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Evaluation of four new studies on the potential toxicity of titanium dioxide used as a food additive (E 171)

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Abstract

The European Commission requested EFSA to carry out a scientific evaluation on four studies on the potential toxicity of titanium dioxide (TiO₂) used as a food additive (E 171) and to indicate whether they would merit re-opening the existing opinion of EFSA on the safety of TiO₂ (E 171) as a food additive. The results of the Bettini et al. (2017) study did not provide enough justification for a new carcinogenicity study, but, should additional useful mechanistic information become available, this could be reconsidered in future. The new *in vitro* findings in the Proquin et al. (2017) study did not modify the conclusion on the genotoxicity of TiO₂ as stated in the previous EFSA opinion of 2016 on the safety of TiO₂ (E 171) as a food additive. The effects of engineered TiO₂ nanoparticles reported by the Guo et al. (2017) study were of uncertain biological significance and therefore of limited relevance for the risk assessment of the food additive TiO₂ (E 171). There was significant uncertainty in the risk assessment performed by Heringa et al. (2016), which did not include a weight of evidence analysis of the whole database. The Panel considered that the four studies evaluated, highlighted some concerns but with uncertainties, therefore their relevance for the risk assessment was considered limited and further research would be needed to decrease the level of uncertainties. Overall, three of the studies, reporting that TiO₂ induced various effects in *in vitro* and *in vivo* models, may be useful for hazard identification of TiO₂. In the fourth study by Heringa et al. (2016), numerous assumptions were made, which resulted in large uncertainty in their conclusion. Altogether, the Panel concluded that the outcome of the four studies did not merit re-opening the existing opinion of EFSA related to the safety of TiO₂ (E 171) as a food additive.

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Summary

The re-evaluation of titanium dioxide (TiO₂, E 171) for use as a food additive had been completed by the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient sources added to food (ANS) in June 2016, and a scientific opinion published in September 2016 (EFSA ANS Panel, 2016). In 2017, the European Commission (EC) launched a call for data on TiO₂ (E 171) inviting interested business operators to generate the additional data requested. In particular, data on particle size and particle size distribution for TiO₂ (E 171) would be submitted by June 2018, whereas a dietary Extended One-Generation Reproductive Toxicity Study with TiO₂ (E 171) would be generated by August 2019.

On 22 March 2018, the EC, in accordance with Article 29(1)(a) of Regulation (EC) No 178/2002, requested EFSA to provide a scientific opinion in relation to four new studies (Heringa et al., 2016; Bettini et al., 2017; Guo et al., 2017; Proquin et al., 2017), published after the scientific opinion in 2016, on the potential toxicity of TiO₂ used as a food additive (E 171). In particular, EFSA is requested to carry out a scientific evaluation of those studies and to indicate whether they would merit re-opening the existing opinion of EFSA related to the safety of TiO₂ (E 171) as a food additive.

The authors of these four publications were also invited to join the ANS plenary meeting open to observers on 16 May 2018 as hearing experts, in order to present an overview on their studies and to answer questions and to comment on their findings. The main points and outcome of that discussion have been considered in developing this document.

The Panel was aware of other publications that have been published after the publication of the scientific opinion on the re-evaluation of TiO₂ (E 171) as a food additive in 2016 (EFSA ANS Panel), but these were not taken into consideration in this evaluation, which is restricted to the four publications included in the EC request. Individual publications should be assessed in respect to the whole data set available, using a weight of evidence approach (EFSA Scientific Committee, 2017b). A proper evaluation of the safety of TiO₂ as a food additive would require considering all data available and not only selected data. The EFSA SC also provided Guidance on when the database should be re-evaluated in light of new evidence (EFSA Scientific Committee, 2017a).

The Panel has assessed the four publications as requested in the last mandate from the EC and made specific comments on each individual studies which have been considered in the context of the conclusions of the EFSA opinion of 2016.

Regarding the Bettini et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the TiO₂ test materials were prepared following the generic Nanogenotox dispersion protocol, which involved coating of the particles with bovine serum albumin (BSA) and ultrasonication. This protocol was designed to prevent agglomeration of the nanoparticles and was especially developed for the hazard identification of nanoparticles in suspension;
- the administration of TiO₂ (E 171) by gavage or drinking water is not fully representative of the use of the food additive E 171 in food. This could introduce some uncertainties in the extrapolation of the results of this study to the assessment of the food additive, because interactions with the food matrix may not occur;
- the effects observed on immunomodulation/inflammation were in line with those occurring in the very early steps of the development of an inflammatory/immunological reaction. Variations of different biomarkers were low (within 10–20% change), thus questioning the biological relevance of the results, also taking into account the lack of historical data for that kind of study;
- BSA coated on particles of TiO₂ (E 171) could itself trigger an immunologic and/or allergic reaction;
- although the model used to investigate the effects of TiO₂ (E 171) in the development of putative preneoplastic lesions in the colon was well described, the Panel noted some limitations, such as the use of a single marker;
- the presence of aberrant crypt foci (ACF) in the absence of an initiator is unusual, but in this study ACF were observed in the colon of a few animals treated with TiO₂ (E 171) alone;
- the initiation potential of TiO₂ reported in this study was not observed in another study using TiO₂ (E 171) (Urrutia-Ortega et al., 2016);

¹ <https://www.efsa.europa.eu/en/events/event/180515>

- a publication by Wijnands et al. (2004), showed that the number and size of ACF induced by different dietary compounds such as wheat bran, curcumin, rutin or benzyl isothiocyanate, were not predictive for the ultimate number of colorectal tumours. Wijnands et al. (2004) concluded that 'the number and size of ACF were not considered to be suitable as biomarkers for colorectal cancer', whereas the expression of some tumour-related genes (e.g. metalloproteinase 1; TIMP-1) 'correlated well with the effects of dietary compounds on the ultimate tumour yield'.

Overall, based on the data provided in the Bettini et al. publication, and the negative results of the carcinogenicity studies in mice and rats performed by NCI (1979), the Panel considered that the new findings were not sufficient to raise a concern on the potential initiation or promotion properties of TiO₂ (E 171) on colon carcinogenesis.

