ENVIRONMENTAL DEFENSE FUND, CENTER FOR FOOD SAFETY, CENTER FOR SCIENCE IN THE PUBLIC INTEREST, AND ENVIRONMENTAL WORKING GROUP

March 13, 2023

Dr. Kristi Muldoon Jacobs
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Center for Food Safety and Applied Nutrition
5100 Campus Drive
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Re: New color additive petition asking FDA to remove approval of synthetically-prepared titanium dioxide (CASRN 13463-67-7) for use as color additive in food pursuant to 21 USC § 379e.

Dear Dr. Muldoon Jacobs:

Petitioners submit this color additive petition pursuant to section 721(b)(1) of the Federal Food, Drug, and Cosmetic Act requesting that the Food and Drug Administration (FDA) remove its approval of the use of synthetically-prepared titanium dioxide (synthetic TiO_2) at 21 C.F.R. § 73.575. Recent scientific studies raise serious questions about the safety of the chemical's use in food such that there is no longer the legally required "convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of the color additive,"[1] after considering the probable consumption of synthetic TiO_2 from its use in food, the cumulative effect of synthetic TiO_2's use after taking into account pharmacologically-related substances in the diet, and appropriate safety factors.[2]

FDA has not reviewed the safety of synthetic TiO_2 since 1973 – a half century ago – when it concluded that its use in food was safe. That conclusion was primarily based on the finding that synthetic TiO_2 was not absorbed through the gastrointestinal tract, and the approval was based primarily on that conclusion.[3] We now know that very small particles can pass through the gastrointestinal tract and accumulate in the human body, something that the agency did not know or consider in 1973.[4]

Over the intervening five decades, the risk posed by very small particles, especially those smaller than 100 nanometers (nm) in any dimension (i.e., nanoparticles), has become substantially clearer.[5] Scientists now recognize that nanoparticles – unlike their larger counterparts – are more likely to bypass the body's natural defenses in the gastrointestinal tract and be absorbed into the bloodstream where the nanoparticles can reach other organs and may cause harm. The risk of gastrointestinal absorption is especially significant if the nanoparticles accumulate in the body instead of being quickly and fully excreted as appears to be the case for TiO_2 nanoparticles present in food-grade, synthetic TiO_2.

Recognizing the emerging evidence of the potential risks posed by nanoparticles, in 2018 the European Food Safety Authority (EFSA) updated its guidance on the risk assessment of nanomaterials in foods to include "a material that is not engineered as nanomaterial but contains a fraction of particles, less than 50% in the number–size distribution … with one or more external dimensions in the size range 1–100 nm."[6] Synthetic TiO_2 used as a food additive fits this description.

Subsequently, EFSA's Expert Panel on Food Additives and Flavourings ("EFSA Expert Panel") applied the 2018 guidance to update its safety assessment of synthetic TiO_2 (designated as the additive "E 171" in the European Union (EU)) for use in food. It concluded that "E 171 can no longer be considered as safe when used as a food additive."[7] It found that although gastrointestinal absorption of synthetic TiO_2 is low,

the nanoparticles that are absorbed may accumulate in the body, and that accumulation may be linked to adverse effects such as immunotoxicity and inflammation and neurotoxicity. It could not rule out genotoxicity.

In response, the European Commission removed its approval for the use of E 171 in January 2022. After August 7, 2022, no food placed on the market in Europe is allowed to use synthetic TiO_2.[8] Any food already on market by that date can be sold "until their date of minimum durability or 'use by' date" passes. It also set a deadline of January 2025 for the European Medicines Agency to evaluate the necessity of E 171 in medicinal products.

The Commission took the necessary steps to protect their constituents; FDA should do the same to protect the American people.

A. Physical properties of synthetic TiO_2

FDA allows synthetic TiO_2 that meets its specifications to be added to human food[9] as a color additive up to one percent by weight of the food at 21 C.F.R. § 73.575[10] and does not limit minimum particle size.

The U.S. Pharmacopeia's Food Chemical Codex (FCC) describes food grade synthetic TiO_2 as a white, amorphous powder that is prepared synthetically and is insoluble in water, in hydrochloric acid, in dilute sulfuric acid, and in alcohol and other organic solvents.[11] FCC's specifications for synthetic TiO_2 are the same as FDA's and include how to analyze a sample to document compliance. As with FDA's, the FCC specifications do not limit minimum particle size.

The EFSA Expert Panel provides the most detailed description of the particle size distribution of commercially available E 171 in its 2019 report.[12] The evaluation is based on "five commercial brands of anatase E 171 and one of rutile E 171 manufactured by the only three EU manufacturers that, according to information submitted by interested business operators, produce food-grade titanium dioxide."[13] For anatase E 171 in this analysis, the maximum percent nanoparticles (< 100 nm) by number was 45.6%, based on an average calculated from the results of three laboratories using scanning electron microscopy (SEM), and the minimum was 11.4%. When measured by transmission electron microscopy (TEM) in a preliminary screen by a single lab, the maximum and minimum were 42% and 5%, respectively.[14]

These products appear consistent with the substance covered by FDA's approval of synthetic TiO_2. It is also consistent with a food grade sample of E 171-E the Titanium Dioxide Manufacturers Association supplied to a Michigan State University research team; the food grade sample was based on "an assessment and characterization of the different grades of E 171 in the market."[15] Lacking any evidence to the contrary, petitioners maintain that the synthetic TiO_2 found in foods sold in the United States is the same as those evaluated by EFSA and Michigan State University as E 171.

The 2021 EFSA Expert Panel Report stated, when describing physico-chemical characterizations of E 171 completed by two other laboratories that the substance, "after applying sample dispersion protocols, consists almost exclusively of near-spherical constituent particles with a median diameter in the order of 100 nm that are often agglomerated (i.e. 50% of the individual particles are at the nanoscale). The crystalline form of this E 171 is anatase."[16] The E 171 sample used by Michigan State University investigators also contained TiO_2 particles less than 100 nm in diameter.[17]

Overall, in its 2021 report, the EFSA Expert Panel stated that "less than 50% of constituent particles by number in E 171 have a minimum external dimension < 100 nm. In addition, the Panel noted that constituent particles < 30 nm amounted to less than 1% of particles by number."[18] The fact that food-grade, synthetic TiO_2 is comprised, in substantial part (i.e., up to 50% by particle number), of

nanoparticles is critical because evidence suggests that these nanoparticles accumulate and induce harm, as discussed in subsequent sections of this petition.

B. Dietary exposure to synthetic TiO_2

In its 2021 report, the EFSA Expert Panel estimated the dietary exposure of E 171. Table 1 contains the exposure estimates from Table 12 of that report.[19] The table shows that on a body weight basis, children 3 to 9 years have greater exposure to E 171.

Table 1. Summary of dietary exposure to E 171 from its use as a food additive in the maximum level exposure assessment scenario and in the refined exposure scenarios, in six population groups (minimum–maximum across the dietary surveys in mg/kg bw per day)

	Infants (12 weeks–11 months)	**Toddlers (12–35 months)**	**Children (3–9 years)**	**Adolescents (10–17 years)**	**Adults (18–64 years)**	**The elderly (≥ 65 years)**
Maximum level exposure assessment scenario						
• Mean	0.06–3.6	0.9–12.8	1.9–11.5	1.3–6.2	0.7–6.7	0.4–4.9
• 95th percentile	0.2–15.8	2.9–31.4	5.9–31.3	4.0–18.6	2.4–15.9	1.9–12.7
Refined exposure assessment scenarios						
Brand-loyal scenario						
• Mean	0.05–3.5	0.8–10.0	1.7–9.7	1.1–5.0	0.6–5.5	0.4–4.2
• 95th percentile	0.1–14.3	2.6–28.0	5.2–25.4	3.3–14.9	2.0–13.1	1.7–10.4
Non-brand-loyal scenario						
• Mean	0.03–2.9	0.6–6.0	0.9–6.9	0.6–3.6	0.3–3.8	0.2–2.8
• 95th percentile	0.1–9.9	1.9–27.5	2.5–23.7	1.6–13.2	1.2–9.5	0.9–7.1

bw: body weight.
Extracted from Table 12 of the 2021 EFSA Expert Panel Report on E 171[20]

Regarding exposure to nanoparticles from E 171, the EFSA Expert Panel said:

> "Taking into account the available data (Verleysen et al., 2021[21]), it can be presumed that **the mass of constituent particles below 100 nm could be up to 30%,** where the mean of the 12 analysed samples is 25%. The Panel noted that **different types of E 171 are used in food and the percentage by number of constituent particles below 100 nm can range from 5% (rutile) to around 50%** (EFSA FAF Panel, 2019[22]). The use levels of the different types of E 171 are unknown, and therefore, it is not possible to estimate accurately the exposure to nanoparticulate TiO2 from the use of E 171 (Table 12)."[23] *[Emphasis and footnotes added]*

Based on a review of the uses[24] described in the EFSA Expert Panel report, we maintain that the U.S. dietary exposure to synthetic TiO_2, and thus its constituent nanoparticles, is likely similar.

C. Other FDA allowed or authorized uses of TiO_2

This petition is focused on FDA's approval of synthetic TiO_2 as a color additive in food at § 73.575. In 2006, FDA approved at § 73.350 the use of titanium salts to produce TiO_2 on mica to make "mica-based pearlescent pigments." The agency expanded the uses in 2013 and 2015. The agency acted in response to a total of five different color additive petitions.[25]

After reviewing FDA's FOIA responses for information on these five petitions,[26] we found that the agency explicitly considered particle sizes and found that the particles ranged from 1,000 to 150,000

nanometers in size. The smallest size was ten times greater than the average particle size in E 171 or synthetic TiO_2. Given the much larger particle size, petitioners think mica-based pearlescent pigments may not be chemically or pharmacologically-related to synthetic TiO_2 or E 171.

Synthetically prepared titanium dioxide may also be used as a food contact substance.

1. TiO_2 as a food contact substance at §§ 175.105, 175.210, 176.170, 177.1200, 177.1400, 177.1460, 177.1650, 177.2260, 177.2600, 177.2800, 178.3297, or 181.30;
2. Silver-chloride coated TiO_2 as a food contact substance at §§ 175.300, 175.320, 175.380, 175.390, 176.170 177.1210, 177.1350, and 177.1680,
3. TiO_2 in a salt with either barium sulfate, calcium sulfate, magnesium silicate, or magnesium as a food contact substance at §§ 175.105, 176.170, and 178.3297;
4. TiO_2 as a component of 15 food contact substances used in food contact materials pursuant to effective Food Contact Substance Notices (FCN) submitted from 2000-2021;[27] and
5. TiO_2 as a component of four food contact substances used in food contact materials pursuant to Threshold of Regulation (TOR) Exemptions authorized from 1996-2010.[28]

FDA's Cumulative Estimated Daily Intake (CEDI) database estimates the cumulative dietary concentration from two types of TiO_2 used in food contact substances as follows:

- Nickel antimony titanium yellow rutile – 27 ppb
- Titanium dioxide-calcium sulfate – 7 ppb.[29]

Petitioners are not asking FDA to remove TiO_2 approvals or authorizations as food contact substances at this time because we suspect that these uses do not present a major source of exposure to TiO2 nanoparticles relative to use as a color additive in food.

D. Presence in foods on the U.S. market

Synthetic TiO_2 is commonly used because of the white color it imparts on food. Environmental Working Group's Food Scores reports[30] that almost 1,500 food products have TiO_2 as a listed ingredient including:

- 457 candy products;
- 272 cake and snack products;
- 176 cookie and biscuit products; and
- 139 dessert and dessert toppings.

