median particle diameter of 99.9 ± 2.0 nm, 50-51% nanoscale) was formulated in the diet.	No adverse effects associated with general toxicity were observed in the EOGRT study with E171 at doses up to 1,000 mg/kg bw per day. The NOAEL is considered to be the highest dose tested, although the EFSA FAF Panel raised uncertainty regarding the extent of exposure to internal TiO_2-NPs across the range of doses tested.
Talamini *et al.* 2019	Male NFR mice (22 per group) were treated **orally** with vehicle or E171 dispersed in water at 5 mg/kg bw/d, 3 d/wk for 3 wk. E171 or water; Dripped into the mouth using a pipette.	**E171** (35% nano), anatase. E171 was "administered with no sonication or de-agglomeration to simulate realistic conditions of use".	RT-PCR was used to examine changes in the pro-inflammatory cytokines IL-1β, TNF-α as well as anti-inflammatory cytokine IL-10 in stomach, intestine and kidney. A statistically significant increase in stomach and intestine IL-1β was observed, as were

Study	Species-strain, dose and dosing regimen	Test Article (source)	Result
			decreases in intestinal TNF-α and liver IL-10. The changes are likely to be adaptive as opposed to adverse. Statistically significant increase in "necroinflammatory foci" in the livers of treated mice, but the toxicological relevance could not be determined (not accompanied by additional endpoints indicative of liver injury; histological evaluation performed on only 2 mice in control group and 4 mice in treatment group; this type of inflammatory foci known to be present in control mice).
Pinget *et al.* 2019	Five to six male C67Bl/6JAusb mice were administered E171 via **drinking water** at doses of 0, 2, 10 or 50 mg E171/kg bw/d for 4 weeks (dose calculated based on water intake measured per cage).	**Food-grade TiO_2** (anatase) from All Color Supplies PTY. TiO_2 was dispersed in drinking water using sonication (no further details provided).	TiO_2 was found to have a minor impact on gut microbiota composition at the highest dose tested. Alterations in bacterial metabolites were observed from 10 mg/kg bw/sd. A reduction in colonic crypt length, increase in colon macrophages and CD8+ cells and an increase in mRNA transcripts for IL-10, TNF-α, and IL-6 were observed.
Bettini *et al.* 2017	Adult male Wistar rats received drinking water (n = 12) or E171 in **drinking water** at a dose of 10 mg/kg bw/d (n = 11) for 100 days. In the 100 day study, E171 was administered alone or following pretreatment with 1,2-dimethylhydrazine (DMH) to initiate colon carcinogenesis.	**E171**: 118 nm anatase (range 20–340 nm). Stable dispersions were generated using the NANOGENOTOX dispersion protocol (particles coated with BSA and ultrasonically dispersed).	Ultrasonicated E171 particles prepared in water did not reagglomerate *in vivo* in the intestinal lumen. In animals exposed to E171, a decrease in % T-helper cells was observed in Peyer's patches. Deviations in Th1/Th17 were also observed in the spleen, indicative of systemic effects. Increases in colonic mucosal levels of TNF-α, IL-8 and IL-10 in the E171 group were indicative of low level inflammation.
Urrutia-Ortega *et al.* 2016	BALB/c male mice were divided into 4 groups (n = 6 per group): a) vehicle control, b) E171 group, c) chemically colitis-associated colorectal cancer (CAC) group, and d) CAC + E171. E171 was administered 5d/wk by **oral gavage** at a dose of 5 mg/kg bw/d for 10 weeks.	**E171**, crystalline form not stated (50 to 600 nm with a maximum peak of 300 nm). E171 was dispersed in water by sonication for 30 minutes.	E171 alone was unable to induce tumour formation, but dysplastic alterations were observed in the distal colon with a statistical significant enhancement of tumour formation in CAC+ E171 group versus CAC group ($p < 0.01$). In the E171 group, despite the absence of tumour formation, a slight but statistically significant increase in the tumour progression markers COX2, Ki67 and b-catenin, and p65 NF-κB were observed.
Han *et al.* 2020	SD rats (10 per sex per group) were administered E171 by **oral gavage** at doses of 0, 10, 100 or 1,000 mg/kg bw/d for 90 days. Study conducted according to OECD TG 408.	**E171** (HOMBITAN® FG; purity 99.5%, 150 nm, anatase) from Venator Germany GmbH. E171 suspensions were prepared at least once per week by dispersing the particles in distilled water for at least 10 minutes by sonication (energy not specified), which were reported to be stable for 7 days when stored in a refrigerator	Statistically significantly decreases in GM-CSF plasma levels (~30%) and plasma IgM (~12% in females and 9% in males) were observed at the highest dose. There was no evidence of a dose response and the magnitude of the effect was small given natural variability in these parameters. No change in immune organ weights or histology.
Heo *et al.* 2020	GLP- and OECD-compliant study conforming to TG 407 (28-d) and 408 (90-d) repeated dose oral toxicity studies. SD	**TiO_2-NPs** (AEROXIDE® TiO_2 P25, KRISS CRM 301–03-001, anatase/rutile (80/20), 99.9%; average primary particle size range 14–	No systemic toxicity was observed in either study up to the highest dose tested, which was considered the NOAEL. Authors state that particles were not detected in target organs, although

