EFSA Re-Assessment of Titanium Dioxide (E 171) , 2021

In this guide

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This is a paper for discussion.

This does not represent the views of the Committee and should not be cited.

50. The following section of this paper discusses the EFSA re-evaluation. It briefly addresses the data considered by EFSA and presents the main conclusions. The underlying data on the endpoints of toxicokinetics and absorption, developmental and reproductive toxicity and aberrant crypt foci are further discussed in detail in paragraphs 81 onwards to allow the COT to independently assess them. While a comment has been included on the conclusions around genotoxicity, these will not be considered further in this paper.

51. Concerning absorption and toxicity of TiO2 particles that are present in E 171, the Panel concluded that:

- The absorption of TiO2 particles is low but they may accumulate in the body due to their long half-life.
- No studies appropriately designed and conducted to investigate the potential carcinogenicity of TiO2 nanoparticles were available.

Data & Methodology of the EFSA 2021 Opinion

52. The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA Scientific Committee, 2009), and relevant existing Guidance from the EFSA Scientific Committee, including the Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health (EFSA Scientific Committee, 2018a).

53. The 2021 EFSA evaluation is based on the following data:

- Information from publications retrieved in the literature search (see Annex B for criteria).
- Data submitted in response to the call for data from European Commission as follow-up of there-evaluation of E 171.
- Toxicokinetic studies considered in the re-evaluation of titanium dioxide (E 171).
- Exposure data available in the re-evaluation 2016 and additional relevant information published since that time.
- In-vitro and in-vivo studies reported in the OECD dossier (2016) and submitted to EFSA.

54. Food consumption data used to estimate the dietary exposure to titanium dioxide (E 171) were derived from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database 1). Dietary data from the UK were included in the EFSA Comprehensive European Food Consumption Database for the period in which UK was a member of the European Union.

55. The Mintel’s Global New Products Database (GNPD) was used to verify the use of titanium dioxide (E 171) in food and beverage products and food supplements within the EU’s food market. The Mintel’s GNPD is an online database that contains the compulsory ingredient information present on the label of numerous products.

56. With regards to toxicity, a literature search was performed following the approach and information on the criteria for inclusion and exclusion of publications based on information from the abstract and title, and material used in the study is described in Annex B. Toxicokinetic and toxicity studies considered 'included' were assessed for their relevance and reliability. The Panel further assessed the EOGRTS data submitted by industry, which also included an endpoint investigating aberrant crypt foci induction, following the Panel's recommendations in the 2016 evaluation.

57. Nanoscale considerations for the assessment of the study design and study results in toxicity studies classified with reliability 1 and 2 (see Annex B for criteria).

Dietary Exposure Data

58. Dietary exposure to E 171 from its use as a food additive was estimated combining the food consumption data available within the Comprehensive Database with reported use levels submitted to the EFSA ANS Panel (2016) and information extracted from a report of the Netherlands National Institute for Public Health and the Environment (RIVM) (Sprong et al., 2015). The exposure was estimated according to different exposure scenarios (EFSA ANS Panel, 2017). Uncertainties in the exposure assessment were identified and discussed. The current paper does not expand on this information due to the fact that, because of the Panel conclusions on genotoxicity, the exposure information was not further considered in the risk assessment.

Toxicity

59. With regard to the genotoxicity studies, combining the available lines of evidence, the FAF Panel concluded that "TiO2 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 TiO2 particles – such as crystalline form, size of constituent particles, shape and 7 agglomeration state – and the outcome of in vitro or in vivo genotoxicity assays" (i.e a cut-off value for TiO2 particle size with respect to genotoxicity could not be identified). The Panel also concluded that "several modes of action (MOA) may operate in parallel and the relative contributions of the different molecular mechanisms resulting in the genotoxicity of TiO2 particles are unknown. Based on the available data, no conclusion could be drawn as to whether the genotoxicity of TiO2 particles is mediated by a mode (s) of action with a threshold(s)".

Therefore, the Panel concluded that a concern for genotoxicity of TiO2 particles cannot be ruled out. The underlying data on genotoxicity is currently being reviewed by the COM, as part of the UK independent review of the safety of TiO2.