These numbers likely underestimate the extent of the TiO_2's presence in food. If synthetically prepared TiO_2 is an ingredient in a food, it may be listed as "artificial color"[31] and therefore not included in EWG's database. Therefore, the counts are unlikely to represent all uses in products on the marketplace.

E. Absorption, distribution, metabolism, and excretion for E 171 and synthetic TiO_2

When FDA originally approved synthetic TiO_2 as a color additive, it concluded that the substance was not absorbed in the gastrointestinal tract and was excreted unchanged. For example, FDA's scientist said:

> "Pure Titanium Dioxide is considered an innocuous material. Titanium dioxide is chemically and physiologically inert. Animal and human experiments show that Titanium Dioxide when ingested is not absorbed at all from the alimentary tract but is excreted unchanged and totally in the feces, and causes no harmful effects."[32]

However, a closer review of a 1963 industry study reveals a different story. The study, submitted as part of the color additive petition, shows that TiO_2 was absorbed through the gastrointestinal tract in young

male and female albino rats that were fed a dietary concentration of 10% titanium dioxide for a period of about 32 days. They concluded:

> "(a) a significant titanium retention of between 0.06 and 0.11 micrograms per gram wet weight of tissue was found in muscle (95% confidence interval).
> (b) significant retention of titanium could not be shown in any of the other organs or tissues tested: liver, spleen, kidney, bone, plasma, and red cells."[33]

The authors also stated that there were problems with the recovery of added titanium in spleen and red blood cells, "where no simple relationship between amount added and amount recovered could be demonstrated."[34] This indicates that the measurements in blood and spleen were unreliable and thus the agency cannot, from these data, dismiss the possibility that there was accumulation of titanium in these tissues as well.

We also found no evidence that FDA knew of or considered the particle size distribution in synthetic TiO_2. It is not surprising because in the early 1970s the tools to identify particle size distribution for nanoparticles were extremely limited.

Given these gaps, the EFSA Expert Panel's 2019 and 2021 reports are critical because they examined the data and risks more rigorously using modern tools, especially on nanoparticles. In the 2021 report, the EFSA Expert Panel noted that the toxicokinetics of E 171 were addressed in three studies in mice and in two studies in humans. The Panel also noted that other studies examined the toxicokinetics of TiO2 nanoparticles specifically in rats and humans. It found that:

- "E 171 has a low oral systemic availability, probably not greater than 0.5%."
- "It may pass the placenta and may be transferred to the fetus."
- "The oral systemic availability of these materials was low (most probably < 1%) but higher than for E 171."
- "Rat studies with TiO2 [nanoparticles], consisting of nanoparticles with primary particle sizes between 7 and 90 nm, showed long half-lives (roughly 200–450 days), a potential for accumulation (accumulation factor of 290 to 450) and long time to reach steady state (3–5 years). The oral systemic availability of these materials was low (most probably < 1%) but higher than for E 171."
- "In tissues from deceased subjects, TiO2 particles were identified in liver, spleen, kidney and intestinal tissues."
- "The low Ti amount of the investigated organs indicated low oral systemic availability of TiO2 ingested from a number of sources, including dietary exposure to E 171."
- "None of the studies were sufficiently long to cover the time needed for reaching the steady-state for accumulation."[35] *[References excluded]*

The EFSA Expert Panel acknowledged that the studies with synthetic TiO_2 nanoparticles < 30 nm were of limited relevance because the particles made up less than 1% of the material; however the evidence of long half-life and bioaccumulation led the panel to conclude that the health risk of TiO_2 could be exacerbated by the presence of particles less than 30 nm in size due to their tendency to accumulate in the various organs including the liver.[36]

The EFSA Panel's overall conclusion regarding toxicokinetics was, the "absorption of TiO2 particles is low, however they can accumulate in the body due to their long half-life." Despite the low systemic availability of E 171 TiO_2 nanoparticles, the bioaccumulative nature of TiO_2 nanoparticles combined with the evidence of toxicity for both E 171 and TiO_2 nanoparticles, discussed in the next section, led EFSA

This reinforced their overall conclusions that E 171 "can no longer be considered as safe when used as a food additive."[37]

F. Assessment of health risks posed by synthetic TiO_2

In its 2021 report,[38] the EFSA Expert Panel evaluated the impacts of various forms of synthetic TiO_2 on eight specific categories health endpoints (in addition to general toxicity) and found cause for concern for four of them. Table 1 describes each of the health endpoints, the Panel's finding, the Panel's rationale for its finding, and the type of TiO_2 studied.

Table 2: Categories of health endpoints assessed by EFSA Panel

Health endpoints	Panel concern	Rationale	Type of TiO_2
Immunotoxicity and inflammation	Yes	Adult animal studies indicate immune dysregulation activity evidenced by several immune-related and inflammatory markers.[39]	E 171 and nanoparticles <30 nm.
Neurotoxicity and neurodevelopmental toxicity	Yes	Adverse effects were observed in brain tissues of animals exposed during gestation and early lactation, and in adult animals.[40]	Nanoparticles <30 nm.
Gastrointestinal toxicity	Yes	Possible induction of aberrant crypt foci in the gut.	E 171
Genotoxicity	Yes	• A concern for genotoxicity of nanoparticles present in the TiO_2 additive (E171) could not be ruled out based on evidence these particles can potentially cause DNA strand breaks and chromosomal damage. • The Panel could not identify cut-off value for TiO2 particle size with respect to genotoxicity could not be identified.	Nanoparticles
Reproductive and developmental toxicity	Yes	Adverse effects in testis and sperm quality.	Nanoparticles <30 nm.
Reproductive and developmental toxicity	No	No effects observed in the Extended One-Generation Reproductive Toxicity (EOGRT) study.[41]	E 171
Carcinogenicity	No opinion	Lack of appropriately designed studies.[42]	Nanoparticles
Gut microbiota	No conclusion	"Panel was unable to come to any conclusion regarding the effects of E 171 on [gastrointestinal tract] microbiota and related effects on health."	Nanoparticles <30 nm.
Endocrine function	No	There were no consistent treatment-related effects on thyroxine, triiodothyronine, and thyroid-stimulating hormone, estradiol, estrone and testosterone levels either in the parents or the offspring.	E 171

Overall, the EFSA Expert Panel concluded that:

- "The absorption of TiO_2 particles is low, however they can accumulate in the body due to their long half-life;
- Studies on general and organ toxicity, including the newly performed EOGRT study with E 171, did not indicate adverse effects up to a dose of 1,000 mg/kg bw per day. Also no effects were seen in studies retrieved from the literature with TiO_2 NP > 30 nm up to the highest dose tested of 100 mg/kg bw per day;
- No effects on reproductive and developmental toxicity up to a dose of 1,000 mg/kg bw per day, the highest dose tested, were observed in the EOGRT study with E 171. No other reliable studies were found in the literature addressing these effects with E 171;
- Some findings regarding immunotoxicity and inflammation with E 171 as well as neurotoxicity with TiO_2 NPs may be indicative of adverse effects;
- There are indications of the induction of aberrant crypt foci with E 171;
- No studies appropriately designed and conducted to investigate the potential carcinogenicity of TiO_2 nanoparticles were available;
- Combining the available lines of evidence on genotoxicity, TiO_2 particles have the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations. No clear correlation was observed between the physico-chemical properties of TiO_2 particles – such as crystalline form, size of constituent particles, shape and agglomeration state – and the outcome of either in vitro or in vivo genotoxicity assays;
- A concern for genotoxicity of TiO_2 particles that may be present in E 171 could not be ruled out;
- Several modes of action for the genotoxicity may operate in parallel. The relative contributions of different molecular mechanisms elicited by TiO_2 particles are unknown and there is uncertainty whether a threshold mode of action could be assumed; and
- A cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified."[43]

In summary, the Panel said: "Overall, on the basis of all currently available evidence along with all the uncertainties, in particular the fact that genotoxicity concern could not be ruled out, the Panel concluded that E 171 can no longer be considered as safe when used as a food additive."[44]

G. Carcinogenicity

Regarding carcinogenicity, we recognize the ongoing legal challenges to the designation of TiO_2 as a carcinogenic substance by inhalation in certain powder forms.[45] Inhalation is a fundamentally different route of exposure than ingestion, it is not directly relevant to this petition.

We also recognize that the National Cancer Institute studied the cancer risks of TiO_2 in 1979.[46] That study did not consider the impact of particle size and is not directly relevant to this petition.

H. Studies published after EFSA Expert Panel review began

We also reviewed the scientific evidence published since 2018 when EFSA completed its literature search and we found nothing that contradicted EFSA's conclusions. See Appendix 1 for details.

I. FDA's communication to industry regarding the 2021 report by EFSA's Expert Panel

We note that Titanium Dioxide Manufacturers Association (TDMA) reported that it received the following statement from FDA in response to the EFSA Expert Panel report.

> The FDA reviewed the findings of EFSA's 2021 Opinion on titanium dioxide. The FDA notes that EFSA's 2021 Opinion continued to confirm no general and organ toxicity, as well as no effects on reproductive and developmental toxicity. In its 2021 Opinion, EFSA noted that it could not rule out genotoxicity and included genotoxicity tests on titanium dioxide nanomaterials. Some of the genotoxicity tests included test materials not representative of the color additive, and some tests included administration routes not relevant to human dietary exposure. The available safety studies do not demonstrate safety concerns connected to the use of titanium dioxide as a color additive. The FDA continues to allow for the safe use of titanium dioxide as a color additive in foods generally according to the specifications and conditions, including that the quantity of titanium dioxide does not exceed 1% by weight of the food, found in FDA regulations at 21 CFR 73.575.[47]

We respectfully disagree with this analysis of the EFSA Expert Panel Report. Without explanation, the agency's statement ignores the evidence that synthetic TiO_2 bioaccumulates in humans with a long half-life and dismisses studies conducted with TiO_2 nanoparticles as irrelevant to the color additive, despite the fact that the color additive is inherently comprised, in substantial part, of nanoparticles.

In addition, the agency's statement fails to address the similar evidence of bioaccumulation ("significant titanium retention…in muscle") in its original safety assessment as it is described in Section E above on absorption, distribution, metabolism and excretion for E 171 and synthetic TiO_2.

It also does not consider the findings regarding immunotoxicity, inflammation, and aberrant crypt foci with E 171 as well as neurotoxicity with TiO_2 nanoparticles (less than 100 nm) which may be indicative of adverse effects. The high level of detail and transparency provided in the EFSA Expert Panel Report warrants a much more thorough and rigorous assessment from FDA than that provided to the Titanium Dioxide Manufacturers Association.

It is unclear to petitioners whether the FDA statement reported by TDMA reflects the totality of information that FDA shared with TDMA or whether this is the full extent of FDA's analysis of the EFSA Panel report and associated evidence. It is possible that FDA intends to conduct a full reassessment of the safety of synthetic TiO_2 used in food. Indeed, the agency has the authority to review the safety of, and revoke approvals for, color additives without prompting by petition. However, without evidence to suggest that a more detailed and rigorous analysis and description is forthcoming, we submit this petition to prompt FDA to undertake such assessment and request such a revocation.

The combination of the evidence that TiO_2 bioaccumulates, the serious questions about health effects, and their potential exacerbation due to the presence of nanoparticles mean that there is no longer convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of synthetic TiO_2 in food[48] For these reasons, FDA is obligated to revoke its approval for the chemicals use as a color additive in food.