Study	Species-strain, dose and dosing regimen	Test Article (source)	Result
	rats were administered vehicle control or TiO_2-NPs by **oral gavage** at doses of 250, 500 or 1000 mg/kg bw/d (*n* = 5 per sex per group for the 28-d study and 10-15 per sex per group for the 90 d study).	21 nm) from Evonik Industries AG (Essen, Germany). Formulated in 5 mM PBS. Particles had no surface modification and were administered in agglomerated/aggregated form.	it is unclear how this was determined. Test article is of uncertain relevance to hazard characterization of E171.
Mohamed 2015	Male Swiss Webster mice (15 per group) were administered water (control) or TiO_2-NP suspensions by **oral gavage** at doses of 5, 50, or 500 mg/kg bw/d for 5 days. Following dosing, animals from each group were sacrificed at 24 h, 7 d and 14 d.	The **TiO_2-NPs** used in this study were a mixture of rutile and anatase forms purchased from Sigma. The average size of particles was estimated to be 46.23 ± 3.45 nm. Particles were suspended by ultrasonication (no further details).	At the lowest dose tested, submucosal edema was identified at 24 h that progressed to ulcerations and ultimately necrosis by 14 days. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Li *et al.* 2019	Male C57BL/6 mice (7 per group) were administered by **oral gavage** either vehicle or one of three TiO_2-NP preparation at a dose of 1 mg/kgbw/d for 7 days. Mice were sacrificed following 3- or 7-days of dosing.	Three test articles were used: 1. **TiO_2-NPs**, anatase, 25 nm; 2. **TiO_2-NPs**, anatase, 50 nm; 3. **TiO_2-NPs**, anatase, 80 nm. Particles were suspended in PBS by ultrasonication for 30 minutes (power not stated).	Study determined the smallest particles (25 nm) lead to colonic epithelial injury and alterations in gut microbiota composition. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Hashem *et al.* 2020	Male Wistar rats were dosed by **oral gavage** with 0.5% hydroxypropyl methylcellulose (HPMC), or TiO_2 suspended in HPMC at doses of 20 or 40 mg/kg bw/d for 90 days.	**"E171"** was said to be obtained from Sigma (no information of particle size distribution or crystal form). Suspensions were prepared daily although no other details are provided.	At the end of treatment, bw of treated animals was 25% lower than controls. The authors also report hematological and immunological alterations but given the reduction in body weight the claim that the test article is E171 is not considered plausible. Sigma carries a variety of TiO_2 particles of various sizes but to Health Canada Food Directorate's knowledge they do not offer E171.
Yu *et al.* 2016	Female CD-1 (ICR) mice (*n* = 20 per group) were dosed by **oral gavage** with 0.5% hydroxypropyl methylcellulose (HPMC), or TiO_2 suspended in HPMC at doses of 2.5, 5 or 10 mg/kg bw/d for 90 days.	Anatase **TiO_2-NPs** were prepared via controlled titanium tetrabutoxide hydrolysis. Average particle size was 5–6 nm. Dispersion protocol not stated.	A dose dependent increase in inflammatory cytokines was observed as was cardiac toxicity. However, Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Li *et al.* 2018	Male C57BL/6 mice were divided into 3 groups (*n* = 10 per group). Animals received either vehicle or the rutile or anatase TiO_2-NPs at 100 mg/kg bw/d by **oral gavage** for 28 days.	Rutile and anatase **TiO_2-NPs** from Alfa Aesar (Ward Hill, MA, USA) were suspended in water. Mean diameter of anatase particles was 20.13 ± 0.18 nm and rutile was 15.91 ± 0.05 nm. Characterized particles by XRD, SEM, and DLA. Zeta potentials and sizes were also determined in different conditions.	Differential accumulation in spleen, lung and kidney of rutile and anatase forms was observed that was not accompanied by histopathological changes. Effects on gut microbiota were observed, particularly with the rutile form and significant increase in vili length and irregular arrangement of villus epithelial cells was found in animals exposed to rutile TiO_2. No immunological parameters other than histopathology of the spleen were included. Health Canada's Food Directorate does not