60. With regards to other endpoints the FAF Panel concluded “that the absorption of TiO2 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 E171, did not indicate adverse effects up to a dose of 1,000 mg/kg bw per day. In addition, no effects were seen in literature studies employing TiO2 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 E171. No other reliable studies were found in the literature addressing these effects with E171; some findings regarding immunotoxicity and inflammation with E171 as well as neurotoxicity with TiO2 NPs may be indicative of adverse effects. They also considered that there are indications of the induction of aberrant crypt foci in the small intestine with E171 and that no studies appropriately designed and conducted to investigate the potential carcinogenicity of TiO2 nanoparticles were available.”

Uncertainty

61. The Panel identified uncertainties related to the following points:

- The size distribution of the particles in marketed E171 that consumers are exposed to, related to the different types of E171, as presented in the EFSA ANS Panel (2019) opinion.
- The processes used by industry when using E171 in food and to what extent these processes may affect the degree of agglomeration and thus internal exposure.
- The state of agglomeration i.e. presence of ‘free’ (non-agglomerated) particles of tested material in gastrointestinal tract of the animals and its effect on absorption.
- The representativity of different tested materials used in toxicity studies for the food additive E171 when used in food.
- Differences in the physico-chemical properties of the different tested materials and the extent of their impact on the observed results.
- Interference in the measurements of Ti/TiO2 in blood, tissues or organs with the most widely used analytical technique, i.e. ICP-MS, and its impact on the reliability of tissue concentration data.

- Confidence in the limited kinetic data as the basis for estimating half-lives and accumulation and for assessment of internal exposure and, related to that, the extent of systemic availability based on the proposed amendment for EU specifications of titanium dioxide.
- None of the rodent studies were sufficiently long to cover the time needed for reaching the steady state for accumulation and this impacted the interpretation of the study results.

62. The Panel identified uncertainties regarding the EOGRT study with respect to its validity to fully identify all potential adverse effects of E 171 when used as a food additive:

- The extent to which the particle size distribution of the E 171 used in the EOGRT study is reflective of the particle size distributions of E 171 when added to foods.
- The extent to which the particle size distribution of E 171 in transit through the gastrointestinal tract in the EOGRT study was affected by the concentration in the diet (i.e. dose).The selected test material was representative of E 171 containing a large proportion (around 50% by number) of constituent particles below 100 nm (E 171 sample E reported in EFSA FAF Panel, 2019).The particle size distribution of the E 171 in samples of the test diet was also analysed after applying a sample dispersion protocol that aims to extract E 171 particles from the feed matrix and the results show that the particle size distribution of the constituent Safety assessment of the food additive titanium dioxide (E 171) was similar to that of pristine E 171 after dispersion (EFSA FAF Panel, 2019; Verleysen et al.,2020). However, neither of these procedures were considered by the Panel to reliably determine the particle size distribution of E 171 in the feed.

63. The Panel acknowledged that the methods for determining particle size distributions in complex foods and feeds in-situ are not currently available. Accordingly, the Panel considers that the extent to which the particle size distribution of the E 171 used in the EOGRT study is sufficiently reflective of the particle size distributions of E 171 when added to foods remains uncertain. The interested business operator considered that mixing of two dry components (feed and E 171) was the best possible option to retain the particle size distribution properties of the original E 171 sample, and that the use of liquid dispersion would add further superfluous unknowns.

64. The Panel considered that E 171 has a broad size distribution of constituent particles (from about 40 to 250 nm); considered that in dry form, this

size distribution of the constituent particles is expected to be stable and further, that homogenous mixing of E 171 with dry diet is a pragmatic approach to adopt in terms of performing an animal study over an extended time frame such as the EOGRT study. The Panel considered this approach to be representative of some uses of E 171 in food (e.g. E 171 in confectionary coatings and fillings and in ready to use sauces. However, the Panel also noted that this approach may not be fully representative for all uses of E 171 in food since liquid dispersion of E 171 was reported to be used, potentially along with additional processes, to reduce the formation of agglomerates in suspension in some products (e.g. incorporation of E 171 into a tablet coating or capsule.