Regarding the Proquin et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- this *in vitro* study adds to a number of studies which provided some evidence of reactive oxygen species (ROS) formation and *in vitro* genotoxicity by microparticulate (MP) TiO₂ and TiO₂ nanoparticles (NP) in a variety of cell lines of human and rodent origin (as reported in EFSA ANS Panel, 2016). These authors reported for the first time genotoxic activity *in vitro* in a Comet assay and cytokinesis-block micronucleus assay using the food additive TiO₂ (E 171). The Panel noted that, in view of the composition of TiO₂ (E 171) (containing around 40% of nanoparticles by number), and of the available evidence of genotoxicity *in vitro* of both TiO₂ MP and TiO₂ NP, a genotoxic activity of E 171 under *in vitro* conditions could already be anticipated. In this respect, the Panel also noted that overall negative results were obtained in studies *in vivo* with both TiO₂ NP and MP (EFSA ANS Panel, 2016), indicating that TiO₂ MP and NP, and consequently E 171, did not raise concern for *in vivo* genotoxicity;
- two cell lines derived from human colon (adeno)carcinomas were selected as model systems to investigate the possible implication of ROS generation and genotoxicity in the promoting activity of TiO₂ (E 171) on chemically-induced colon carcinogenicity in mice reported in a previous study (Urrutia-Ortega et al., 2016). Based on the discussion at the technical hearing at the open Plenary, the Panel noted that this model appeared suitable for hazard characterisation but not for the purpose of risk assessment;
- undifferentiated Caco-2 cells, harvested after only 3 days of culture, were used for the assessment of DNA damage induced by TiO₂. As to the relevance of the cell system used for hazard characterisation, the Panel noted that another recent *in vitro* study highlighted a comparable lower incorporation of TiO₂ in differentiated compared to undifferentiated Caco-2 cells, and no induction of DNA damage in differentiated Caco-2 cells monolayers exposed to TiO₂ NP was observed (Vila et al., 2018). The Panel also noted that under *in vivo* conditions, the uptake of TiO₂ particles (including E 171) may be substantially decreased by the mucus layer of the intestinal epithelium (Garcia-Rodriguez et al., 2018);
- concerning the claimed colocalisation of TiO₂ (E 171) with 'kinetochore poles, mitotic genome and α -tubulin', the Panel noted that no clear conclusion can be drawn from the figures presented, which may be either indicative of a true colocalisation and functional interaction, or arise from the unspecific overlap of TiO₂ particles on cellular structures in the microscopic preparation.

Overall, the Panel considered that the results reported in the Proquin et al. (2017) study may be useful for an evaluation of the hazard of TiO₂ NP under the specific conditions of the study protocol, including the cell model used and the conditions of culture. However, the Panel also considered that the relevance of the results for risk assessment of the food additive E 171 has not been established.

During the discussion at the technical hearing at the open Plenary, one of the authors mentioned that consistent results appeared to be observed in mouse colon in ongoing *in vivo* transcriptomics studies. The Panel considered that these studies could be evaluated when completed, and if deemed necessary, the overall database reassessed considering the entire literature available at that time.

Regarding the Guo et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the study was performed with engineered TiO₂ NP and not with TiO₂ as a food additive;
- the TiO₂ NP suspension was sonicated to minimise agglomeration and/or aggregation which may not occur in more realistic environments;

- in addition to the transmission electron microscopy (TEM) analysis of primary particles, the dynamic light scattering (DLS) results suggested that the hydrodynamic sizes were considerably larger which suggested that the TiO₂ NP may be aggregated and agglomerated;
- the authors assumed that all ingested particles would be in contact with the wall surface but without considering the potential interaction of nanoparticles with luminal contents, e.g. their estimated number of nanoparticles was comparable with the number of microorganisms per gram of intestinal contents (ranges from 10⁵⁻⁶ in intestine to 10¹³ in colon);
- the glycocalyx on cultured Caco-2 cells is different from that on normal human intestinal cells;
- the cells were rinsed in phosphate-buffered saline (PBS) before exposing them to TiO₂ NP which could alter the glycocalyx and the interactions of Caco-2 cells with TiO₂ NP;
- changes in the expression of proinflammatory genes seen after 5-days of treatment with TiO₂ NP were small, not dose-related and uneven;
- no information was available about possible markers of inflammation such as production/secretion of cytokines, changes in the expression of membrane antigens;
- no transport of TiO₂ NP across the Caco-2/Ht29 MTX monolayers was reported;
- as more physiological conditions were approached the effects were attenuated, and it was difficult to extrapolate these results to the *in vivo* situation.

Regarding the Heringa et al. (2016) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the National Institute for Public Health and the Environment (RIVM) provided this and two other unpublished reports at the time of the re-evaluation of the TiO₂ (E 171) as a food additive in 2016 (EFSA ANS Panel, 2016);
- these unpublished studies did not affect the Panel's conclusions drawn from the available database at that time;
- Heringa et al. assumed that, although there was no information available on the absorption of TiO₂ particles, any toxicity was caused by TiO₂ NP;
- the conclusions of the authors that, because of uncertainties and assumptions in their analysis, further studies would be needed, for example, on the actual concentration of nano-TiO₂ in human organs and on the effects in liver and the reproductive system after chronic exposure to well-characterised TiO₂ NP;
- data on reproductive toxicity were scarce;
- the results on effects on testosterone levels in the two studies (Jia et al., 2014; Tassinari et al., 2014) used by Heringa et al. (2016) were contradictory;
- significant uncertainties were associated with extrapolation from short term to lifetime exposures;
- although the application of uncertainty factors for extrapolation from short-term studies is a policy choice, Heringa et al. used uncertainty factors from REACH rather than those recommended by the EFSA SC (EFSA Scientific Committee, 2012);
- in deriving acceptable Margin of internal Exposure (MoEi), the kinetic element of the inter- and intraspecies uncertainty factors was only removed from the interspecies but not from the intraspecies uncertainty factor;
- effects seen in short-term studies with no functional effect in longer term studies are usually not considered a robust basis for risk assessment.

Overall, the Panel considered that the aforementioned evaluations and considerations indicated that there was significant uncertainty in the assessment by Heringa et al. (2016) and noted that there was not a weight of evidence analysis of the whole database on E 171. The Panel considered that the assessment was consistent with a hazard from TiO₂ NP when dosed as in the selected studies but the relevance to nanoparticles in a food matrix could not be assessed. The Panel concluded that the additional studies called for in its 2016 opinion should provide a more robust basis for addressing the reported effects in reproductive organs in the studies used by Heringa et al. (2016).