J. Summary

We maintain that EFSA's rigorous and transparent scientific analysis is sufficient to show that there is no longer "convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of the color additive" – the definition of safety for color additives at 21 C.F.R. § 70.3(i)[49] – for synthetic TiO_2 in food. This is especially the case after considering the probable consumption of synthetic TiO_2, the cumulative effect of the substance in the diet, taking into account the same or any chemically or pharmacologically related substance or substances in such diet, and appropriate safety factors as required by 21 U.S.C. § 379e(b)(5)(A).[50]

We have submitted this color additive petition electronically. Appendix 1 provides our responses to elements required by § 71.1.[51] Appendix 2 provides our proposed changes to FDA approvals.

Should FDA file the petition, we request that the agency include the petition and appendices in the docket and request public comment.

If you have questions or comments, please contact Tom Neltner at tneltner@edf.org and Dr. Maricel Maffini at drmvma@gmail.com on all responses.

Sincerely,

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Index to Appendices:

Appendix I
Responses to elements required by 21 C.F.R. § 71.1

In this color additive petition, we ask FDA to alter an existing regulation issued pursuant to Section 721(b) of the Federal Food Drug and Cosmetic Act (21 U.S.C. § 379e(b)).[52] Specifically, we ask that FDA revoke its approved color additive uses of synthetically-prepared titanium dioxide ("synthetic TiO_2") in foods and delete 21 C.F.R. § 73.575.[53]

Pursuant to § 71.1(c)(H),[54] we are providing full information on each proposed change that is to be made in the original regulation. Accordingly, we may omit statements made in the original petition for which we are proposing no change.

Note that we provide responses to the requested elements of a color additive petition based on FDA's July 2009 "Guidance for Industry: Color Additive Petitions[55] - FDA Recommendations for Submission of Chemical and Technological Data on Color Additives for Food, Drugs, Cosmetics, or Medical Devices."

I.A. Name and all pertinent information concerning the color additive.

1. Identity

The identity of the color additive is as follows:

- Name: Titanium dioxide
- Chemical formula: TiO_2
- Formula weight: 79.866
- Chemical Abstract Service No.: 13463-67-7
- INS No.: 171
- UNI No.: 15FIX9V2JP

In its "Substances Added to Food (formerly EAFUS)"[56] FDA lists the following other names for titanium dioxide.

- TITANIUM DIOXIDE
- TITANIUM PEROXIDE
- TITANIUM OXIDE (TIO_2)
- C.I. PIGMENT WHITE 6
- TITANIUM(IV) OXIDE
- PIGMENT WHITE 6
- C.I. 77891
- SYNTHETIC TiO_2

For synthetic TiO_2 approved by FDA pursuant to § 73.575[57], the color additive is synthetically prepared TiO2, free from admixture with other substances. Color additive mixtures for food use made with titanium dioxide may contain only those diluents that are suitable and that are listed in this subpart as safe in color additive mixtures for coloring foods, and the following: Silicon dioxide (SiO2) and/or aluminum oxide, (Al2O3), as dispersing aids - not more than 2 percent total.

2. Physical, Chemical, and Biological Properties

This petition relies on the physical, chemical, and biological properties of synthetic TiO_2 that FDA considered in its approvals. We provide FDA's response to a Freedom of Information Act request by the Environmental Working Group[58] provided as Attachment A to this petition for documentation.

3. Manufacturing Process Description:

This petition relies on the manufacturing process description of synthetic TiO_2 that FDA considered in its approvals. We provide FDA's response to a Freedom of Information Act request by the Environmental Working Group provided as Attachment A to this petition for documentation.

4. Stability Data:

This petition relies on the stability data of synthetic TiO_2 that FDA considered in its approvals. We provide FDA's response to a Freedom of Information Act request by the Environmental Working Group[59] for documentation.

I.B. Amount of the color additive proposed for use and the color effect intended to be achieved, together with all directions, recommendations, and suggestions regarding the proposed use.

We request that FDA revoke its approved color additive uses of synthetic TiO_2 in foods at § 73.575.[60]

I.C. Description of practicable methods to determine the pure color and all intermediates, subsidiary colors, and other components of the color additive.

We request that FDA revoke its approved color additive uses of synthetic TiO_2 in foods at § 73.575.[61] Therefore, no practical methods are needed to determine the pure color, intermediates, subsidiary colors, and other components of the color additive.

I.D. Full reports of investigations made with respect to the safety of the color additive.

We base our request on the March 2021 safety assessment of synthetic TiO_2 used in food (known as "E 171") by the European Food Safety Authority[62] (EFSA) that evaluated all new relevant data available to EFSA since it completed an assessment in 2016. The EFSA Expert Panel "concluded that E 171 can no longer be considered as safe when uses as a food additive." EFSA based this conclusion on scientific evidence that:

- "Although gastrointestinal absorption of TiO_2 particles is low, they may accumulate in the body."
- "Observations of potential immunotoxicity and inflammation with E 171 and potential neurotoxicity with TiO_2 [nanoparticles], together with the potential induction of aberrant crypt foci with E171, may indicate adverse effects."
- "A concern for genotoxicity of TiO_2 particles that may be present in E 171 could therefore not be ruled out."[63]

We performed an update literature search from December 3, 2020 to October 3, 2022 using as a reference the search terms used by EFSA. We reviewed the studies haven't been able to find new

evidence contracting EFSA's conclusions. Rather, we noticed evidence that reinforces or expands EFSA concerns.

1. Evidence of gastrointestinal absorption and accumulation in the body of TiO_2 particles.

The EFSA Expert Panel reached the following conclusions regarding the toxicokinetics:

> "The Panel considered that E 171 has a **low oral systemic availability**, probably not greater than **0.5%.** It may pass the placenta and may be transferred to the fetus. Furthermore, the Panel considered that rat studies with TiO2NPs, consisting of nanoparticles with primary particle sizes between 7 and 90 nm, showed **long half-lives** (roughly **200–450 days**), a potential for **accumulation** (accumulation factor of **290 to 450**) and **long time to reach steady state** (3–5 years) (Geraets et al., 2014; Disdier et al., 2015). The oral systemic availability of these materials was low (most probably<1%) but higher than for E 171. In tissues from deceased subjects, TiO2 particles were identified in liver and spleen, the low Ti amount of the investigated organs indicating low oral systemic availability of TiO2 ingested from a number of sources, including dietary exposure to E 171."[64] *[Emphasis added]*

> "The **absorption of TiO2 particles is low**; however, they may **accumulate** in the body due to their **long half-life**"[65]

> "In rats, two intravenous studies (Disdier et al., 2015; Kreyling et al., 2017a) demonstrated long half-lives and, hence the potential for accumulation. Together with data from an intravenous study (Geraets et al. (2014) already addressed in the EFSA opinion on the re-evaluation of E 171 (EFSA ANS Panel, 2016), **half-lives** of **83 days** (for liver) and of **450 days** (for whole body) were estimated and **accumulation factors** between **135 and 450**. Based on these data, the **steady state** would be reached between 1.5 and 5 **years**."[66]

2. Evidence neurotoxicity and neurodevelopmental toxicity.

The EFSA Expert Panel reached the following conclusions regarding neurotoxicity and neurodevelopmental toxicity:

> "Overall for neurotoxicity, **adverse effects were seen with TiO2NPs<30 nm**. In mice, Zhou et al.(2017; scoring 3 for NSC), reported adverse effects (i.e. inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function) in *ex vivo* hippocampal neurons of weanling mice after dosing TiO2NPs (6–7 nm) during gestation and early lactation at a dose of 1 mg/kg bw per day, the lowest dose tested. **In adult female rats** (Canli et al., 2020; scoring 3 for NSC), adverse effects (reduced brain cholinesterase, and increased brain Na/K-ATPase activity) were observed with TiO2NPs (21 nm) at 0.5 mg/kg bw per day, the lowest of three doses tested, in a 14-day study"[67]

For TiO2 nanoparticles smaller than 30nm, the EFSA Expert Panel found effects in mice and rats stating:

> "Zhou et al., 2017 **reported inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function**, in ex vivo hippocampal neurons of the offspring with TiO2NPs (6=7 nm) at all doses tested(1, 2 or 3 mg/kg bw per day)"

> "gestational and/or lactational maternal rat exposure to TiO2NPs (10 nm) at 100 mg/kg bw per day altered **passive avoidance behaviour**, **increased hippocampal apoptosis** and **reduced hippocampal neurogenesis** in the offspring."[68]

It also found effects in *adult* mice and rats:

> "The most sensitive endpoint in adult mice was **reduced volume of hippocampus** and dentate gyrus granular layer, and density and number of dentate gyrus granular cells observed with TiO2NPs (21 nm) at 2.5 mg/kg bw per day, the lowest dose tested, in males dosed for35 days (Rahnama et al., 2020)" [69]

> "The most sensitive endpoint in adult rats was reduced (dose related) brain cholinesterase activity and increased brain Na/K-ATPase activity, observed at 0.5 mg/kg bw per day (in females dosed for 14 days), the lowest of three doses tested, reported by Canli et al. (2020) with TiO2NPs (21 nm). However, Grissa et al. (2016) reported reduced brain cholinesterase activity at 100 but not 50 mg/kg bw per day (in males dosed for 60 days with TiO2NPs (5–10 nm)). This apparent 200-fold difference in potency adds to **uncertainty**." [70]

> "Oral TiO2NPs administered to rats during embryofetal and early postnatal development **reduced hippocampal neurogenesis** at 100 mg/kg bw per day, and that oral administration to adult rats produced adverse effects in the brain consistent with oxidative stress at 500 mg/kg bw per day."[71]

3. Evidence of aberrant crypt foci in the gut.

The EFSA panel considered evidence relating to aberrant crypt foci (ACF) in the gut from two key studies (Bettini et al, 2017[72] and Blevins et al 2019[73]) and a new study on extended one-generation reproduction toxicity (EORGT)[74] requested by the European Commission in 2017. The EORGT study was carried out according to the current OECD guidelines and was later also specifically tasked to address concerns about precancerous lesions in the colon and rectum, ACF raised in Bettini et al., 2017[30]. The EFSA Expert Panel considered:

> "that the effect of E 171 alone (without prior initiation) in producing ACF reported by Bettini et al., has not been replicated in later investigations (EOGRT and Blevins et al., 2019), but one of these investigations (Blevins et al., 2019) had methodological limitations. Furthermore, it is unclear to what extent animals were exposed to NPs in the EOGRT and Blevins et al. (2019). The Panel considered that E 171 may induce ACF in male rats at a dose of 10 mg/kg bw per day when it is dispersed in test vehicle preventing agglomeration of NPs prior to administration. The Panel noted that there is literature indicating that ACFs may be a risk factor for human colorectal cancer (Anderson et al., 2012; Drew et al., 2018; Quintanilla et al., 2019; Hong et al., 2019; Clapper et al., 2020; Kowalczyk et al., 2020; Siskova et al., 2020)."[75]

4. Evidence of genotoxicity and carcinogenicity.

The EFSA Expert Panel reached the following conclusion regarding the genotoxicity:

> "A concern for genotoxicity could not be ruled out" because TiO2 particles had the "potential to induce **DNA strand breaks and chromosomal damage**, but not gene mutations."[76]

"There is evidence for several **modes of action** for genotoxicity that may operate in parallel:

- **Direct interaction** of TiO2 nanoparticles with DNA (there is no proof of covalent bonding)
- **Direct formation of reactive (oxygen) species** due to intrinsic properties of TiO2 nanoparticles
- Reactive (oxygen) species formation via TiO2particles-induced **inflammation**
- Reactive (oxygen) species formation via interference of TiO2nanoparticles with **mitochondrial function.**