65. The Panel considered that investigations of TiO2 levels in tissues would have reduced uncertainty regarding dose dependency of internal exposure. However, the Panel noted that the EOGRT study demonstrated unequivocally low levels of internal exposure to TiO2 in animals that were fed a diet prepared by addition of E 171 to dry feed. Dispersed Nanoparticles show a greater tendency to agglomerate when suspended in liquid media at higher concentrations. This concentration effect on agglomeration and/or resistance to de-agglomeration may also exist in the gastro-intestinal tract at high-dose levels The Panel therefore considered that there remains an uncertainty regarding the effects of dose levels/concentrations in feed and the extent to which agglomeration occurred in the gastrointestinal tract. However, the Panel considered the propensity for this agglomeration is likely reduced when exposure is via feed rather than through bolus gavage administration of E 171

Conclusion:

66. Considering all currently available evidence and uncertainties, the Panel concluded that E 171 can no longer be considered as safe when used as a food additive due to genotoxicity considerations. This applies to E 171 as described in Commission Regulation (EU) No 231/2012 and E 171 specified in the EFSA Food Additive and Flavourings Panel opinion in 2019.

COT comments on the 2021 EFSA opinion

67. The COT considered the EFSA Opinion on titanium dioxide at their July 2021 meeting. The Committee considered a summary of the EFSA opinion as well as the preliminary comments from the COM meeting; these are noted in the introduction and not considered further in this paper.

68. The COT also noted that in several parts of the Opinion, published papers were presented at face value, and there was no discussion of the results nor the overall Weight of Evidence to support the conclusions being made. They furthermore noted discrepancies and conflicts between the results of the studies reported and the overall conclusions.

69. Overall, the COT considered that there was a lack of internal consistency and of objective weighing of the evidence. While some of this might have been due to differences in the nature of the TiO2 tested, this was not clear in the Opinion.

70. Members also noted that it was difficult to draw any conclusions from the studies and a closer look in terms of material characterisation was needed in order to understand some of the effects reported. Members also considered that follow up was needed on the reproductive toxicity study as only the presence or absence of an effect was measured.

71. The large variation in the specifications of E 171 was also discussed based on the analytical data for pristine E 171 that indicated that more than 50% of the constituents were in the nano-range so the COT considered that more clarification was needed on the actual composition of E 171. It was noted that the EFSA definition of nanomaterials lacked clarity with regard to materials that were not engineered as nanomaterials but contained particles in the nano range. The possibility and plausibility of removing the nano fraction from E 171 in order to mitigate the risk was also discussed by the COT.

72. With regard to absorption, it was noted that there was no reason to believe that titanium dioxide particles behaved differently to other particles in the gastrointestinal tract.

73. Members were advised that newer studies used in the previous evaluation were re-considered (evidence from deceased humans and indications that titanium dioxide could cross the placenta). The duration of the animal studies was not sufficient to evaluate at which levels steady state would be reached and therefore it was considered that absorption had previously been underestimated.

74. The extended one generation reproductive toxicity (EOGRT) study provided indirect evidence for systemic exposure following administration of titanium dioxide. Members were informed that EFSA had indications that when used by industry, E171 was dispersed into nanoparticles by sonication and therefore also considered data on materials made solely of nanoparticles for the

assessment. However, this was questioned by Members as it was noted that pure nano titanium dioxide would lose its technical function in the food (as it would not provide colour) and would therefore not be of use.

75. The COT also questioned the conclusions with regards to the ability of TiO2 to induce aberrant crypt foci. On this point, the Committee were advised that because of the above consideration by EFSA, only one study that used sonication of the material was considered, as the material tested was undispersed in the other available studies.

76. The findings of the studies on neurotoxicity were considered inconsistent by the COT. It was noted that the EOGRT study did not report any effects and that most of the other studies on this endpoint were of nanomaterials.

77. In the EFSA evaluation, the issue of the test material in the EOGRT not being dispersed was taken into consideration with regards to the conclusions on this endpoint, as they considered that had it been dispersed and stabilised in the nano form some effects could possibly have been observed. The COT, as previously, questioned the relevance of such dispersion to real world use. Members noted that the histopathology tests performed for the EOGRT study were standard and were not sensitive enough in comparison to other studies on this endpoint that performed specific neuro-histopathology testing.

78. On balance, the Committee considered that the weight of evidence did not support the conclusions drawn by EFSA. The COT also agreed with the comments of the COM with regards to risk communication that “As it stands the conclusion is highly risk adverse based on the weak evidence available, and it might create unnecessary concern to the public.” They considered that care should be taken when expressing the conclusions as they might cause unnecessary concern and they were uncomfortable with EFSA’s binary communication on a dataset with a lot of uncertainties. They highlighted that the COT does not follow the precautionary approach.

79. When considering whether they agreed with EFSA’s conclusion that no differentiation could be made with regards to size/form of titanium dioxide and different aspects of toxicity, the COT erred towards the view that nanoparticles were driving the toxicity. It was decided that an interim position paper, capturing the COT’s view and the proposed next steps should be published.