Based on the evaluation of the four studies concerning the potential adverse health effects of titanium dioxide the Panel considered that:

- the results of the Bettini et al. (2017) study did not provide enough justification for a new carcinogenicity study, but, should additional useful mechanistic information become available, this could be reconsidered in future;

- the new *in vitro* findings in the study by Proquin et al. (2017) did not modify the conclusion on the genotoxicity of TiO₂ as stated in the previous EFSA opinion (EFSA ANS Panel, 2016) on the safety of TiO₂ (E 171) when used as a food additive;
- the effects of engineered TiO₂ nanoparticles reported by the Guo et al. (2017) study were of uncertain biological significance and therefore of limited relevance for the risk assessment of the food additive TiO₂ (E 171);
- there was significant uncertainty in the risk assessment performed by Heringa et al. (2016), which did not include a weight of evidence analysis of the whole database;
- the four studies evaluated, highlighted some concerns but with uncertainties, therefore their relevance for the risk assessment was considered limited and further research would be needed to decrease the level of uncertainties.

Overall, three of the studies assessed in this opinion reported that TiO₂ was able to induce various effects in *in vitro* and *in vivo* models. These studies may be useful for hazard identification of TiO₂. The Panel considered that the limited relevance of the protocols of these studies to the use of E 171 under realistic conditions in food, hampered the use of the data in the risk assessment of the food additive E 171. In the fourth study by Heringa et al. (2016), numerous assumptions were made, which resulted in large uncertainty in their conclusion.

More research exploring the possible effects observed in three of the four studies could address their applicability to the risk assessment of the food additive E 171 under realistic conditions of use.

Altogether, the Panel concluded that the outcome of the four studies did not merit re-opening the existing opinion of EFSA related to the safety of TiO₂ (E 171) as a food additive.

The Panel recommended that:

- in order to substantiate the observations in the Bettini et al. (2017), biomarkers for putative preneoplastic lesions in the colon as additional parameters should be examined in the extended one-generation reproductive toxicity study recommended by EFSA (EFSA ANS Panel, 2016);
- further studies on TiO₂ NP should include administration in a food matrix.

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1. Introduction

Upon request of the European Commission (EC), the EFSA Food Additive and Nutrient sources added to Food (ANS Panel) has assessed four new publications (Heringa et al., 2016; Bettini et al., 2017; Guo et al., 2017; Proquin et al., 2017) concerning the potential adverse health effects of titanium dioxide (TiO₂). The Panel is aware of other publications that have been published after the publication of the scientific opinion on the re-evaluation of TiO₂ (E 171) as a food additive in 2016 (EFSA ANS Panel), but these were not taken into consideration in this evaluation, which is restricted to the four publications included in the EC request.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

The use of food additives is regulated under the European Parliament and Council Regulation (EC) No 1333/2008 on food additives.² Only food additives that are included in the Union list, in particular in Annex II to that regulation, may be placed on the market and used in foods under the conditions of use specified therein. Moreover, food additives shall comply with the specifications as referred to in Article 14 of that Regulation and laid down in Commission Regulation (EU) No 231/2012³.

Titanium dioxide is authorised for use as a food additive (food colour) in the Union. Since titanium dioxide (E 171) was permitted in the Union before 20 January 2009, it belongs to the group of food additives which are subject to a new risk assessment by the European Food Safety Authority (EFSA), according to Commission Regulation (EU) No 257/2010⁴, and in line with the provisions of Regulation (EC) No 1333/2008.

The re-evaluation of titanium dioxide as a food additive (E 171) was completed by EFSA in June 2016 and a scientific opinion was published in September 2016 (EFSA ANS Panel, 2016). In that opinion, EFSA concluded, on the basis of the available evidence, that titanium dioxide (E 171) when used as a food additive does not raise a concern with respect to genotoxicity and that it is not carcinogenic after oral administration. However, several data gaps were also identified by EFSA in the opinion. These warranted a follow-up by the European Commission⁵ and new scientific evidence is being generated by interested parties in order to address the uncertainties highlighted by EFSA in its scientific opinion.

Nevertheless, authorised food additives should be kept under continuous observation and must be re-evaluated whenever necessary in the light of changing conditions of use and new scientific information.

Four new scientific articles (Heringa et al., 2016; Bettini et al., 2017; Guo et al., 2017; Proquin et al., 2017a) describing a potential adverse health effect of titanium dioxide used as a food additive (E 171) have recently been published, after the adoption and publication of EFSA's scientific opinion on the re-evaluation of titanium dioxide as a food additive (E 171) in 2016. Consequently, the European Commission has decided to consult EFSA on this matter.

1.1.2. Terms of Reference

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002⁶, the European Commission requests the European Food Safety Authority (EFSA) to provide a scientific opinion in relation to four new studies on the potential toxicity of titanium dioxide used as a food additive (E 171). In particular, EFSA is requested to carry out a scientific evaluation of those studies and to indicate whether they would merit re-opening the existing opinion of EFSA related to the safety of titanium dioxide (E 171) as a food additive.

2. Data and methodologies

2.1. Data

The data considered in this scientific opinion are those identified by the requestor of this mandate, which concerns the four publications identified in the background to this request:

² OJ L 354, 31.12.2008, p. 16.

³ OJ L 83, 22.3.2012, p. 1.

⁴ OJ L 80, 26.3.2010, p. 19.

⁵ https://ec.europa.eu/food/sites/food/files/safety/docs/fs-iron_titanium_dioxide-overview-deadlines-milestones-20170730.pdf

⁶ OJ L 31, 1.2.2002, p. 1.

- Bettini et al. (2017). Food-grade TiO₂ impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. *Sci Rep.* 2017, 7:40373.
- Proquin et al. (2017). Titanium dioxide food additive (E 171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions. *Mutagenesis*, 2017, 32, 139–149.
- Guo et al. (2017). Titanium dioxide nanoparticle ingestion alters nutrient absorption in an *in vitro* model of the small intestine. *NanoImpact*, 5, 70–82, 2017.
- Heringa et al., 2016. Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations. *Nanotoxicology* Vol. 10, Iss. 10, 2016.

When necessary for the interpretation of the results of the studies, also references cited in the publications above have been retrieved and assessed.

Additional information was provided by the authors of the four publications upon request of the Working Group (Appendices B, C, D and E).

The authors of the four publications above were invited to join the ANS plenary meeting open to observers on 16 May 2018 as hearing experts,¹ in order to present an overview on their studies and to answer questions and to comment on their findings. The main points and outcome of that discussion have been considered in developing this document.