Additionally, there are indications that TiO2particles may:

- **induce epigenetic modifications** affecting the expression of genes involved in the maintenance of genome function (e.g. downregulation of some genes involved in DNA repair pathways)
- **interact with proteins** involved in the control of chromosome segregation and the spindle apparatus."[77]

"The relative contribution of the modes of action mentioned above to the genotoxicity elicited byTiO2particles is unknown and there is uncertainty on whether a threshold mode of action could be assumed. Even if it was assumed that all modes of action would be indirect, the available data would not allow identification of a threshold dose. Therefore, the Panel concluded that **a concern for genotoxicity of TiO2particles that may be present in E 171 cannot be ruled out.** A cut-off value forTiO2particle size with respect to genotoxicity could not be identified"[78]

The EFSA Panel evaluated a carcinogenicity bioassay published by the U.S. National Cancer Institute in 1979 and determined that "this study was not appropriate to ascertain the absence of a potential to elicit chronic toxicity and carcinogenicity by TiO2 nanoparticles."[79] The Panel did not identify any new publications on chronic toxicity or carcinogenicity via their literature search. Ultimately the Panel concluded that "no studies appropriately designed and conducted to investigate the potential carcinogenicity of TiO2 nanoparticles were available."[80]

5. Evidence of immunotoxicity and inflammation

The EFSA evaluated 15 studies of various reliability scoring that evaluated diversity of immune effects of E171 and other TiO2 in mouse and rat animal models and concluded that:

"these studies indicate **immune dysregulatory activity of E 171**, evidenced by several immune-related and inflammatory markers. These effects were not observed up [sic] 50 mg E 171/kg bw per day. In three single dose level studies with E 171, effects were noted at lower doses, i.e. 2, 5 and 10 mg/kg bw per day. Effects of E 171 may, at least in part, stem from the activity of the fraction of the smaller TiO2particles, as studies with these particles also indicate **inflammatory effects of exposure to TiO2 NPs** (5–6 nm) at 2.5 mg/kg per day."[81]

6. Evidence on sex-related alterations

In their 2021 conclusion, EFSA stated that:

"In rats orally exposed to TiO2 NPs < 30 nm inconsistent and/or unexplained sex differences in some parameters were reported (e.g. hypobilirubinaemia in females (Chen et al. (2015a);

heart rate and blood pressure changes in females (Chen et al. (2015b); leucocyte changes in females (Heo et al.,2020); higher absolute pituitary weights in males (Heo et al., 2020); lower blood insulin levels in females, lower C-peptide levels in males and differences in blood concentrations compared to controls in a glucose tolerance test in males (Chen et al., 2020b)."[82]

7. Recent studies of key endpoints beyond the timeframe considered by EFSA Expert Panel

We performed a literature search for studies published after 2020, EFSA's cutoff date for new studies in its March 2021 report.

Appendix A of the EFSA report provides a detailed methodology to define the scope of the literature search and meticulous line of search in one public and one private databases.[83] We used EFSA's search strings for our updated literature search in PubMed to identified relevant studies based on the name of the substance and their possible synonyms, type of study, toxicokinetics, and adverse effects. Date data filters were applied starting on the day following the last EFSA Panel's search (December 3, 2020) and ending on October 3, 2022).

We retrieved 1771 manuscripts that were manually searched for relevance and reliability closely following EFSA's guidance provided in EFSA Expert Panel's Appendices B-M.[84] Twenty-one publications were found fitting the criteria, which we read in detail and assessed in the context of the EFSA evaluation to determine whether the literature published since the EFSA report would likely alter the conclusions reached by the EFSA panel:

7.1. Exposure assessment in the U.S.

7.1.1. Putra C et al., 2022**.** *Estimation of Titanium Dioxide Intake by Diet and Stool Assessment among US Healthy Adults*. J Nutr. 152(6):1525-1537. doi: 10.1093/jn/nxac061. PMID: 35266002.

Background: Titanium dioxide (TiO2/E171) is used in foods primarily as a whitening agent. Little is known regarding TiO2 exposure in the United States.

Objectives: To quantify stool TiO2 content among US adults and evaluate its association with estimated intake.

Methods: Adults participated in phase 1 [three 24-h dietary recalls (DRs) and stool TiO2 measured from 3 matched samples (n = 52)] and/or phase 2 [tailored FFQ and stool TiO2 measured from 3 samples over 3 mo (n = 61)]. TiO2 in foods was estimated from a database, and concentration in 49 additional foods and 339 stool samples were quantified using inductively coupled plasma mass spectrometry. Associations between dietary and stool TiO2 were assessed by log-linear multivariable regression. USDA food groups (n = 49, servings/d) were related to stool TiO2 by stepwise regression.

Results: TiO2 food content varied by brand. Mean TiO2 intake from three 24-h DRs [0.19 ± 0.31 mg/(kg body weight · d)] was lower than from the FFQ [0.30 ± 0.21 mg/(kg body weight · d)]. Dietary TiO2 was not predictive of stool TiO2, in phase 1 or phase 2, 10^(β) per 10 times higher dietary TiO2: 1.138 [10^(95% CI): 0.635, 2.037, P = 0.66] and 0.628 [10^(95% CI): 0.206, 1.910, P = 0.41],

respectively. Food groups related to stool TiO2 were 1) milk desserts, sauces, and gravies [10^(β) per servings/d: 3.361; 10^(95% CI): 0.312, 36.163; P = 0.002] and 2) yeast breads [10^(β): 1.430; 10^(95% CI): 0.709, 2.884; P = 0.002] in phase 1 and 1) cream and cream substitutes [10^(β) = 10.925; 10^(95% CI): 1.952, 61.137; P = 0.01] and 2) milk and milk drinks [10^(β) = 0.306; 10^(95% CI): 0.086, 1.092, P = 0.07] in phase 2.

Conclusions: Intake of certain foods was associated with higher stool TiO2 content. There is a need for valid estimation of TiO2 intakes via the improvement of a dietary assessment method and a TiO2 food composition database. Future research should assess whether high stool TiO2 content is related to adverse health outcomes.

***Petitioners' Assessment**: A study by the American Society for Nutrition demonstrating Americans are currently exposed to TiO_2.*

7.2. Absorption of TiO2 particles

7.2.1. Duan SM et al. 2021. *The Influence of Long-Term Dietary Intake of Titanium Dioxide Particles on Elemental Homeostasis and Tissue Structure of Mouse Organs.* J Nanosci Nanotechnol. 21(10):5014-5025. doi: 10.1166/jnn.2021.19351. PMID: 33875086.

Background: Titanium dioxide (TiO2), consisting of nanoparticles and sub-microparticles, were widely used as food additive and consumed by people every day, which has aroused a public safety concern. Some studies showed TiO2 can be absorbed by intestine and then distributed to different tissues after oral intake, which is supposed to affect the content of various elements in the body whereas led to tissue damage. However, knowledge gaps still exist in the impact of TiO2 on the disorder of elemental homeostasis. Thus, this study aimed to explore the oral toxicity of TiO2 by assessing its influence on elemental homeostasis and tissues injury.

Method: ICR mice were fed with normal feed, TiO2 nanoparticles (NPs)-mixed feed or TiO2 submicron particles (MPs)-mixed feed (1% mass fraction TiO2 NPs or MPs were mixed in commercial pellet diet) for 1, 3, and 6 months. Particles used in this study were characterized. The distribution of Ti and other 23 elements, the correlation among elements, and pathological change in the liver, kidney, spleen and blood cells of the mice was determined.

Result: **Ti accumulation only appeared in blood cells of mice treated with TiO2 MPs-mixed feed for 6 months,** but TiO2 cause 12 kinds of elements (boron, vanadium, iron, cobalt, copper, zinc, selenium, sodium, calcium, magnesium, silicon, phosphorus) content changed in organ tissue. The changed kinds of elements in blood cells (6 elements), liver (7 elements) or kidney (6 elements) were more than in the spleen (1 element). The TiO2 NPs induced more elements changed in blood cells and liver, and the TiO2 MPs induced more elements changed in kidney. Significantly positive correlation between Ti and other elements was found in different organs except the liver. Organ injuries caused by TiO2 NPs were severer than TiO2 MPs. Liver exhibited obvious

pathological damage which became more serious with the increase of exposure time, while kidney and spleen had slight damages.

Conclusion: These results indicated long-time dietary intake of TiO2 particles could induce element imbalance and organ injury. The liver displayed more serious change than other organs, especially under the treatment with TiO2 NPs. Further research on the oral toxicity of TiO2 NPs should pay more attention to the health effects of element imbalances using realistic exposure methods.

***Petitioners' Assessment**: The study correlates with EFSA's conclusion regarding potential bioaccumulation and chronic effects of TiO_2 particles with emphasis on liver toxicity.*

7.2.2. Mbanga O,. et al, 2022. *Dissolution of titanium dioxide nanoparticles in synthetic biological and environmental media to predict their biodurability and persistence.* Toxicol In Vitro. 2022 Oct;84:105457. doi: 10.1016/j.tiv.2022.105457. PMID: 35987448.

Investigating the biodurability and persistence of titanium dioxide nanoparticles (TiO2 NPs) is of paramount importance because these parameters influence the particles' impact on human health and the environment. Contrary to most research conducted so far, the present study elucidates the dissolution kinetics, namely the dissolution rates, rate constants, order of reaction and half-times of TiO2 NPs in five different simulated biological fluids and two synthetic environmental media to predict their behaviour in real life situations. Results have shown that the dissolution of TiO2 NPs in all simulated fluids was limited. Of all the simulated biological media tested, acidic media such as phagolysosomal and gastric fluid produced the highest dissolution of TiO2 NPs compared to alkaline media such as blood plasma, Gamble's fluid, and intestinal fluid. Furthermore, when the particles were exposed to simulated environmental conditions, the dissolution was higher in high ionic strength seawater compared to freshwater. The dissolution kinetics of titanium dioxide nanoparticles followed first order reaction kinetics and were generally characterized by low dissolution rates and long half-times. These findings indicate that TiO2 NPs are very insoluble and will remain unchanged in the body and environment over long periods of time. Therefore, these particles are most likely to cause both short and long-term health effects and will remain persistent following release into the environment.

***Petitioners' Assessment**: The study contributes to EFSA's concerns about TiO_2's bioaccumulation and its persistence in tissues.*

7.2.3. Li M et al, 2022. *Bioaccumulation and biomagnification effects of nano-TiO2 in the aquatic food chain.* Ecotoxicology. 31(6):1023-1034. doi: 10.1007/s10646-022-02572-0. PMID: 35831721.

The increasing production of nano-TiO2 has attracted extensive concerns about the ecological consequence and health risk of these compounds in natural ecosystem. However, little is known about its toxicity on zooplankton, especially its possibility to access to the food chain via dietary exposure. To address this concern, the toxic and cumulative effects of nano-TiO2 on an aquatic food chain

were explored through two trophic levels independently or jointly including producer and consumer. The results revealed that exposure to suspensions of nanomaterials had negative effects on both producers and consumers. Specifically, nanoparticles reduced the density of algal cells in a concentration-dependent way, and hatching life expectancy, average lifespan, net reproductive rate, and population intrinsic growth rate of rotifers decreased significantly with the concentration of nanomaterials increased ($P < 0.05$). Notably, nanoparticles accumulated in algal cells and were transferred to consumers through dietary exposure. Biomagnification of nano-TiO2 was observed in this simplified food chain, as many of the biomagnification factor (BMF) values in this study were >1. Exposure concentration, exposure time and their interactions play a strong part in the accumulation of nanoparticles in algae and rotifers. Overall, the present findings confirmed that nano-TiO2 was deleterious to plankton, posing a significant environmental threat to aquatic ecosystems.