Study	Species-strain, dose and dosing regimen	Test Article (source)	Result
		In simulated gastric fluid and simulated intestinal fluid the particles were aggregated (anatase: 135 nm, 420 nm; rutile: 148 nm, 360 nm, respectively).	consider the test article relevant to the hazard characterization of food-grade TiO_2.
Chen *et al.* 2015	SD rats (5 per sex per group) were administered suspensions of TiO_2-NPs (0, 2, 10 or 50 mg/kg bw/d), glucose (1.8 g/kg bw/d), or TiO_2-NPs + glucose at the same doses by **oral gavage** for 30 (interim sacrifice) or 90 days.	Anatase **TiO_2-NPs** from Shanghai Aladdin Reagent Co. Ltd, China. Average size of the TiO_2-NPs was 24 ± 5 nm. Particles were suspended in water or water + glucose by sonication for 15 minutes (no further details stated).	The authors reported histopathological findings in the liver at the highest dose but no changes in aminotransferases (ALT, AST) that would be indicative of liver damage. The 50 mg/ kg bw/d dose can therefore be considered a NOAEL, although Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.
Grissa *et al.* 2020	Male Wistar rats (8 per group) were administered by **oral gavage** a vehicle control or TiO_2-NPs at doses of 50, 100 or 200 mg/kg bw/d five d/wk for 8 weeks.	Anatase **TiO_2-NPs** from AZ tech (Italy); mean particle size was 8.5 ± 3.5. Particles were dispersed by ultrasonic bath for 30 minutes (no further details stated).	Brain histopathology was noted at the mid- and high-dose. SOD and CAT activities were significantly decreased at all doses and other markers of oxidative stress at the mid- and high-dose. Health Canada's Food Directorate does not consider the test article relevant to the hazard characterization of food-grade TiO_2.

Appendix J – Summary of Studies Investigating TiO_2 Effects on Gut Microbiota (not an exhaustive list)

Table 12. Summary table of test results of gut microbiota studies via the oral route.