2.2. Methodologies

In order to address this mandate, the approach described in the 'Scientific opinion on scientific motivations and criteria to consider updating EFSA scientific assessments' adopted by the EFSA Scientific Committee in 2017 (EFSA Scientific Committee, 2017a) was followed.

In particular, with respect to the approach followed for reaching conclusions on whether there is a need to update the previous ANS Panel scientific opinion, the new scientific evidence has been evaluated against the following criteria:

- Are the new data relevant to the safety of TiO₂ used as a food additive?
- Does the new scientific evidence address important data gaps previously identified?
- Are the new data likely to change the overall conclusions of the scientific opinion (EFSA ANS Panel, 2016), in a weight of evidence approach?

The screening of the new data has been carried out by a Working Group (WG) under the responsibility of the EFSA ANS Panel, which was involved in the original assessment.

2.2.1. Bettini et al. (2017). Food-grade TiO₂ impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon

In 2017, EFSA was informed that the French Food Safety Agency (ANSES) was asked to assess the publication by Bettini et al. (2017). EFSA collaborated with ANSES with respect to their ongoing assessment, following the provisions of Article 30(1) and (2) of the Regulation EC No 178/2002, in order to identify at an early stage any potential source of divergence between the conclusions of EFSA and ANSES opinions. In March 2017, a technical hearing was organised by ANSES with the authors of this study. ANSES experts and staff, EFSA staff, two experts from the ANS Panel and one expert from the EFSA FEEDAP Panel participated in this meeting and the joint scientific discussion that followed. The ANS Panel was informed about the outcome of those discussion and that the main conclusions of the ANSES WG of experts were going to be included in a scientific opinion of ANSES, which was published in April 2017 (ANSES, 2017; an English translation of the conclusions of the ANSES WG of experts is provided in Appendix A). The Panel agreed with ANSES that the publication by Bettini et al. (2017) would not justify reopening the EFSA opinion on TiO₂ (E 171) (EFSA ANS Panel, 2016).

Bettini et al. (2017) investigated the potential effects of titanium dioxide (TiO₂, E 171, containing 44.7% by number of nanoparticles as measured by transmission electron microscopy (TEM))⁷ on inflammation, genotoxicity and initiation/promotion of putative preneoplastic colon lesions in rats.

In a first set of toxicological studies, adult male Wistar rats (10 rats/group) were administered nano titanium dioxide (NM-105) (JRC, 2014), or TiO₂ (E 171) or vehicle (water), by gavage at a dose of 10 mg/kg body weight (bw) per day, for 7 days. TiO₂ test materials were prepared following the

⁷ One commercially available titanium dioxide food additive (E 171) provided from a French supplier of food colouring.

generic Nanogenotox dispersion protocol, which involved coating of the particles with bovine serum albumin (BSA) and ultrasonication.

In a second series of experiments, adult male Wistar rats (11–12 rats/group) were treated with or without 1,2-dimethylhydrazine (DMH) (180 mg/kg, one single intraperitoneal (i.p.) injection) to initiate colon carcinogenesis, and were then administered with TiO₂ (E 171) at 200 µg or 10 mg/kg bw per day in drinking water, for 100 days. Control animals (n = 12) received water only.

The dispersion state of TiO₂ particles was determined in the luminal content of the jejunum and colon (4 h after the administration of a single dose of TiO₂ (E 171)). The results indicated that TiO₂ particles did not re-agglomerate *in vivo* when transiting along the gastrointestinal tract. TiO₂ particles were found in the Peyer's Patches (PP) of the small intestine, colonic mucosa and liver of rats administered TiO₂ (E 171) by gavage for 7 days, but not in the controls. In the same treated rats, titanium was detected in the gut lumen, PP, colon mucosa and liver. A similar distribution pattern in PP (highest in the nuclei) was found in animals treated with NM-105 or TiO₂ (E 171). No significant changes in the gut permeability were found, suggesting that the particle absorption did not result from a dysfunction of the epithelial barrier integrity. No increase in DNA damage was found in PP of rats treated with TiO₂ (E 171) or NM-105.

A significant increase in dendritic cells (DC) frequency in PP was found after 7 days in TiO₂ (E 171) or NM-105 treated rats, but not after 100 days in rats exposed to TiO₂ (E 171) in drinking water, indicating that this effect was transient. Other regulatory T cells (i.e. Tregs, expressing the biomarkers CD4⁺, CD25⁺, FoxP3⁺) were decreased in animals treated with TiO₂ (E 171) for 7 or 100 days, and this was concomitant with a decrease in T helper (Th) cells (i.e. CD4⁺; CD25⁺). A decrease in interferon-γ (IFN-γ) secretion in the PP, and an increase in IFN-γ secretion and interleukin-17 (IL-17) level in the spleen, were reported in all treated animals.

In rats, i.p. injected with a single dose of 180 mg/kg bw DMH and treated with TiO₂ (E 171) at 10 mg/kg bw per day for 100 days in drinking water, the total number of aberrant crypts per colon as well as the number of large aberrant crypt foci (ACF) per colon (i.e. more than three aberrant crypts per ACF) was significantly increased compared to controls or the 200 µg/kg bw per day group. No ACF were observed in the colon of control rats (without initiation with DMH). However, in the TiO₂ (E 171) group without initiation with DMH, 4 out of 11 animals spontaneously developed one to three ACF per colon, and 3 out of 4 of these rats developed lesions of 1–3 aberrant crypts per ACF, whereas one rat developed one ACF consisting of 12 aberrant crypts. In the colonic mucosa of the treated animals with TiO₂ (E 171), a significant increase of tumour necrosis factor-α (TNF-α), IL-8 and IL-10 was also observed compared to the controls. There was no evidence of inflammasome activation in the mucosa.

Altogether, according to the authors, 'food-grade TiO₂ particles cross the gut barrier and reach the liver without altering intestinal permeability or causing DNA damage in Peyer's patches'; 'food-grade TiO₂ particles affect dendritic cell frequencies and T cell populations in the Peyer's patches and cause imbalances in intestinal and systemic immune responses'; and 'food-grade TiO₂ particles initiate and promote preneoplastic lesion formation in the colon and induce mucosal low-grade inflammation'.