Petitioners' Assessment: *This study suggests algae (seaweed) could be a novel source of oral exposure to TiO_2 nanoparticles. Capability of seaweed to accumulate TiO_2 nanoparticles may demonstrate a relevant source of human exposure if TiO_2 nanoparticles are present in the marine ecosystem. The research also supports EFSA's concerns about bioaccumulation of TiO_2.*

7.3. Neurotoxicity

7.3.1. Ribeiro LW et al, 2022. *Titanium dioxide and carbon black nanoparticles disrupt neuronal homeostasis via excessive activation of cellular prion protein signaling.* Part Fibre Toxicol.19(1):48. doi: 10.1186/s12989-022-00490-x. PMID: 35840975; PMCID: PMC9284759.

Background: Epidemiological emerging evidence shows that human exposure to some nanosized materials present in the environment would contribute to the onset and/or progression of Alzheimer's disease (AD). The cellular and molecular mechanisms whereby nanoparticles would exert some adverse effects towards neurons and take part in AD pathology are nevertheless unknown.

Results: Here, we provide the prime evidence that titanium dioxide (TiO2) and carbon black (CB) nanoparticles (NPs) bind the cellular form of the prion protein (PrPC), a plasma membrane protein well known for its implication in prion diseases and prion-like diseases, such as AD. The interaction between TiO2- or CB-NPs and PrPC at the surface of neuronal cells grown in culture corrupts PrPC signaling function. This triggers PrPC-dependent activation of NADPH oxidase and subsequent production of reactive oxygen species (ROS) that alters redox equilibrium. Through PrPC interaction, NPs also promote the activation of 3-phosphoinositide-dependent kinase 1 (PDK1), which in turn provokes the internalization of the neuroprotective TACE α-secretase. This diverts TACE cleavage activity away from (i) TNFα receptors (TNFR), whose accumulation at the plasma membrane augments the vulnerability of NP-exposed neuronal cells to TNFα -associated inflammation, and (ii) the amyloid precursor protein APP, leading to overproduction of neurotoxic amyloid Aβ40/42 peptides. The silencing of PrPC or the pharmacological inhibition of PDK1 protects neuronal cells from TiO2- and CB-NPs effects regarding ROS production, TNFα hypersensitivity,

and Aβ rise. Finally, we show that dysregulation of the PrPC-PDK1-TACE pathway likely occurs in the brain of mice injected with TiO2-NPs by the intra-cerebro-ventricular route as we monitor a rise of TNFR at the cell surface of several groups of neurons located in distinct brain areas.

Conclusion: Our in vitro and in vivo study thus posits for the first time normal cellular prion protein PrPC as being a neuronal receptor of TiO2- and CB-NPs and identifies PrPC-coupled signaling pathways by which those nanoparticles alter redox equilibrium, augment the intrinsic sensitivity of neurons to neuroinflammation, and provoke a rise of Aβ peptides. By identifying signaling cascades dysregulated by TiO2- and CB-NPs in neurons, our data shed light on how human exposure to some NPs might be related to AD.

Petitioners' Assessment: *This study shows strong evidence of neurotoxicity and neuroinflammation associated with TiO_2 nanoparticles and demonstrates a plausible mechanism of neurological effects. Altogether, the research adds to the EFSA Expert Panel's conclusions about TiO_2 neurotoxicity.*

7.3.2. Medina-Reyes EI et al. 2022. *Food-grade titanium dioxide decreases hematocrit and hemoglobin and increases compulsive-like behavior in male mice.* J Appl Toxicol. 42(8):1411-1419. doi: 10.1002/jat.4296. PMID: 35128702.

Food-grade titanium dioxide (E171) is widely used as a food additive, and it is known that after oral consumption, E171 is translocated into the bloodstream reaching the highest titanium level at 6 h. E171 is accumulated in some organs triggering toxicity, but the effects on the blood parameters after oral consumption have been less studied. Recently, evidence shows that oral exposure to E171 induces behavioral signs of anxiety and depression. The relation between blood alterations and psychiatric disorders has been previously demonstrated. However, the oral exposure to E171 effects on alterations in blood parameters and effects linked to alterations in animal behavior has not been explored. In this short communication, we aimed to investigate the effects of E171 on specific blood parameters (hematocrit, hemoglobin, number of erythrocytes, and leukocytes) and anxiety and compulsive-like behavior in males and females orally exposed to ~5 mg/kg for 4 weeks. The results showed that E171 decreased hematocrit and hemoglobin in male but not in female mice while leukocyte and erythrocyte count remained unaltered. Oral consumption of E171 decreased the levels of anxiety-like behavior in females but not in male mice, while compulsive-like behavior was increased in both male and female mice.

Petitioners' Assessment*: This is a short communication demonstrating sex-dependent neurobehavioral effects of TiO_2.*

7.3.3. Mortensen NP et al. 2022. *Oral administration of TiO2 nanoparticles during early life impacts cardiac and neurobehavioral performance and metabolite profile in an age- and sex-related manner.* Part Fibre Toxicol. 19(1):3. doi: 10.1186/s12989-021-00444-9. PMID: 34986857; PMCID: PMC8728993.

Background: Nanoparticles (NPs) are increasingly incorporated in everyday products. To investigate the effects of early life exposure to orally ingested TiO2 NP, male and female Sprague-Dawley rat pups received four consecutive daily doses of 10 mg/kg body weight TiO2 NP (diameter: 21 ± 5 nm) or vehicle control (water) by gavage at

three different pre-weaning ages: postnatal day (PND) 2-5, PND 7-10, or PND 17-20. Cardiac assessment and basic neurobehavioral tests (locomotor activity, rotarod, and acoustic startle) were conducted on PND 20. Pups were sacrificed at PND 21. Select tissues were collected, weighed, processed for neurotransmitter and metabolomics analyses.

Results: Heart rate was found to be significantly decreased in female pups when dosed between PND 7-10 and PND 17-20. Females dosed between PND 2-5 showed decrease acoustic startle response and when dosed between PND 7-10 showed decreased performance in the rotarod test and increased locomotor activity. Male pups dosed between PND 17-20 showed decreased locomotor activity. The concentrations of neurotransmitters and related metabolites in brain tissue and the metabolomic profile of plasma were impacted by TiO2 NP administration for all dose groups. Metabolomic pathways perturbed by TiO2 NP administration included pathways involved in amino acid and lipid metabolism.

Conclusion: Oral administration of TiO2 NP to rat pups impacted basic cardiac and neurobehavioral performance, neurotransmitters and related metabolites concentrations in brain tissue, and the biochemical profiles of plasma. The findings suggested that female pups were more likely to experience adverse outcome following early life exposure to oral TiO2 NP than male pups. Collectively the data from this exploratory study suggest oral administration of TiO2 NP cause adverse biological effects in an age- and sex-related manner, emphasizing the need to understand the short- and long-term effects of early life exposure to TiO2 NP.

***Petitioners' Assessment**: The study provides evidence that bolsters EFSA Panel's concerns about nano-TiO_2 neurotoxicity. Further, it adds to expanding concern about cardiovascular effects nanoparticles, and acknowledges novel but important sex-dependent effects of TiO_2 NPs.*

7.3.4. Yang, C. et al., 2022. *Prenatal exposure to titanium dioxide nanoparticles induces persistent neurobehavioral impairments in maternal mice that is associated with microbiota-gut-brain axis.* Food Chem Toxicol. 2022 Sep 13;169:113402. doi: 10.1016/j.fct.2022.113402. PMID: 36108982.

Gestational exposure to titanium dioxide nanoparticles (TiO2NPs) has been widely reported to have deleterious effects on the brain functions of offspring. However, little attention has been paid to the neurotoxic effects of TiO2NPs on maternal body after parturition. The pregnant mice were orally administrated with TiO2NPs at 150 mg/kg from gestational day 8-21. The potential effects of TiO2NPs on the neurobehaviors were evaluated at postnatal day 60. The gut microbiota, morphological alterations of intestine and brain, and other indicators that involved in gut-brain axis were all assessed to investigate the underlying mechanisms. The results demonstrated that exposure to TiO2NPs during pregnancy caused the persistent neurobehavioral impairments of maternal mice after delivery for 60 days, mainly including behavioural changes, pathological changes in hippocampus, cortex and intestine. Our data also showed that persistent dysfunction and tissue injuries were probably associated with the disruption of gut-brain axis, manifested by the shift in the composition of gut microbial community, alteration of Sstr1, inhibition of enteric neurons and reduction of diamine oxidase contents in maternal mice. These findings provide a novel insight that regulation of gut

microecology may be an alternative strategy for the protection against the neurotoxicity of TiO2NPs in pregnant women.

Petitioners' Assessment: *The study indicates that ingested TiO_2 nanoparticles during pregnancy cause neurobehavioral problems in maternal mice potentially resulting from alterations to the maternal gut-brain axis, including the gut microbiome.*

7.4. Carcinogenicity

7.4.1. Bischoff, NS et al., 2022. *Effects of the Food Additive Titanium Dioxide (E171) on Tumor Formation and Gene Expression in the Colon of a Transgenic Mouse Model for Colorectal Cancer*. Nanomaterials (Basel). 12(8):1256. doi: 10.3390/nano12081256. PMID: 35457963; PMCID: PMC9027218.

Titanium dioxide (TiO2) is present in many different food products as the food additive E171, which is currently scrutinized due to its potential adverse effects, including the stimulation of tumor formation in the gastrointestinal tract. We developed a transgenic mouse model to examine the effects of E171 on colorectal cancer (CRC), using the Cre-LoxP system to create an Apc-gene-knockout model which spontaneously develops colorectal tumors. A pilot study showed that E171 exposed mice developed colorectal adenocarcinomas, which were accompanied by enhanced hyperplasia in epithelial cells, lymphatic nodules at the base of the polyps, and increased tumor size. In the main study, tumor formation was studied following the exposure to 5 mg/kgbw/day of E171 for 9 weeks (Phase I). E171 exposure showed a statistically nonsignificant increase in the number of colorectal tumors in these transgenic mice, as well as a statistically nonsignificant increase in the average number of mice with tumors. Gene expression changes in the colon were analyzed after exposure to 1, 2, and 5 mg/kgbw/day of E171 for 2, 7, 14, and 21 days (Phase II). Whole-genome mRNA analysis revealed the modulation of genes in pathways involved in the regulation of gene expression, cell cycle, post-translational modification, nuclear receptor signaling, and circadian rhythm. The processes associated with these genes might be involved in the enhanced tumor formation and suggest that E171 may contribute to tumor formation and progression by modulation of events related to inflammation, activation of immune responses, cell cycle, and cancer signaling.

Petitioners' Assessment*: The study provides some answers to the EFSA Panel's call for research appropriately designed to investigate the effects of TiO_2 with regard to carcinogenicity and immunotoxicity. However, the study period (9 weeks) was exceptionally short relative to accepted carcinogenicity bioassay procedures (2 years) and focused exclusively colorectal cancer using a transgenic animal model of colorectal cancer. Thus, this study and its null results regarding the effect of TiO2 on colorectal cancer does not fully satisfy EFSA's call for additional research on the carcinogenicity of TiO_2.*

7.5. Genotoxicity

7.5.1. Vieira A. et al. 2022**.** *Investigation of the genotoxicity of digested titanium dioxide nanomaterials in human intestinal cells***.** Food Chem Toxicol. doi: 10.1016/j.fct.2022.112841. PMID: 35093430.