Detailed breakdown on studies considered by EFSA

80. This section expands on the underlying data of toxicokinetics and absorption, the EOGRT study and ACF. For context, the EFSA Panel conclusions have been included in the relevant sections, for the COT's information.

Toxicokinetics and Absorption

81. The toxicokinetics of E 171 was addressed in five studies in total, three studies in mice and in two studies in humans.

E171 studies in mice

Talamini et al (2019)

82. The test material used was: E171 (food grade titanium dioxide), 99.3% pure anatase, 35% nanoparticles.

83. Groups of 8 week old male NRF mice (n=22/group) were treated with 5 mg/kg bw of E171 dispersed in water (no sonication or deagglomeration). The animals were treated for 3 days/week for 3 weeks receiving a total of 9 treatments in 21 days. The average daily dose of 2 mg/kg bw. The actual treatment concentration was verified by inductively coupled plasma mass spectrometry (ICP-MS). The test material: E171 or water (control) was slowly dripped with pipette in the mouths of the mice, allowing for each drop to be swallowed.

84. The animals were weighed at the beginning of the experiment and observed daily. No signs of general toxicity were observed. On day 21, the animals were sacrificed and the lungs, liver, stomach, spleen. Kidney, brain, testes and whole intestine were removed. The concentrations of titanium were determined in 4 animals.

85. In the brain, kidney and testes, titanium levels were 0.03 µg/g, the quantification limit of the analytical method for solid tissue samples. In lungs and spleen the levels were low, with a not statistically significant, but slightly higher deposition in spleen of E171 treated animals compared to the controls. The authors reported that "Titanium concentration was one order of magnitude greater in the small intestine compared to the above tissues and distinctly higher in the stomach, large intestine and liver". The concentrations of titanium in treated animals were: 1.07 ± 0.38 µg Ti/g tissue in the large intestine and 0.94 ± 0.57 µg Ti/g tissue in the liver. These levels were 1.8 and 3.6 times higher compared to the controls, respectively.

Comera et al (2020)

86. The test material used was: E 171, > 95% anatase, 20–340 nm (transmission electron microscopy- TEM); 44.7% nanoparticles.

87. In the first round of experiments adult C57BL/6 mice (12–18 weeks) were treated with a single gavage dose of 40 mg/kg of E171 suspended in water and sonicated or water. In the second round of experiments, 300µg/mL of E171 was suspended in buffer and used to fill a closed mid- jejunal loop of 10cm, pre-treated with inhibitors of tight junctions, micropinocytosis, clathrin-mediated endocytosis or raft-dependent endocytosis.

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

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

90. An increase in the reflective particle content was observed in the jejunal and ileal villi, Peyer’s Patches and colon crypts. The overall particle content in jejunal villi increased from 2 hours after gavage, peaked at 4 hours, and returned to basal values at 8 hours. A statistically significant increase (p 0.001) 4 hours after E171 administration was observed in the titanium dioxide particle density in the jejunal mucosa (increased by 3.4-fold over the controls). A lower and non-significant trend of increased particle content was also observed at 4 hours in the ileum and colon, with the values decreasing close to control levels at the time 8 hours in all three intestinal sections. In the jejunum, the reflective TiO2 particle spots displayed a mean diameter of 700 ± 59 nm (n = 70) and were mostly observed in the lamina propria and in goblet cells (GCs) distributed in the epithelium, with some of them also found in enterocytes lining the gut lumen. Analysis by transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy (TEM-EDX) indicated the presence of both Ti and O in particles

detected in jejunal GCs and enterocytes. These appeared as primary particles or aggregates with respective sizes of 450 and 170 nm. . In the Peyer's patches, a statistically significant increase in laser-reflecting particles was found only at 8 hours (increased by 5.4-fold over that of controls (p 0.001)). In blood, the number of particles significantly increased by 3.5- and 4.1-fold at 4 and 8 hours, respectively, but the titanium concentrations remained below the limit of detection (LOD0.02 ng Ti/kg) at all timepoints. From the content in the intestines and the weight of the mice tissues, the authors calculated that approximately 0.007% of the titanium administered was present in the entire intestine at the 4 hours timepoint. The authors concluded that titanium dioxide was absorbed predominantly in the ileum, partly in jejunum and that small amount absorbed in the colon. Based on the surface area information it was concluded that titanium dioxide is predominantly absorbed by the small intestinal villi and to a lesser extent through Peyer's patches.