2.2.2. Proquin et al. (2017). Titanium dioxide food additive (E 171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions

Proquin et al. (2017) evaluated cytotoxicity, reactive oxygen species (ROS) production and *in vitro* genotoxicity of TiO₂ in two human colon cancer cells lines (Caco-2 and/or HCT116) using three different materials: TiO₂ (E 171, containing 39% nanoparticles by number; analysed by scanning electron microscopy (SEM)),⁸ microparticulate TiO₂ (MP, average size 535 nm, analysed by TEM) and TiO₂ nanoparticles (NP, 10–30 nm, analysed by SEM). For the experiments, particles were dispersed at a concentration of 1 mg/mL in Dulbecco's modified Eagle's medium (DMEM) with 0.05% BSA, Hank's balanced salt solution (HBSS), or McCoy medium with 10% fetal bovine serum (FBS) and sonicated 30 min. Characterisation of hydrodynamic size distribution and zeta potential by dynamic light scattering (DLS) showed that serum, and to a lower extent BSA, reduced the agglomeration of particles after sonication, improving the dispersion and stability of suspensions. Cytotoxicity was evaluated in Caco-2 cells by the trypan blue viability assay after 24 h exposure to 1–1,000 µg/mL (equivalent to 0.143–143 µg/cm²) of TiO₂ (all three materials) dispersed in DMEM plus 0.05% BSA. Cytotoxicity was observed at 1,000 µg/mL with all three materials and at 100 µg/mL with TiO₂ (E 171) only; the two lowest doses

⁸ Sensient Technologies company (Mexico).

(1 and 10 $\mu\text{g/mL}$) were non-cytotoxic and were selected for further experiments with Caco-2 cells. The capacity of TiO_2 (all three materials) to generate ROS was investigated by electron spin resonance (ESR)/paramagnetic resonance spectroscopy under cell-free conditions and in Caco-2 cells. In cell-free experiments, both TiO_2 (E 171) (at 1,000 $\mu\text{g/mL}$) and TiO_2 NP (at 100 and 1,000 $\mu\text{g/mL}$) generated ROS when suspended in HBSS, but ROS generation was inhibited in medium plus 0.05% BSA, suggesting a scavenging or inhibitory effect by the protein corona. Differently from what observed in cell-free conditions, only TiO_2 MP (1–10 $\mu\text{g/mL}$) significantly increased intracellular ROS levels in Caco-2 cells. Exposure of Caco-2 cells to TiO_2 (E 171), as well as to TiO_2 NP and TiO_2 MP (24 h, 1–10 $\mu\text{g/mL}$) induced significant, although not dose-related, increases of DNA damage in comet assays. The potential of TiO_2 (E 171) to affect chromosome integrity and/or segregation was evaluated with the cytokinesis-block micronucleus assay in HCT116 cells, which show a more stable karyotype compared to Caco-2 cells and are more suitable for micronucleus analysis. Cells were exposed for 24 h to 50, 100 and 500 $\mu\text{g TiO}_2$ (E 171)/mL: no cytotoxicity was observed in treated cultures, which showed a statistically significant and dose-related increases of micronucleated cells (up to threefold over negative control). Finally, the capacity of TiO_2 (E 171) to interfere with mitotic chromosome segregation in HCT116 cells was preliminary investigated by the immunostaining of HCT116 cells with a mouse monoclonal anti- α -tubulin antibody. According to the study authors, TiO_2 (E 171) 'seemed to interact with the centromere region of kinetochore poles during mitosis', and 'appear to co-localize with α -tubulin', suggesting an interaction with chromosomes or the mitotic apparatus.

The authors of the study concluded that this was the first demonstration of the induction of ROS formation and DNA damage in cultured colon cells by TiO_2 (E 171), and that this would raise concerns about potential adverse effects associated with TiO_2 (E 171) in food.

The authors also made a tentative estimation of exposure of human colon cells to TiO_2 following the ingestion of 1 mg TiO_2 /kg bw per day using their specified exposure scenario and assumptions. The authors estimated that the concentration of TiO_2 reaching colon cells would be in the same order of magnitude as the concentrations of TiO_2 used in the *in vitro* experiments (1–10 $\mu\text{g/mL}$).

2.2.3. Guo et al. (2017). Titanium dioxide nanoparticle ingestion alters nutrient absorption in an *in vitro* model of the small intestine

Guo et al. (2017) assessed the effect of 4 h and 5 days exposure to TiO_2 nanoparticles (NP, 20–40 nm, analysed by TEM), using an *in vitro* Caco-2/HT29-MTX cell culture model, on the intestinal barrier function, nutrient absorption, formation of ROS, and proinflammatory signal. *In vitro* doses of TiO_2 NP were formulated to represent potential real-life exposure. The daily intake of TiO_2 NP has been estimated by the authors to be 10^{12} – 10^{14} NP per day (approximately 10^{11} – 10^{13} particles per meal; Lomer et al., 2002). Low (10^6 particles/ cm^2), medium (10^8 particles/ cm^2) and high (10^{10} particles/ cm^2) concentrations of TiO_2 NP were used for the 4 h exposure. The authors estimated the concentrations of nanoparticle by dividing the estimated number of nanoparticles by the surface area of the small intestine. The daily dose used in the 5 days exposure was three times the single dose concentration. The 4 h exposure of the cells to TiO_2 NP did not show any significant difference on the tight junction functionality compared to the control; however, 5 days exposure (at the three concentrations) enlarged the gap between cells and increased the tight junction permeability. The authors reported that despite efforts to measure TiO_2 transport to the basolateral chamber with ICP-MS, no TiO_2 transport across the Caco-2/HT29-MTX monolayers was observed.

Both the 4 h and 5 days exposure (at the three concentrations) resulted in an increase in ROS production in the intestinal epithelial cells, as well as in a decrease in iron and zinc transport and uptake. Only the 5 days exposure (high concentration) to TiO_2 NP significantly reduced the fatty acid uptake, whereas both the 4 h and 5 days exposure (medium and high concentrations) altered the brush border membrane enzyme functionality by significantly increasing the intestinal alkaline phosphatase (IAP) activity. Gene expression of iron transport proteins as well as of proinflammatory cytokines (i.e. IL-8, TNF- α , nuclear factor kappa B (NF- κ B)) was altered, in response to 5 days exposure to TiO_2 NP.

According to the authors, the results of this study suggested that physiologically relevant concentrations of TiO_2 NP may affect the ROS generation in an *in vitro* model of the small intestine, inducing proinflammatory signalling, which in turn could have an impact on the intestinal epithelial cells function.