The widespread use of titanium dioxide nanomaterials (TiO2 NMs) in food and consumer products such as toothpaste or food contact materials, suggests the relevance of human oral exposure to these nanomaterials (NMs) and raises the possibility of adverse effects in the gastrointestinal tract (GIT). We previously showed that the in vitro digestion of TiO2 NMs may increase their toxicity in intestinal cells. In this work, we analyzed the genotoxicity and the intracellular reactive oxygen species induction by physiologically relevant concentrations of three different TiO2 NMs (NM-102, NM-103 and NM-105) in Caco-2 and HT29-MTX-E12 intestinal cells, while considering the potential influence of the digestion process in the NMs' physiochemical characteristics. The results evidenced a DNA-damaging effect dependent on the NM, more relevant for the rutile/anatase NM-105, possibly due to its lower hydrodynamic size in the cells medium. In addition, the results of the micronucleus assay suggest effects on chromosomal integrity, an indicator of cancer risk, in the HT29-MTX-E12 cells, for all the tested TiO2 NMs, especially after the in vitro digestion. This work supports the evidence for concerns on the use of TiO2 NMs as a food additive, recently reported by EFSA, and for their use in applications in consumer products that may drive human exposure through ingestion.

Petitioners' Assessment*: A follow-up research of a study considered by the EFSA Panel in 2021. Results of this later investigation strengthen the argument of intestinal genotoxicity of TiO_2 nanoparticles.*

7.5.2. Rodríguez-Ibarra C., et al, 2022. *Food grade titanium dioxide accumulation leads to cellular alterations in colon cells after removal of a 24-hour exposure.* Toxicology. 2022 Aug;478:153280. doi: 10.1016/j.tox.2022.153280.. PMID: 35973603.

Titanium dioxide food grade (E171) is one of the most used food additives containing nanoparticles. Recently, the European Food Safety Authority indicated that E171 could no longer be considered safe as a food additive due to the possibility of it being genotoxic and there is evidence that E171 administration exacerbates colon tumor formation in murine models. However, less is known about the effects of E171 accumulation once the exposure stopped, then we hypothesized that toxic effects could be detected even after E171 removal. Therefore, we investigated the effects of E171 exposure after being removed from colon cell cultures. Human colon cancer cell line (HCT116) was exposed to 0, 1, 10 and 50 μg/cm2 of E171. Our results showed that in the absence of cytotoxicity, E171 was accumulated in the cells after 24 of exposure, increasing granularity and reactive oxygen species, inducing alterations in the molecular pattern of nucleic acids and lipids, and causing nuclei enlargement, DNA damage and tubulin depolymerization. After the removal of E171, colon cells were cultured for 48 h more hours to analyze the ability to restore the previously detected alterations. As we hypothesized, the removal of E171 was unable to revert the alterations found after 24 h of exposure in colon cells. In conclusion, exposure to E171 causes alterations that cannot be reverted after 48 h if E171 is removed from colon cells.

***Petitioners' Assessment**: The research focusing on the cellular effects of short-term exposure to good grade TiO_2 shows lasting impairment in gut cells including adverse impact on the nuclei and DNA. The effects demonstrated are consistent with EFSA's concerns about TiO_2's genotoxicity.*

7.5.3. Rolo et al. 2022. *Adverse Outcome Pathways Associated with the Ingestion of Titanium Dioxide Nanoparticles—A Systematic Review* Nanomaterials 2022, 12, 3275. https://doi.org/10.3390/nano12193275.

Titanium dioxide nanoparticles (TiO2 -NPs) are widely used, and humans are exposed through food (E171), cosmetics (e.g., toothpaste), and pharmaceuticals. The oral and gastrointestinal (GIT) tract are the first contact sites, but it may be systemically distributed. However, a robust adverse outcome pathway (AOP) has not been developed upon GIT exposure to TiO2 -NPs. The aim of this review was to provide an integrative analysis of the published data on cellular and molecular mechanisms triggered after the ingestion of TiO2 -NPs, proposing plausible AOPs that may drive policy decisions. A systematic review according to Prisma Methodology was performed in three databases of peer-reviewed literature: Pubmed, Scopus, and Web of Science. A total of 787 records were identified, screened in title/abstract, being 185 used for data extraction. The main endpoints identified were oxidative stress, cytotoxicity/apoptosis/cell death, inflammation, cellular and systemic uptake, genotoxicity, and carcinogenicity. From the results, AOPs were proposed where colorectal cancer, liver injury, reproductive toxicity, cardiac and kidney damage, as well as hematological effects stand out as possible adverse outcomes. The recent transgenerational studies also point to concerns with regard to population effects. Overall, the findings further support a limitation of the use of TiO2 -NPs in food, announced by the European Food Safety Authority (EFSA).

***Petitioners' Assessment:** The review provides evidence parallel to the EFSA's evaluation, data on possible mechanisms of TiO_2's action. In their conclusions, the authors side with the European precautionary approach to TiO_2.*

7.5.4. El Yamani N. et al. M. *Lack of mutagenicity of TiO_2 nanoparticles in vitro despite cellular and nuclear uptake*. Mutat Res Genet Toxicol Environ Mutagen. 2022 Oct;882:503545. doi: 10.1016/j.mrgentox.2022.503545. PMID: 36155144.

The potential genotoxicity of titanium dioxide (TiO2) nanoparticles (NPs) is a conflictive topic because both positive and negative findings have been reported. To add clarity, we have carried out a study with two cell lines (V79-4 and A549) to evaluate the effects of TiO2 NPs (NM-101), with a diameter ranging from 15 to 60 nm, at concentrations 1-75 μg/cm2. Using two different dispersion procedures, cell uptake was determined by Transmission Electron Microscopy (TEM). Mutagenicity was evaluated using the Hprt gene mutation test, while genotoxicity was determined with the comet assay, detecting both DNA breaks and oxidized DNA bases (with formamidopyrimidine glycosylase - Fpg). Cell internalization, as determined by TEM, shows TiO2 NM-101 in cytoplasmic vesicles, as well as close to and inside the nucleus. Such internalization did not depend on the state of agglomeration, nor the dispersion used. In spite of such internalization, no cytotoxicity was detected in V79-4 cells (relative growth activity and plating efficiency assays) or in A549 cells (AlamarBlue assay) after exposure lasting for 24 h. However, a significant decrease in the relative growth activity was detected at longer exposure times (48 and 72 h) and at the highest concentration 75 μg/cm2. When the modified enzyme-linked alkaline comet assay was performed on A549 cells,

although no significant induction of DNA damage was detected, a positive concentration-effects relationship was observed (Spearman's correlation = 0.9, p 0.0001). Furthermore, no significant increase of DNA oxidized purine bases was observed. When the frequency of Hprt gene mutants was determined in V79-4 cells, no increase was observed in the exposed cells, relative to the unexposed cultures. Our general conclusion is that, under our experimental conditions, TiO2 NM-101 exposure does not exert mutagenic effects despite the evidence of NP uptake by V79-4 cells.

***Petitioners' Assessment**: A similar study on V79-4 cells that observed no mutagenic effects in apoptosis assays was evaluated by the EFSA's Expert Panel (Kazimiorva et al., 2020). Therefore, this study does not contradict the evidence EFSA has used for its conclusion.*

7.6. Novel effects

7.6.1. Microbiome disruption and immunotoxicity

7.6.1.1. Yan J. et al. 2022. *Intestinal toxicity of micro- and nano-particles of foodborne titanium dioxide in juvenile mice: Disorders of gut microbiota-host co-metabolites and intestinal barrier damage***.** Sci Total Environ. doi: 10.1016/j.scitotenv.2022.153279. PMID: 35074372

The wide use of TiO2 particles in food and the high exposure risk to children have prompted research into the health risks of TiO2. We used the microbiome and targeted metabolomics to explore the potential mechanism of intestinal toxicity of foodborne TiO2 micro-/nanoparticles after oral exposure for 28 days in juvenile mice. Results showed that the gut microbiota-including the abundance of Bacteroides, Bifidobacterium, Lactobacillus, and Prevotella-changed dynamically during exposure. The organic inflammatory response was activated, and lipopolysaccharide levels increased. Intestinal toxicity manifested as increased mucosal permeability, impaired intestinal barrier, immune damage, and pathological changes. The expression of antimicrobial peptides, occludin, and ZO-1 significantly reduced, while that of JNK2 and Src/pSrc increased. Compared with micro-TiO2 particles, the nano-TiO2 particles had strong toxicity. Fecal microbiota transplant confirmed the key role of gut microbiota in intestinal toxicity. The levels of gut microbiota-host co-metabolites, including pyroglutamic acid, L-glutamic acid, phenylacetic acid, and 3-hydroxyphenylacetic acid, changed significantly. Significant changes were observed in the glutathione and propanoate metabolic pathways. There was a significant correlation between the changes in gut microbiota, metabolites, and intestinal cytokine levels. These, together with the intestinal barrier damage signaling pathway, constitute the network mechanism of the intestinal toxicity of TiO2 particles.

***Petitioners' Assessment**: The study further supports the Expert Panel's argument of nano-TiO_2 immunotoxicity. Damage to the intestinal barrier and gut microbiome were observed, changes that manifested in inflammation and immune dysfunctions.*

7.6.1.2. Perez L.et al, 2021. *Dietary nanoparticles alter the composition and function of the gut microbiota in mice at dose levels relevant for human exposure.* Food Chem Toxicol. doi: 10.1016/j.fct.2021.112352. PMID: 34153347.

Background: Nanotechnologies provide new opportunities for improving the safety, quality, shelf life, flavor and appearance of foods. The most common nanoparticles (NPs) in human diet are silver metal, mainly present in food packaging and appliances, and silicon and titanium dioxides used as additives. The rapid development and commercialization of consumer products containing these engineered NPs is, however, not well supported by appropriate toxicological studies and risk assessment. Local and systemic toxicity and/or disruption of the gut microbiota (GM) have already been observed after oral administration of NPs in experimental animals, but results are not consistent and doses used were often much higher than the estimated human intakes. In view of the strong evidence linking alterations of the GM to cardiometabolic (CM) diseases, we hypothesized that dietary NPs might disturb this GM-CM axis.

Materials and methods: We exposed male C57BL/6JRj mice (n = 13 per dose group) to dietary NPs mixed in food pellets at doses relevant for human exposure: Ag (0, 4, 40 or 400 μg/kg pellet), SiO2 (0, 0.8, 8 and 80 mg/kg pellet) or TiO2 (0, 0.4, 4 or 40 mg/kg pellet). After 24 weeks of exposure, we assessed effects on the GM and CM health (n = 8 per dose group). The reversibility of the effects was examined after 8 additional weeks without NPs exposure (recovery period, n ≤ 5 per dose group).

Results: No overt toxicity was recorded. The GM β-diversity was dose-dependently disrupted by the three NPs, and the bacterial short chain fatty acids (SCFAs) were dose-dependently reduced after the administration of SiO2 and TiO2 NPs. These effects disappeared completely or partly after the recovery period, strengthening the association with dietary NPs. We did not observe atheromatous disease or glucose intolerance after NP exposure. Instead, dose-dependent decreases in the expression of IL-6 in the liver, circulating triglycerides (TG) and urea nitrogen (BUN) were recorded after administration of the NPs.

Conclusion: We found that long-term oral exposure to dietary NPs at doses relevant for estimated human intakes disrupts the GM composition and function. These modifications did not appear associated with atheromatous or deleterious metabolic outcomes.