91. In the *ex vivo* experiment, under anaesthesia, a closed mid-jejunal loop (10cm) was isolated and pre-treated with either just PBS (control) or a PBS solution with inhibitors as described above for 30 minutes. The contents were then rinsed and replaced with PBS (control) or sonicated E171 and incubated for a further 30 minutes. A significant inhibition of TiO2 absorption (by 66%) was observed and the authors considered that the paracellular route is a major pathway governing transepithelial TiO2 passage. However, as the absorption was not totally blocked by paracellular pathway inhibitors, the authors concluded that besides a paracellular pathway, endocytosis could also be involved in the transport of titanium dioxide from the intestinal lumen to blood.

Riedle et al., (2020)

92. The test material used was: E171, anatase, 119nm (EFSA 2021).

93. C57BL/6 mice were treated with titanium dioxide doses of 0, 6.25, 62.5 and 625 mg/kg of diet. These were equivalent to 0 and approximately equal to 1, 10 and 100 mg E 171/kg bw per day.

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

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

E171 studies in humans

Pele *et al.,* (2015)

96. The test material used was: E171, anatase, d50=250nm (EFSA, 2021)

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

98. Baseline blood samples were also taken at 9 am. Following that, the subjects received two tablets containing 50 mg of E171 (total dose 100 mg). Blood samples were collected at: 30 minutes, 1, 1.5, 2, 3, 6, 8 and 10 hours post E171 ingestion. Of the 8 volunteers, only 7 completed the study as blood could not be withdrawn from the cannula of 1 subject.

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

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

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

Guillard *et al.,* (2020)

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

103. Human placentae and meconium were collected at term from normal pregnancies. The samples were analysed using ICP-MS and scanning transmission electron microscopy (STEM) coupled to EDX spectroscopy for content analysis of titanium and analysis of titanium dioxide particle deposition, respectively. Transplacental passage of titanium dioxide was determined using an *ex vivo* placental transfusion model.

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

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

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

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

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

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

Studies on Titanium dioxide other than E171, or with titanium dioxide nanoparticles

Rats

Disdier *et al.,* (2015)

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

110. Adult Fischer rats were treated with 1 mg/kg titanium dioxide or saline buffer (control); no dispersion protocol was applied for the *in* vivo experiment. The dose was administered intravenously and samples were taken at 30 minutes, 1, 2,6 and 24 hours and 7, 28, 90 and 365 days post treatment from blood, liver, brain, spleen, kidney and lungs. Blood and brain samples were additionally collected at 5 and 15 minutes post injection. Titanium concentrations were determined by ICP-MS.

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

authors estimated that they recovered approximately 44 % of the administered dose in the liver, 10 % in lungs and 2 % in spleen 6 hours post administration.

Kreyling *et al.,* (2017a)

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

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

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

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

Kreylig *et al.,*(2017b)

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

117. Titanium concentrations were determined at five retention time points 1 h, 4 h, 24 h, 7 d and 28 d after gavage in four rats for each time point. However,

after observing in the seven-day experiment that fecal excretion of the test item was complete after 4–5 days, no further animals were sacrificed for a 28-day biodistribution.

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

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

Geraets *et al.*, (2014)

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

121. The test item used was: NM-100, NM101, NM-102, NM-103 and NM-104

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

54.5- 59.9 mg/kg bw for female rats.

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

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

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

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

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

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

Tassinari *et al.*, (2014)

129. The test material used was titanium dioxide nanoparticles (anatase, primary size 25nm, BET surface area 45–55 m^2 /g, purity 99%).

130. Male and female Sprague-Dawley rats (7 rats/sex/dose) were treated with 0, 1, 2 mg/kg body weight (bw) per day by gavage. The controls received distilled water.

131. Twenty-four hours after the last treatment (day 6), male and female rats were anaesthetised and blood samples were. Subsequently, the animals were sacrificed and the uterus, ovary, testes, thyroid and adrenals were excised and weighted. Spleen was sampled both for histopathological examination and for studying tissue deposition of Titanium dioxide nanoparticles.