2.2.4. Heringa et al. (2016). Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations

The publication by Heringa et al. (2016) is not a primary research study but rather a risk assessment of the oral intake of TiO₂ nanoparticles (TiO₂ NP) based on data reported in other publications. The assessment was performed using two approaches: a first one based on human dietary intake estimation (external dose) combined with the lowest no observed adverse effects levels (NOAEL) from the animal toxicity studies, and a second one based on internal, modelled dose.

Both approaches start with an estimation of human dietary intake of TiO₂ NP from food products, food supplements and toothpaste in the Dutch population, as calculated by Rompelberg et al. (2016). Only high level (P95) intake of TiO₂ NP were considered for this risk assessment, ranging from 0.74 µg/kg bw per day in the elderly (70 years and older) to 4.2 µg/kg bw per day in 2- to 6-year-old children. The P95 intake of TiO₂ NP for the population aged 7–69 years was estimated to be 1.6 µg/kg bw per day. Based on the values above, the lifelong daily intake of TiO₂ NP for the P95 of the population was calculated to be 1.7 µg/kg bw per day.

The Panel noted that the estimated intake of TiO₂ from the Rompelberg et al. (2016) study is lower than the estimated exposure calculated by EFSA for TiO₂ (E 171), which is most probably due to the different concentration levels of TiO₂ assumed in food products. In addition, the food categories considered in both the exposure assessments were different. Rompelberg et al. (2016) also took into account intakes of TiO₂ from its use in toothpaste. With respect to the calculation of exposure to TiO₂ nanoparticles, it was noted that the ANS Panel considered that 3.2% (by mass) of the TiO₂ (E 171) were nanoparticles, according to information provided by industry (EFSA ANS Panel, 2016), whereas in the Rompelberg et al. (2016) study the percentage of nanoparticles in TiO₂ was considered to be 0.31% (by mass).

Four toxicity studies (NCI, 1979; Wang et al., 2013; Jia et al., 2014; Tassinari et al., 2014) were identified as pivotal for the risk assessment by the Heringa et al. All of them, with the exception of the study by Wang et al. (2013), had been already considered by the Panel in the re-evaluation of TiO₂ (E 171) as a food additive (EFSA ANS Panel, 2016).

According to Heringa et al. (2016), using the traditional approach for risk assessment, the intake was used for deriving Margins of external Exposure (MoEe) by dividing the NOAEL or the lowest observed adverse effect level (LOAEL) from their identified four key toxicity studies (see more details in Table 1 of Heringa et al., 2016) by this intake estimate.

The study by Wang et al. (2013) was used by Heringa et al. (2016) to derive a Point of Departure (PoD) for effects on the liver, equal to a NOAEL of 10 mg/kg bw per day. This value, divided by the estimated intake of TiO₂ NP for the general population of 1.7 µg/kg bw per day resulted in a MoEe of 5,882, i.e. nearly 10-fold higher than the value considered to be acceptable by the authors (> 600).

Other MoEes were calculated also for the other LOAELs and NOAELs identified from the other three key toxicity studies reported above, all of them resulting in acceptable MoEes with the exception of the effects on the ovary observed in the study by Tassinari et al. (2014), for which Heringa et al. (2016) defined an acceptable MoEe of 3,000 compared to the 300–700 in the three other key studies. Heringa et al. (2016) however, acknowledged that owing to the short duration (5 days) of the Tassinari et al. (2014) study, extrapolation of the results to chronic intake introduces important uncertainties and that the findings would need to be confirmed in long-term studies.

In their second approach for the risk assessment, Heringa et al. (2016) used a kinetic model, based on the data by Geraets et al. (2014) obtained after intravenous (i.v.) administration of TiO₂ NP adjusted for a better fit for the prediction of oral absorption. The authors acknowledged that although they considered this to be the best available model for oral exposures, there might be some inaccuracy in the results due to the differences in toxicokinetic behaviour between the two routes of administration. This kinetic model was used to calculate internal organ doses for the selected rat toxicity studies. However, the authors noted that the predicted values in rat liver were higher than measured ones following oral absorption in the Geraets et al. (2014) study by 1.5- to 4-fold, which was ascribed to using worst case assumptions in the model.

The rat kinetic model was then converted into a human model using physiological data and allometric scaling of kinetic parameters from rat to human. Organ concentrations in humans were estimated using the intake data for the three different age groups from the Rompelberg et al. (2016) study. According to this second approach, the estimated organ concentrations at which effects of TiO₂ NP were observed in the animal studies were compared to the estimated human organ concentrations resulting in Margins of internal Exposure (MoEi). The MoEi calculated by the authors at three ages (20,

40 and 80 years) were then compared to estimated acceptable MoEi derived from the key four studies. For the latter, Heringa et al. (2016) acknowledged that these values have not been subject to international review and, therefore, should only be considered for the sole purpose of their evaluation. There was a decrease in the MoEi with age for all estimated organs except the liver. By using this approach, for the three studies in which TiO₂ NP were tested, the estimated MoEi were lower than the derived acceptable MoEis by Heringa et al. (2016). The acceptable MoEi values were defined by Heringa et al. (2016) using factors of 2.5 for interspecies and 10 for intraspecies differences with default factors for extrapolation for the duration of exposure to chronic exposure and LOAEL to NOAEL extrapolation from REACH Guidance (ECHA, 2012). Heringa et al. (2016) considered that these MoEis indicated a potential risk for effects on the target organs of these studies (liver, testes and ovary). In contrast, assuming a fraction of 0.31% nanoparticles by weight in the test material, the estimated MoEi from the 2-year carcinogenicity study (NCI, 1979) was higher than the acceptable estimated MoEi, indicating a risk was unlikely.

Heringa et al. (2016) also noted that for all the three studies conducted with TiO₂ NP, the predicted levels in human organs were still lower than the organ levels estimated from the kinetic model that were related to toxicity in the corresponding animal studies, the only exception being the predicted levels in the human ovaries. As for the other approach, also in this case, Heringa et al. (2016) considered that extrapolation of the data from the short-term study by Tassinari et al. (2014) represented a 'rather large' source of uncertainty. Heringa et al. (2016) included a discussion of the uncertainties and assumptions used in the model and the risk assessment.