***Petitioners' Assessment**: This investigation is consistent with the EFSA Panel's conclusions on absent overt toxicity of nano-TiO_2 but demonstrates possible long-term impacts on gut microbiota following chronic oral exposure.*

7.6.1.3. Wang S. et al., 2022. *Oral exposure to Ag or TiO2 nanoparticles perturbed gut transcriptome and microbiota in a mouse model of ulcerative colitis.* Food Chem Toxicol. 2022 Sep 7;169:113368. doi: 10.1016/j.fct.2022.113368. PMID: 36087619.

Silver (nAg) and titanium dioxide (nTiO2) nanoparticles improve texture, flavour or anti-microbial properties of various food products and packaging materials. Despite their increased oral exposure, their potential toxicities in the dysfunctional intestine are unclear. Here, the effects of ingested nAg or nTiO2 on inflamed colon were revealed in a mouse model of chemical-induced acute ulcerative colitis. Mice (eight/group) were exposed to nAg or nTiO2 by oral gavage for 10 consecutive days. We characterized disease phenotypes, histology, and alterations in colonic transcriptome (RNA sequencing) and gut microbiome (16S sequencing). Oral exposure to nAg caused only minor changes in phenotypic hallmarks of colitic mice but induced extensive responses in gene expression enriching processes of apoptotic cell death and RNA metabolism. Instead, ingested nTiO2 yielded shorter colon, aggravated epithelial hyperplasia and

deeper infiltration of inflammatory cells. Both nanoparticles significantly changed the gut microbiota composition, resulting in loss of diversity and increase of potential pathobionts. They also increased colonic mucus and abundance of Akkermansia muciniphila. Overall, nAg and nTiO2 induce dissimilar immunotoxicological changes at the molecular and microbiome level in the context of colon inflammation. The results provide valuable information for evaluation of utilizing metallic nanoparticles in food products for the vulnerable population.

Petitioners' Assessment: *This paper shows severe pro-inflammatory effect of TiO_2 nanoparticles in the gut and their impact on the gut microbiome in a murine model of colitis, consistent with EFSA's concerns about intestinal health.*

7.6.2. TiO_2 co-exposure

7.6.2.1. Yang C. et al. 2022**.** *Intestinal Microecology of Mice Exposed to TiO2 Nanoparticles and Bisphenol A***.** Foods.11(12):1696. doi: 10.3390/foods11121696. PMID: 35741895; PMCID: PMC9222895.

Exposure to titanium dioxide nanoparticles (TiO2 NPs) and bisphenol A (BPA) is ubiquitous, especially through dietary and other environmental pathways. In the present study, adult C57BL/6J mice were exposed to TiO2 NPs (100 mg/kg), BPA (0, 5, and 50 mg/kg), or their binary mixtures for 13 weeks. The 16S rDNA amplification sequence analysis revealed that co-exposure to TiO2 NPs and BPA altered the intestinal microbiota; however, this alteration was mainly caused by TiO2 NPs. Faecal metabolomics analysis revealed that 28 metabolites and 3 metabolic pathways were altered in the co-exposed group. This study is the first to reveal the combined effects of TiO2 NPs and BPA on the mammalian gut microbial community and metabolism dynamics, which is of great value to human health. The coexistence of TiO2 NPs and BPA in the gut poses a potential health risk due to their interaction with the gut microbiota.

Petitioners' Assessment: *This study shows the cumulative adverse effects caused by a mixture of BPA and nanoTiO_2 on the gut (i.e., intestinal damage and inflammation) and gut microbiome. BPA and nanoTiO_2 may be toxicologically related, per the authors of this study who state, "When TiO2 NPs are utilised as medication carriers in the human body or when people consume the food containing E171…interactions between TiO2 NPs and BPA may occur. Previous studies have noted that TiO2 NPs could enhance the bioavailability and toxicity of co-existing toxicants in the aquatic phase. The simultaneous presence of BPA and TiO2 NPs causes neurotoxicity, reproductive toxicity, and disturbances in the intestinal microecology of zebrafish."*

7.6.2.2. Cao X. et al. 2021. *Co-exposure to boscalid and TiO2 (E171) or SiO2 (E551) downregulates cell junction gene expression in small intestinal epithelium cellular model and increases pesticide translocation.* NanoImpact. doi: 10.1016/j.impact.2021.100306. PMID: 35559963.

A recent published study showed that TiO2 (E171) and SiO2 (E551), two widely used nano-enabled food additives, increased the translocation of the commonly used pesticide boscalid by 20% and 30% respectively. Such increased absorption of pesticides due to the presence of engineered nanomaterials (ENMs) in food raises health concerns for these food additives. In this companion study, mRNA expression of genes related to cell

junctions in a small intestinal epithelial cellular model after exposure to simulated digest as of fasting food model (phosphate buffer) containing boscalid (150 ppm) with or without either TiO2 or SiO2 (1% w/w) were analyzed. Specific changes in cell barrier function underlying or contributing to the increased translocation of boscalid observed in the previous study were assessed. Results showed that exposure to boscalid alone has no significant effect on cell junction genes, however, co-exposure to boscalid and TiO2 significantly regulated expression of cell-matrix junction focal adhesion-related genes, e.g., downregulating Cav1 (-1.39-fold, $p < 0.05$), upregulating Cav3 (+ 3.30-fold, $p < 0.01$) and Itga4 (+ 3.30-fold, $p < 0.05$). Similarly, co-exposure to boscalid and SiO2 significantly downregulated multiple cell-cell junction genes, including tight junction genes (Cldn1, Cldn11, Cldn16, Cldn18, and Jam3), adherens junction genes (Notch1, Notch3, Pvrl1) and gap junction genes (Gja3 and Gjb2), as well as cell-matrix junction focal adhesion genes (Itga4, Itga6, Itga7). Together, these findings suggest that co-ingestion of boscalid with TiO2 (E171) or SiO2 (E551) could cause weakening of cell junctions and intercellular adhesion, which could result in dysregulation of paracellular transport, and presumably contributed to the previously observed increased translocation of boscalid at the presence of these ENMs. This novel finding raises health safety concerns for such popular food additives.

Petitioners' Assessment: *This study raises concerns about food grade TiO_2 co-ingested with a common, EPA-registered fungicide, and elucidates potential mechanisms of adverse effects of TiO2 in the gut. In addition, it argues that the dietary intake of TiO_2 must be included when estimating systemic boscalid exposure. Similarly, these results argue that when assessing the safety of TiO_2 as a food additive, regulators should take into consideration the potential influence of TiO_2 on the uptake of other chemicals found in the diet.*

7.6.3. Cardiovascular effects of TiO_2

7.6.3.1. Zhu X. et al, 2022. *Dietary titanium dioxide particles (E171) promote diet-induced atherosclerosis through reprogramming gut microbiota-mediated choline metabolism in APOE-/- mice*. J Hazard Mater. doi: 10.1016/j.jhazmat.2022.129179. PMID: 35739712.

Food-grade titanium dioxide (E171) has been reported to induce changes in some intestinal metabolites related to development of atherosclerosis (AS). However, little is known about the effects of chronic dietary intake of E171 on AS development, particularly in AS-prone populations with high-choline western diet (HCD). Herein, we disclosed that E171 obviously exacerbated HCD-induced AS through increasing production of trimethylamine (TMA) and pro-atherogenic trimethylamine-N-oxide (TMAO) via remodeling gut microbiota structure in APOE-/- mice. Oral administration of 40 mg/kg E171 daily for 4 months significantly increased the atherosclerotic lesion area, especially in the HCD group. Mechanistic studies revealed that E171 induced much more TMAO production by increasing the gut microbial expression of choline TMA lyases (CutC/D), which converted dietary choline to TMA by a glycyl radical reaction. The 16S rDNA sequencing analysis demonstrated that bacterial strains expressing CutC/D were enriched by E171 in HCD-fed mice. In contrast, gut microbiota depletion eliminated the impact of E171 on choline/TMA/TMAO pathway and AS progression, indicating gut flora shifts were responsible for the exacerbation effects of E171 ingestion on HCD-induced AS. All the results emphasized the alarming role of E171 on AS progression and stated the importance of reevaluating the impact of food additives on the development of chronic diseases.

Petitioners' Assessment: *This study shows that TiO2 changes in the gut microbiota can have adverse effects on cardiovascular system when combined with meat-based diet. This study bolsters the Panel's concerns about chronic effects of TiO2 added to food.*

7.6.4. Disruption to lipid metabolism

7.6.4.1 Chen et al. 2022. *Landscape of lipidomic metabolites in gut-liver axis of Sprague-Dawley rats after oral exposure to titanium dioxide nanoparticles.* Part Fibre Toxicol. 19(1):53. doi: 10.1186/s12989-022-00484-9. PMID: 35922847; PMCID: PMC9351087.

Background: The application of titanium dioxide nanoparticles (TiO2 NPs) as food additives poses a risk of oral exposure that may lead to adverse health effects. Even though the substantial evidence supported liver as the target organ of TiO2 NPs via oral exposure, the mechanism of liver toxicity remains largely unknown. Since the liver is a key organ for lipid metabolism, this study focused on the landscape of lipidomic metabolites in gut-liver axis of Sprague Dawley (SD) rats exposed to TiO2 NPs at 0, 2, 10, 50 mg/kg body weight per day for 90 days.

Results: TiO2 NPs (50 mg/kg) caused slight hepatotoxicity and changed lipidomic signatures of main organs or systems in the gut-liver axis including liver, serum and gut. The cluster profile from the above biological samples all pointed to the same key metabolic pathway and metabolites, which was glycerophospholipid metabolism and Phosphatidylcholines (PCs), respectively. In addition, absolute quantitative lipidomics verified the changes of three PCs concentrations, including PC (16:0/20:1), PC (18:0/18:0) and PC (18:2/20:2) in the serum samples after treatment of TiO2 NPs (50 mg/kg). The contents of malondialdehyde (MDA) in serum and liver increased significantly, which were positively correlated with most differential lipophilic metabolites.

Conclusions: The gut was presumed to be the original site of oxidative stress and disorder of lipid metabolism, which resulted in hepatotoxicity through the gut-liver axis. Lipid peroxidation may be the initial step of lipid metabolism disorder induced by TiO2 NPs. Most nanomaterials (NMs) have oxidation induction and antibacterial properties, so the toxic pathway revealed in the present study may be primary and universal.

Petitioners' Assessment: *In this paper, oral exposure of rats to titanium dioxide nanoparticles manifested in hepatotoxicity and altered lipid profiles in the gut, serum and liver, providing useful insights into potential adverse effects resulting from use of TiO_2 as a food additive.*

I.E. Complete data which will allow the Commissioner to consider, among other things, the probable consumption of, and/or other relevant exposure from the additive and of any substance formed in or on food, drugs, or cosmetics because of such additive; and the cumulative effect, if any, of such additive in the diet of man or animals, taking into account the same or any chemically or pharmacologically related substance or substances in the diet including, but not limited to food additives and pesticide chemicals for which tolerances or exemptions from tolerances have been established.

We request that FDA revoke its approved color additive uses of synthetic TiO_2 in foods at § 73.575.[85] Therefore, this petition proposes to eliminate the probable dietary consumption of TiO2 particles from current levels and reduce the cumulative effect of the dietary intake.

I.F. Proposed tolerances and other limitations on the use of the color additive, if tolerances and limitations are required in order to insure its safety. A petitioner may include a proposed regulation.