Study	Species-strain, doses, and dosing regimen	Test Article (source)	Result
Cao *et al.* 2020	6 week-old male C57BL/6 mice (*n* = 15 per subgroup); Exposure via **diet**; Mice divided into 2 groups: low-fat diet (10 kcal% as fat) and high-fat diet (60kcal% as fat). These two groups were divided into 3 subgroups of 15 each: control diet, diet containing TiO_2-NPs, and diet containing E171; Mice were exposure to a diet with 0.1 wt% TiO_2 for 8 weeks and then sacrificed	Two test articles: 1. **E171**, anatase, mean size 111.9 nm (range 20 to 230) with 44% nano, Source: Precheza Inc. (Czech Republic) 2. **TiO_2-NPs**, anatase, mean size 32.7 nm (range 10 to 90 nm), Source: US Research Nanomaterial Inc. (Houston, TX, USA)	No general toxicity observed in obese or non-obese mice fed E171 (potential adverse effects of TiO_2-NPs in liver and kidney). Both E171 and TiO_2-NPs altered the composition of microbiota in the gut of obese and non-obese mice compared to control (increase in *Firmicutes* phylum and decrease in *Bacteroidetes* phylum and *Bifidobacterium* and *Lactobacillus* genera). Effects were more pronounced in obese mice compared to non-obese mice and in obese mice fed TiO_2-NPs compared to E171. Both test articles decreased the cecal levels of short-chain fatty acid. Both test articles increased pro-inflammatory immune cells and cytokines in the colonic mucosa (suggestive of an inflammatory state), but the immune response was stronger in mice exposed to TiO_2-NPs. The effects are also more pronounced in mice fed the high-fat diet (i.e., obese mice)
Li *et al.* (2019)	Male C57BL/6 mice (8 weeks old) (28 per group); Treated by **oral gavage** for 3 or 7 d; TiO_2-NPs suspended in PBS; Rats divided in 4 groups of 28: control or TiO_2-NPs (25 nm) , TiO_2-NPs (50 nm) , TiO_2-NPs (80 nm) at a dose of 1 mg/kg bw/d	Three test articles: 1. **TiO_2-NPs**, anatase, 25 nm; Source: Shanghai Naiou Nanotechnology Co., Ltd., China 2. **TiO_2-NPs**, anatase, 50 nm; Source: Shanghai Naiou Nanotechnology Co., Ltd., China 3. **TiO_2-NPs**, anatase, 80 nm; Source: Shanghai Naiou Nanotechnology Co., Ltd., China	Short-term oral exposure to TiO_2-NPs (25 nm) at a dose of 1 mg/kg bw/d for 7 days led to colonic epithelial injury, reduced expression levels of tight junction proteins and reduced thickness of the 'luminal mucus layer'. This was associated with altered gut microbiota composition, with reduction in number of Bifidobacterium compared with controls.
Pinget *et al.* (2019)	Male C67BL/6JAusb mice (5-6 per group); Exposure via **drinking water** for 4 wks at doses of 0, 2, 10, 50 mg/kg bw/d (dose calculated based on water intake measured per cage).	**E171**, anatase, 30-300 nm Source: All Color Supplies PTY. TiO_2 was dispersed in drinking water using sonication (no further details provided).	Examined microbiota populations in fecal samples or the small intestine through 16S rRNA sequencing. Exposure to E171 had minimal impact on their compositions of gut microbiota, but altered the release of bacterial metabolites.