3. Discussion

As requested in this mandate by the EC, in this opinion the Panel concentrated on the evaluation of four publications concerning the potential adverse health effects of TiO₂ (Heringa et al., 2016; Bettini et al., 2017; Guo et al., 2017; Proquin et al., 2017). The Panel was aware of these publications and of a number of other new publications on TiO₂ since its last opinion (EFSA ANS Panel, 2016), but these other publications were not taken into consideration in the present evaluation. Individual publications should be assessed in respect to the whole dataset available, using a weight of evidence approach (EFSA Scientific Committee, 2017b). A proper evaluation of the safety of titanium dioxide as a food additive would require considering all data available and not only selected data. The EFSA SC also provided Guidance on when the database should be re-evaluated in light of new evidence (EFSA Scientific Committee, 2017a).

The re-evaluation of TiO₂ (E 171) for use as a food additive had been completed by the EFSA ANS Panel in June 2016, and a scientific opinion published in September 2016 (EFSA ANS Panel, 2016). The Panel concluded that the use of TiO₂ as a food additive did not raise a genotoxic concern. From a carcinogenicity study with TiO₂ in mice and in rats (NCI, 1979), the Panel identified the lowest NOAEL which was 2,250 mg TiO₂/kg bw per day for males from the rat study, the highest dose tested in this species and sex. However, the Panel was unable to reach a definitive conclusion on the reproductive and developmental toxicity endpoints, due to the lack of an extended 90-day study or a multigeneration or extended one-generation reproduction toxicity study with the food additive (E 171). Therefore, the Panel did not establish an acceptable daily intake (ADI). Taking into account the toxicological database available at that time and the considerations that the absorption of orally administered TiO₂ was extremely low, the Panel considered that the margins of safety (MoS) calculated from the NOAEL of 2,250 mg TiO₂/kg bw per day and the exposure calculated from the reported use/analytical levels of TiO₂ (E 171) would not be of concern. The Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI).

From the data provided from interested parties and the ones publicly available, the Panel noted that the fraction of titanium dioxide nanoparticles (with one dimension less than 100 nm) measured in E 171 is method-dependent (EFSA ANS Panel, 2016). The Panel further noted that there were no set limits for the particle size of titanium dioxide in the EU specifications (Commission Regulation (EU) No 231/2012), and recommended that the characterisation of the particle size distribution as well as the percentage (in number and by mass) of particles in the nanoscale present in the food additive E 171 should be included among the EU specifications.

The EC, as part of the follow up activities for those food additives whose safety re-evaluation by EFSA was hindered by limited data availability but which were not expected to pose an immediate food safety concern, launched a call for data on TiO₂ (E 171) inviting interested business operators to

generate the additional data requested by a certain deadline.⁹ Following this call for data, interested parties have committed to provide the requested information according to a defined timeline. In particular, data on particle size and particle size distribution for TiO₂ (E 171) would be submitted by June 2018, whereas a dietary Extended One-Generation Reproductive Toxicity Study with TiO₂ (E 171) in rats (carried out according to the current OECD guidelines and with TiO₂ (E 171) complying with the EU specifications and including a characterisation of the particle size distribution) would be generated by August 2019. The study design should include cohort 1 (extension by mating of F1 animals to the F2 generation), cohort 2 (for developmental neurotoxicity) and cohort 3 (for developmental immunotoxicity).

The Panel has assessed the four publications as requested in the last mandate from the EC and made the following specific comments on each individual studies which have been considered in the context of the conclusions of the EFSA opinion of 2016.

Regarding the Bettini et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the TiO₂ test materials were prepared following the generic Nanogenotox dispersion protocol, which involved coating of the particles with BSA and ultrasonication. This protocol was designed to prevent agglomeration of the nanoparticles and was especially developed for the hazard identification of nanoparticles in suspension;
- the administration of TiO₂ (E 171) by gavage or drinking water is not fully representative of the use of the food additive E 171 in food. This could introduce some uncertainties in the extrapolation of the results of this study to the assessment of the food additive, because interactions with the food matrix may not occur;
- the effects observed on immunomodulation/inflammation were in line with those occurring in the very early steps of the development of an inflammatory/immunological reaction. Variations of different biomarkers were low (within 10–20% change), thus questioning the biological relevance of the results, also taking into account the lack of historical data for that kind of study;
- BSA coated on particles of TiO₂ (E 171) could itself trigger an immunologic and/or allergic reaction;
- although the model used to investigate the effects of TiO₂ (E 171) in the development of putative preneoplastic lesions in the colon was well described, the Panel noted some limitations, such as the use of a single marker;
- the presence of ACF in the absence of an initiator is unusual, but in this study ACF were observed in the colon of a few animals treated with TiO₂ (E 171) alone;
- the initiation potential of TiO₂ reported in this study was not observed in another study using TiO₂ (E 171) (Urrutia-Ortega et al., 2016);
- a publication by Wijnands et al. (2004), showed that the number and size of ACF induced by different dietary compounds such as wheat bran, curcumin, rutin or benzyl isothiocyanate, were not predictive for the ultimate number of colorectal tumours. Wijnands et al. (2004) concluded that 'the number and size of ACF were not considered to be suitable as biomarker for colorectal cancer', whereas the expression of some tumour-related genes (e.g. metalloproteinase 1; TIMP-1) 'correlated well with the effects of dietary compounds on the ultimate tumour yield'.

Overall, based on the data provided in the Bettini et al. publication, and the negative results of the carcinogenicity studies in mice and rats performed by NCI (1979), the Panel considered that the new findings were not sufficient to raise a concern on the potential initiation or promotion properties of TiO₂ (E 171) on colon carcinogenesis.

The Panel noted that determination of the presence of biomarkers for putative preneoplastic lesions in the colon as additional parameters to be examined in the ongoing extended one-generation reproductive toxicity study should be considered to substantiate the observations in the Bettini et al. (2017).

The Panel considered that there was not enough justification at this stage for a new carcinogenicity study as this would involve a large number of animals, but that, if additional mechanistic studies provided useful information on the relevance of the reported results, this could be reconsidered in future.