132. The titanium content in the samples was determined by ICP-MS using an Elan DRC II spectrometer. The highest titanium concentration was detected in the thyroid, however the levels was no statistical significance between treated animals and controls (higher dose, 2 mg/kg bw per day and at 0.24 ± 0.09 vs. 0.22 ± 0.04 mg/g in controls). A significant increase was observed at the higher dose in the ovary (0.28 ± 0.07 vs. 0.12 ± 0.04 mg/g fresh weight). Levels in uteri were low (0.051 ± 0.006 vs. 0.49 ± 0.04 mg/g fresh weight). A significant increase in the concentration of titanium in the spleen concentration was observed at a dose of 2 mg/kg bw per day.

134. Overall, the authors considered that these results indicated the potential for titanium dioxide bioaccumulation.

Hendrickson *et al*., (2016)

134. The test material used was: NM-101 (5-10nm) and NP-25 (20-25nm).

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

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

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

138. In NP-25 treated animals accumulation of titanium dioxide nanoparticles were detected in the small intestines and liver (0.29 µg/g in the liver) and at low levels (0.01 µg/g) in the kidneys. In the spleen, it was detected at levels of 0.29 µg/g of the organ. No titanium dioxide nanoparticles were detected in the lungs, brain, testicles, heart or blood.

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

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

Ammendolia *et al.*,2017

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

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

143. A day (24 hours) after the last dose, the animals were sacrificed and the small intestine was excised. A piece of jejunum was used for histological analysis and the remaining part of small intestine was sampled for studying either tissue accumulation of titanium dioxide nanoparticles, determined as titanium by

ICP-MS.

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

Hendrickson *et al.,* 2020

145. The test material used was titanium dioxide nanoparticles, rutile, rod/ needle like shape, 5930 nm.

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

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

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

Chen *et al.,*2020a

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

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

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

152. Significantly increased titanium dioxide nanoparticle levels were only detected in the colon of rats exposed to 50 mg/kg test material, compared with the control group. There was no dose-response relationship, however. The authors hypothesised that the significant increase of titanium dioxide in colon tissue was due to the titanium dioxide nanoparticles attaching on the surface of the colonic mucosa tissue and not in mucosa cells. As most of orally ingested test material was excreted through feces, it resulted to long-term retention in large intestine. The titanium dioxide nanoparticles did not enter the colon epithelial cells and were mainly deposited in the intestinal cavity or between villi. The content of Ti in all tissues was very low, which was approximately 0.0001%-0.00001% or 100-1000 ng/g tissue, except for the colon of the high dose group. All spleen and heart tissue samples from rats contained very low titanium levels, which were below the limit of detection (LOD) of 0.032 µg/g. Finally, they concluded that the results indicate that the absorption and distribution of titanium dioxide nanoparticles was very low after low-dose and long-term oral administration.

153. The EFSA evaluation also cites a second study by Chen *et al¸(2020*b*)*, however it has not been described in the current paper as, following evaluation by the Secretariat it appears to investigate effects on lipid metabolism rather than addressing toxicokinetics/absorption.

Human studies

Heringa *et al* (2018)

154. Titanium was measured using high resolution ICP-MS in liver and spleen from 15 deceased human subjects (nine women and six men) who had donated their bodies for research and educational purposes. The LOD of the method was 10 ng/g tissue.

155. TiO2 particles were detected in 7/15 liver and 13/15 spleen samples. The number-based TiO2 particle size distributions in liver and spleen were comparable and had a size range of 85–550nm and 85–720nm, respectively. In the tissues, 24% of the TiO2 particles in the number-based size distribution was 100 nm, but this fraction may be underestimated considering that the smallest titanium dioxide particle that could be detected with the method used was 85 nm.

156. The particle mass concentration in liver ranged from 0.01 to 0.3 mg titanium/kg tissue. In the spleen, the concentration ranged from 0.01 to 0.4 mg titanium/kg tissue. The average concentration in samples where titanium could be

determined was 40 ng/g in the liver and 80 ng/g in the spleen.

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

Peters *et al* (2020)

158. Post-mortem human liver, spleen, kidney, jejunum and ileum samples were analyzed from 15 human subjects, 7 male and 8 female, who died at the age of 64–98 years. From these persons, written informed consent was obtained during life that allowed the use of their entire bodies for educational and research purposes.

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

160. The particle sizes were measured by spICP-MS and ranged between 50 and 500 nm in the different tissues (50 nm was the lower size detection limit). The titanium dioxide particle concentrations were considered by the authors to represent about 80% of the total titanium concentration.