Study	Species-strain, doses, and dosing regimen	Test Article (source)	Result
Talbot *et al.* (2018)	Adult male Wistar rats (*n* = 8 per group) were dosed daily with E171 by **intragastric gavage** (10 mg/kg bw/d) for 7 d. In a second experiment, rats (*n* = 10 per group) were exposed to E171 via **drinking water** at doses of 0, 0.1 or 10 mg/kg bw/d for 60 days.	**E171** obtained from a French commercial supplier of food coloring (the same batch as that used in the study of Bettini *et al.* 2017). Particles were ultrasonically dispersed in water and 0.05% BSA per the NANOGENOTOX protocol.	At the end of the exposure periods, rats were sacrificed and the cecal contents collected for analysis of short-chain fatty acids. Tissues from the small intestine and distal colon were sampled for mucin *O*-glycosylation. The authors reported no changes in microbial production of short-chain fatty acid or gut mucin O-glycosylation patterns after either period of exposure, suggesting the absence of mucus barrier impairment.
Waller *et al.* (2017)	***In vitro* model of human colon reactor**; Experimental conditions including control, the 5 different food-grade TiO_2 samples, and the industrial grade TiO_2 sample; Testing spanned 5 days and each condition performed in triplicate; Dose administered was equivalent to ~0.3 to 0.7 mg/kg bw/d (based on previously published estimates of TiO_2 exposure for adults consuming a western diet); Particles were dispersed by sonication prior to introduction into the media	Two test articles: 1. **Commercially available TiO_2** acquired from Arizona State University that represents a form likely consistent with that used in food, 122 ± 22 nm (used TEM), surface was coated with inorganic phosphate, 98% anatase, 2% rutile 2. **Industrial grade TiO_2**; nominal size of 21 nm, 100% of particles in nano range; no surface coating; mixture of 75% anatase and 25% rutile (Aeroxide® P25, Evonik Degussa)	Inhibition of expected, natural shift in microbial composition from *Proteobacteria* to *Firmicutes* phyla (observed during control conditions) in the presence of industrial and 'food-grade' TiO_2 (effect large with 'food-grade TiO_2) Industrial and 'food-grade' TiO_2 led to lower colonic pH (<5) compared to the control (>5) (largest reduction seen with 'food-grade TiO_2) Exposure to industrial or 'food-grade' TiO_2 may have little effect on microbial stability Authors conclude that the inherent physical and chemical properties of industrial and 'food-grade' TiO_2 may produce different microbial responses
Yan *et al.* (2020)	Male ICR mice (8 per group); Treat by **oral gavage** for 28 d; Test articles suspended in physiological saline by sonication); Mice divided into 7 groups of 8: 10, 40, or 160 mg/kg bw/d (TiO_2 250 nm) or 10, 40, or 160 mg/kg bw/d (TiO_2-NPs 25 nm) or control (saline)	Two test articles: 1. **TiO_2-NPs**, anatase, 20 nm Source: Shanghai Jianghu Co. Ltd, China 2. **TiO_2**, anatase, 250 nm Source: Shanghai Jianghu Co. Ltd., China	Colon tissues were isolated under aseptic conditions and contents were excised for 16S rRNA analysis. Exposure to TiO_2 or to TiO_2-NPs led to changes in the composition of the gut microbiota, especially the microbiota associated with mucus.
Zhang *et al.* (2020)	Thirty male C57BL/6J mice were treated with either vehicle control or 150 mg/kg bw TiO_2NPs for 30 days. Particles were dispersed by sonication in 2% heat-inactivated mouse serum and controls were dosed with sonicated vehicle. Group sizes not stated but presumably *n* = 15.	**TiO_2-NPs**, 21 nm primary particle size, were purchased from Sigma Aldrich Chemical Co.	The endpoints examined included small intestine and brain histopathology, effects on gut microbiota (faecal bacterial 16S rRNA gene sequencing), gut and cerebral cortex transcriptomics, as well as locomotor activity, spatial learning and memory ability. No histopathological lesions were observed, although significant changes in the richness and evenness of gut microbial composition were observed in the TiO_2-treated group. These changes were accompanied by changes in gut markers of serotonergic activity although inflammatory cytokines were unaffected. In an open field test, centre-field activity, a measurement of anxiety-like behaviour,

Study	Species-strain, doses, and dosing regimen	Test Article (source)	Result
			was reduced in the TiO_2-treated group, although spatial learning and memory were not affected.

Appendix K – Extended One-Generation Reproductive Toxicity Study with Additional Endpoints (LPT 2020 as cited in EFSA 2021a)

Table 13. Summary table of test results of the extended one-generation reproductive toxicity (EOGRT) study with additional endpoints for aberrant crypt foci (ACF), neurotoxicity, and immunotoxicity.

Species-strain (group size)	Test article, doses, and dosing regimen	Result
Male and female rats (CD/Crl:CD(SD)) F0 (20 per sex per group) F0 satellite - assessed ACF (30 per sex per group) F1-1A - reproductive & developmental toxicity cohort (20 per sex per group) F1-1B – reproductive & developmental toxicity cohort (20 per sex per group) F1-2A - developmental neurotoxicity cohort (10 per sex per group) F1-2B - developmental neurotoxicity cohort (10 per sex per group)	**E171-E** (anatase) was formulated in the diet. The pristine form of E171-E had a median particle diameter of 99.9 ± 2.0 nm with 50-51% of particles < 100 nm. In the diet preparations, the median diameter ranged from 109.2 ± 1.4 to 113.7 ± 4.9 nm and approximately 31-43% of particles < 100 nm. Exposure via **diet** in F0 generation for 10 wks prior to mating until weaning of F1 generation; F0 generation divided into 4 groups of 20 per sex: 0, 100, 300, and 1000 mg/kg bw/d Exposure via **diet** in F1 generation from weaning until PND 4 or PND 8 of F2 generation; Exposure via **milk** in F2 generation until PND 4 or PND 8 (i.e., study termination); Duration of dosing dependent on endpoint investigated, with longest	Study conducted according to OECD TG 443; **Internal Exposure (F0, F1-1A, F1-1B, F2)** The basal diet contained an amount of Ti equivalent to approximately 1.4 mg TiO_2/kg bw/d and there were variable measurable levels of Ti in blood and urine of control animals ranging from 0.008 to 0.046 µg/L (blood) and 0.010 to 0.036 µg/g (urine); Ti concentrations in blood of F2 pups elevated above background (dose-dependent) at doses ≥300 mg/kg bw/d indicating exposure via placenta and possibly via milk; also indicates that the F1 dams had absorbed at least a small fraction of Ti from the diet (slight increase in blood Ti in F0 cohort in 1000 mg/kg bw/d group, but not F1-1A or F1-1B). However, blood Ti concentrations may have been elevated in F2 pups due to inhalation of E171 in dust from powdered feed and/or limited fecal excretion. Urine Ti concentrations low in F2 pups with no clear increase with dose whereas urine Ti concentrations in F0 and F1 generations may have been contaminated by Ti in feces and therefore, difficult to interpret. **Clinical observation, body weight, food intake (F0, F1-1A, F1-1B, F1-2A, F1-2B)** No mortality and no treatment-related effects reported at any dose for any generation. **Hematology, clinical biochemistry, urinalysis (F0, F1-1A)** No treatment-related effects reported at any dose for any generation. **Thyroid hormones (F0, F0 satellite, F1 pups, F1-1A)** No treatment-related effects reported at any dose for any generation. **Sex hormones (F0, F0 satellite, F1-1A, F1-1B, F1-2A, F1-2B)**