We request that FDA revoke its approved color additive uses of synthetic TiO_2 in foods at § 73.575.[86] Therefore, there is no need for a tolerance or other limitations.

IG. If exemption from batch certification is requested, the reasons why it is believed such certification is not necessary (including supporting data to establish the safety of the intended use).

We are not proposing to alter the certification status of color additive uses of synthetic TiO_2 in foods at § 73.575.[87] It should continue to be exempt from batch certification.

I.H. If submitting a petition to alter an existing regulation issued pursuant to section 721(b) of the act, full information on each proposed change that is to be made in the original regulation must be submitted. The petition may omit statements made in the original petition concerning which no change is proposed. A supplemental petition must be submitted for any change beyond the variations provided for in the original petition and the regulation issued on the basis of the original petition.

See Appendix II for proposed changes to be made to Sec. 73.575.

I.I. The prescribed fee of $3,000 for admitting the color additive to listing.

Pursuant to 21 C.F.R. § 70.19(q), petitioners request a waiver of the color additive petition fees and deposit requirements. The petitioners are non-profit organizations and individuals who submit this petition because it is in the public interest to protect public health by reducing exposure to lead. They have no financial interests in synthetic TiO_2 or any of the alternatives that may benefit from removing this color additive from the market.

I.J. The petitioner is required to submit either a claim for categorical exclusion under § 25.30 or 25.32 of this chapter or an environmental assessment under § 25.40 of this chapter.

This color additive petition is categorically excluded from the need to prepare an Environmental Assessment under 21 C.F.R. § 25.32(m) as an "action to prohibit or otherwise restrict or reduce the use of a substance in food, food packaging, or cosmetics." We have identified no extraordinary circumstances as defined at 21 C.F.R. § 25.21 for the action requested in this petition which would require the submission of an Environmental Assessment because the use of synthetic TiO_2 as a color additive is primarily to make the food more attractive and, therefore, is not an essential. No substitutes are needed.

Appendix II
Proposed Changes to FDA Approvals

PART 73 – LISTING OF COLOR ADDITIVES EXEMPT FROM CERTIFICATION

Delete Sec. 73.575 – Titanium dioxide

Appendix III
List of References

A) References

1. FDA's response to a Freedom of Information Act (FOIA) request at https://blogs.edf.org/health/files/2022/08/TiO2-EWG-FOIA-Response-June-20221-Redacted-CAP-4C0019.pdf.
    a. Footnote 3: FDA, Letter from C.J. Kokoski to Richard Marx, October 7, 1970. See page 339 of 393 in the PDF of FDA's response to a Freedom of Information Act (FOIA) request at https://blogs.edf.org/health/files/2022/08/TiO2-EWG-FOIA-Response-June-20221-Redacted-CAP-4C0019.pdf.
    b. Footnote 33: American Cyanamid Company, Investigations of the Possible Absorption of Titanium Dioxide from the Gastrointestinal Tract, 1963, page 130 of 391 in the PDF of FDA's response to a Freedom of Information Act (FOIA) request at https://blogs.edf.org/health/files/2022/08/TiO2-EWG-FOIA-Response-June-20221-Redacted-CAP-4C0019.pdf.
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10. EFSA 2021 TiO_2 exposure assessment. Supporting Information Appendices Q-V. https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2021.6585&file=efs26585-sup-0014-Appendices_Q-V.xlsx
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[1] As defined by FDA at 21 C.F.R. § 70.3(i).
[2] As required by the three factors at 21 U.S.C. § 379e(b(5)(A).
[3] FDA, Letter from C.J. Kokoski to Richard Marx, October 7, 1970. See page 339 of 393 in the PDF of FDA's response to a Freedom of Information Act (FOIA) request at https://blogs.edf.org/health/files/2022/08/TiO2-EWG-FOIA-Response-June-20221-Redacted-CAP-4C0019.pdf. FDA reached this conclusion despite evidence in the petition showing that the concentration of titanium in muscle tissue increased in rats fed TiO_2 relative to control animals.
[4] Based on FDA's response to a Freedom of Information Act (FOIA) request at https://blogs.edf.org/health/files/2022/08/TiO2-EWG-FOIA-Response-June-20221-Redacted-CAP-4C0019.pdf.
[5] FDA, Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology, June 2014 at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/considering-whether-fda-regulated-product-involves-application-nanotechnology. FDA states that "Materials in the nanoscale range (i.e., with at least one dimension in the size range of approximately 1 nanometer (nm) to 100 nm) can exhibit different chemical or physical properties, or biological effects compared to larger-scale counterparts."
[6] EFSA, 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, https://doi.org/10.2903/j.efsa.2018.5327.
[7] EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[8] European Commission Regulation (EU) 2022/63, January 14, 2022 at https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32022R0063&from=EN.
[9] Unless it would violate a standard of identity for the food. See 21 C.F.R. §§ 131-169.
[10] 21 C.F.R. § 73.575.
[11] USP, Food Chemicals Codex, Thirteenth Edition, Monograph for Titanium Dioxide, downloaded February 11, 2023. See https://www.foodchemicalscodex.org/.
[12] EFSA Panel on Color additives and Flavourings, Scientific opinion on the proposed amendment of the EU specifications for titanium dioxide (E 171) with respect to the inclusion of additional parameters related to its particle size distribution, 2019, https://doi.org/10.2903/j.efsa.2019.5760.
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[14] *Id.* Table 3.
[15] Blevins LK et al. 2019. Evaluation of immunologic and intestinal effects in rats administered an E 171-containing diet, a food grade titanium dioxide (TiO_2). Food and Chemical Toxicology 133:110793. https://doi.org/10.1016/j.fct.2019.110793.
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[16] EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[17] "[A]nalysis using scanning electron microscopy (SEM) showed 36% of TiO_2 particles were < 100 nm in diameter with the average diameter recorded being between 110 and 115 nm. Volume/mass based approaches showed similar average particle diameter (150 nm) but showed only 1–2% or particles falling below 100 nm in diameter." Blevins LK et al. 2019. Evaluation of immunologic and intestinal effects in rats administered an E 171-containing diet, a food grade titanium dioxide (TiO_2). Food and Chemical Toxicology 133:110793. https://doi.org/10.1016/j.fct.2019.110793.
[18] EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[19] *Id.*
[20] *Id.*
[21] 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. See https://efsa.onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2021.EN-6678.
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[23] EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[24] *Id.* Supporting Information, Appendices Q-V. https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2021.6585&file=efs26585-sup-0014-Appendices_Q-V.xlsx.
[25] CAP 8C0262 by EM Industries in 1998 for food, CAP 2C0294 by E & J Gallo Winery in 2012 for distilled spirits, CAP 4C0299 by EM Millipore in 2014 for cocktails, CAP 5C0301 by Signature Brands in 2014 for egg dying kits, and CAP 5C0302 by E & J Gallo Winery in 2015 for distilled spirits.
[26] CAP 8C0262 at https://blogs.edf.org/health/files/2023/02/CAP-8C0257-Documents-related-to-Titanium-dioxide-coated-mica-COMBINED.pdf, CAP 2C0294 at https://blogs.edf.org/health/files/2023/02/CAP-2C0294-Mica-based-pearlescent-pigments-distilled-spirits-2012-filing-COMBINED-11-07-22-compressed.pdf, CAP 4C0299 at https://blogs.edf.org/health/files/2023/02/CAP-4C0299-Mica-based-pearlescent-pigments-alcoholic-and-non-alcoholic-beverages-COMBINED-11-07-22.pdf, CAP 5C0301 at https://blogs.edf.org/health/files/2023/02/CAP-5C0301-Mica-based-pearlescent-pigments-egg-shells-COMBINED-11-07-22.pdf, and CAP 5C0302 at https://blogs.edf.org/health/files/2023/02/CAP-5C0302-Mica-based-pearlescent-pigments-distilled-spirits-COMBINED-11-07-22.pdf.
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[33] American Cyanamid Company, Investigations of the Possible Absorption of Titanium Dioxide from the Gastrointestinal Tract, 1963, page 130 of 391 in the PDF of FDA's response to a Freedom of Information Act

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[34] American Cyanamid Company, Investigations of the Possible Absorption of Titanium Dioxide from the Gastrointestinal Tract, 1963. See page 130 of 393 in FDA FOIA response.
[35] EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[36] *Id.*
[37] *Id.*
[38] *Id.*
[39] Technical limitations prohibited the EFSA panel from assessing of developmental immunotoxicity of E 171. EFSA Panel on Color additives and Flavourings, Safety assessment of titanium dioxide (E71) as a color additive, May 6, 2021, https://doi.org/10.2903/j.efsa.2021.6585.
[40] There were no neurotoxicity studies using E 171.
[41] EFSA Expert Panel found "no effects on reproductive and developmental toxicity up to a dose of 1,000 mg/kg bw per day, the highest dose tested, were observed in the Extended One Generation Reproductive Toxicity Study with E 171. No other reliable studies were found in the literature addressing these effects with E 171."
[42] Among the studies EFSA found insufficient for assessing carcinogenicity of TiO2 was a National Cancer Institute study of the cancer risks of TiO2 (National Toxicology Program. Bioassay of titanium dioxide for possible carcinogenicity. Natl Cancer Inst Carcinog Tech Rep Ser. 1979;97:1-123. PMID: 12806394. https://pubmed.ncbi.nlm.nih.gov/12806394/). That study did not consider the impact of particle size, hence why EFSA found it insufficient and why it is not directly relevant to this petition.
[43] *Id.*
[44] *Id.*
[45] Court of Justice of the European Union, The General Court annuls the Commission Delegated Regulation of 2019 so far as it concerns the harmonised classification and labelling of titanium dioxide as a carcinogenic substance by inhalation in certain powder forms, Press Release of Judgment of the General Court in Joined Cases T-279/20, T-288/20 and T-283/20 | CWS Powder Coatings and Others v Commission, November 2022 at https://curia.europa.eu/jcms/upload/docs/application/pdf/2022-11/cp220190en.pdf.
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[49] 21 C.F.R. § 70.3(i).
[50] 21 U.S.C. § 379e(b)(5)(A).
[51] 21 C.F.R. § 71.1.
[52] 21 U.S.C. § 379e(b)).
[53] 21 C.F.R. § 73.575.
[54] 21 C.F.R. § 71.1(c)(H).
[55] Guidance for Industry: Color Additive Petitions
[56] FDA, Substances Added to Food (*formerly EAFUS*), accessed on August 15, 2022, at https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&id=TITANIUMDIOXIDE.
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[62] EFSA Panel, Abstract.

[63] *Id.*
[64] EFSA Panel, 2021 at page 21.
[65] EFSA Panel, 2021 at page 4.
[66] EFSA Panel, 2021 at page 21.
[67] EFSA Panel, 2021 at pages 28-29.
[68] EFSA Panel, 2021 at both paragraphs on page.28.
[69] EFSA Panel, 2021 at page 27.
[70] EFSA Panel, 2021 at page 28.
[71] EFSA Panel, 2021 at page 26.
[72] Bettini S et al, 2017. Food-grade TiO2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. Scientific Reports, 7, 40373.
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[74] EFSA Panel, 2021.
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[76] EFSA Panel, 2021 at page 62.
[77] *Id.*
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[79] EFSA Panel, 2021 at page 74.
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[81] EFSA Panel, 2021 at page 30.
[82] EFSA Panel, 2021 at page 25.
[83] https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2021.6585&file=efs26585-sup-0001-Appendix_A.pdf.
[84] https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6585.
[85] 21 C.F.R. § 73.575.
[86] *Id.*
[87] *Id.*