Species-strain (group size)	Test article, doses, and dosing regimen	Result
F1-3 - developmental immunotoxicity cohort (10 per sex per group)	duration of treatment up to 18 weeks Concentration adjusted weekly using food consumption values from previous weeks to maintain constant dose level in relation to bw E171-E intake reported in mg/kg bw/d for each test week calculated based on relative food consumption and nominal E171-E concentration used in previous weeks Analyses of the basal diet revealed low background levels of Ti ranging from 11 to 31 mg Ti/kg diet (mean: 17 mg Ti/kg diet) which was estimated by the EFSA FAF Panel (2021a) to be equivalent to a dose of approximately 1.4 mg TiO_2/kg bw/d.	No treatment-related effects reported at any dose for any generation. **Pathology (F0, F1-1A, F1-2A, F1-2B)** All animals survived until scheduled necropsy; No treatment-related effects on gross pathology or histopathology (including a qualitative and quantitative neurohistopathological examination of F1-2A and F1-2B). **Aberrant crypt foci (F0 satellite 10/sex per group)** Study did not include a positive control group for the development of ACF (i.e., treatment with known GI tract tumour initiator such as DMH). No ACF were found in the colons of animals in any dose group. **Reproductive toxicity (F0, F1-1A (males), F1-1B (females))** No treatment-related effects on sexual function or fertility in males or females **Developmental toxicity (F0, F1, F2)** No treatment-related pre- or postnatal loss observed in F0 or F1 generations, no external or internal abnormalities detected in F1 or F2 pups at termination, and no treatment-related effects on growth or sexual development in F1 or F2 generations. **Developmental neurotoxicity (F1-2A)** No treatment-related effects on neurofunctional endpoints at any dose. **Developmental immunotoxicity** *For KLH assay (F1-3, F1 satellite 10/sex per group):* In F1-3 cohort, a slight, but 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. Satellite animals of F1 (10/sex, PND 55) acted as positive control (treated with cyclophosphamide) for the KLH assay, but assay was not performed at the same time as the F1-3 cohort and at a different age from the F1-3 cohort (PND 87-96) and therefore, the positive control was not considered valid by the EFSA FAF Panel (2021a) although Health Canada's Food Directorate considered this to be a relatively mild deficiency; No other control included. *Other immune parameters (F1-1A; F1-3):* No treatment-related differences in weight or histopathology of spleen, thymus, lymph nodes, bone marrow histopathology, total and differential peripheral WBC count, and splenic lymphocyte subpopulation distribution in F1-1A, but isolated observations in this cohort not sufficient to conclude on potential immunotoxicity.

Species-strain (group size)	Test article, doses, and dosing regimen	Result
		No treatment-related differences in splenic lymphocyte subpopulation distribution were observed in the F1-3 cohort.