⁹ http://ec.europa.eu/food/safety/food_improvement_agents/additives/re-evaluation_en

Regarding the Proquin et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- this *in vitro* study adds to a number of studies which provided some evidence of ROS formation and *in vitro* genotoxicity by TiO₂ MP and TiO₂ NP in a variety of cell lines of human and rodent origin (as reported in EFSA ANS Panel, 2016). These authors reported for the first time genotoxic activity *in vitro* in a Comet assay and cytokinesis-block micronucleus assay using the food additive TiO₂ (E 171). The Panel noted that, in view of the composition of TiO₂ (E 171) (containing around 40% of nanoparticles by number), and of the available evidence of genotoxicity *in vitro* of both TiO₂ MP and TiO₂ NP, a genotoxic activity of E 171 under *in vitro* conditions could already be anticipated. In this respect, the Panel also noted that overall negative results were obtained in studies *in vivo* with both TiO₂ NP and MP (EFSA ANS Panel, 2016), indicating that TiO₂ MP and NP, and consequently E 171, did not raise concern for *in vivo* genotoxicity;
- two cell lines derived from human colon (adeno)carcinomas were selected as model system to investigate the possible implication of ROS generation and genotoxicity in the promoting activity of TiO₂ (E 171) on chemically-induced colon carcinogenicity in mice reported in a previous study (Urrutia-Ortega et al., 2016). Based on the discussion at the technical hearing at the open Plenary, the Panel noted that this model appeared suitable for hazard characterisation but not for the purpose of risk assessment;
- undifferentiated Caco-2 cells, harvested after only 3 days of culture, were used for the assessment of DNA damage induced by TiO₂. As to the relevance of the cell system used for hazard characterisation, the Panel noted that another recent *in vitro* study highlighted a comparable lower incorporation of TiO₂ in differentiated compared to undifferentiated Caco-2 cells, and no induction of DNA damage in differentiated Caco-2 cells monolayers exposed to TiO₂ NP was observed (Vila et al., 2018). The Panel also noted that under *in vivo* conditions, the uptake of TiO₂ particles (including E 171) may be substantially decreased by the mucus layer of the intestinal epithelium (García-Rodríguez et al., 2018);
- concerning the claimed colocalisation of TiO₂ (E 171) with 'kinetochore poles, mitotic genome and α -tubulin', the Panel noted that no clear conclusion can be drawn from the figures presented, which may be either indicative of a true colocalisation and functional interaction, or arise from the unspecific overlap of TiO₂ particles on cellular structures in the microscopic preparation.

Overall, the Panel considered that the results reported in the Proquin et al. (2017) study may be useful for an evaluation of the hazard of TiO₂ nanoparticles under the specific conditions of the study protocol, including the cell model used and the conditions of culture. However, the Panel also considered that the relevance of the results for risk assessment of the food additive E 171 has not been established.

During the discussion at the technical hearing at the open Plenary, one of the authors mentioned that consistent results appeared to be observed in mouse colon in ongoing *in vivo* transcriptomics studies. The Panel considered that these studies could be evaluated when completed, and if deemed necessary, the overall database reassessed considering the entire literature available at that time.

The Panel concluded that the new *in vitro* findings provided in the study by Proquin et al. (2017) do not modify the conclusion on the genotoxicity of TiO₂ as stated in the previous EFSA opinion (2016) on the safety of TiO₂ (E 171) when used as a food additive.

Regarding the Guo et al. (2017) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the study was performed with engineered TiO₂ NP and not with TiO₂ as a food additive;
- the TiO₂ NP suspension was sonicated to minimise agglomeration and/or aggregation which may not occur in more realistic environments;
- in addition to the TEM analysis of primary particles, the DLS results suggested that the hydrodynamic sizes were considerably larger which suggested that the TiO₂ NP may be aggregated and agglomerated;
- the authors assumed that all ingested particles would be in contact with the wall surface but without considering the potential interaction of nanoparticles with luminal contents, e.g. their estimated number of nanoparticles was comparable with the number of microorganisms per gram of intestinal contents (ranges from 10⁵⁻⁶ in intestine to 10¹³ in colon);

- the glycocalyx on cultured Caco-2 cells is different from that on normal human intestinal cells;
- the cells were rinsed in PBS before exposing them to TiO₂ NP which could alter the glycocalyx and the interactions of Caco-2 cells with TiO₂ NP;
- changes in the expression of proinflammatory genes seen after 5-days of treatment with TiO₂ NP were small, not dose-related and uneven;
- no information was available about possible markers of inflammation such as production/secretion of cytokines, changes in the expression of membrane antigens;
- no transport of TiO₂ NP across the Caco-2/Ht29 MTX monolayers was reported;
- as more physiological conditions were approached the effects were attenuated, and it was difficult to extrapolate these results to the *in vivo* situation;

Therefore, the Panel concluded that the results from the Guo et al. (2017) study cannot be extrapolated to the human situation and cannot be used for the risk assessment of the food additive TiO₂ (E 171).

Regarding the Heringa et al. (2016) study and the technical hearing in the Open ANS Plenary,¹ the Panel noted that:

- the National Institute for Public Health and the Environment (RIVM) provided this and two other unpublished reports at the time of the re-evaluation of the TiO₂ (E 171) as a food additive in 2016 (EFSA ANS Panel, 2016);
- these unpublished studies did not affect the Panel's conclusions drawn from the available database at that time;
- Heringa et al. assumed that, although there was no information available on the absorption of TiO₂ particles, any toxicity was caused by TiO₂ NP;
- the conclusions of the authors that, because of uncertainties and assumptions in their analysis, further studies would be needed, for example, on the actual concentration of nano-TiO₂ in human organs and on the effects in liver and the reproductive system after chronic exposure to well-characterised TiO₂ NP;
- data on reproductive toxicity were scarce;
- the results on effects on testosterone levels in the two studies (Jia et al., 2014; Tassinari et al., 2014) used by Heringa et al. (2016) were contradictory;
- significant uncertainties were associated with extrapolation from short term to lifetime exposures;
- although the application of uncertainty factors for extrapolation from short term studies is a policy choice, Heringa et al. used uncertainty factors from REACH rather than those recommended by the EFSA SC (EFSA Scientific Committee, 2012);
- in deriving acceptable MoEi, the kinetic element of the inter- and intraspecies uncertainty factors was only removed from the interspecies but not from the intraspecies uncertainty factor;
- effects seen in short term studies with no functional effect in longer term studies are usually not considered a robust basis for risk assessment.

Overall, the Panel considered that the aforementioned evaluations and considerations indicated that there was significant uncertainty in the assessment by Heringa et al. (2016) and noted that there was not a weight of evidence analysis of the whole database on E 171. The Panel considered that the assessment was consistent with a hazard from TiO₂ NP when dosed as in the selected studies but the relevance to nanoparticles in a food matrix could not be assessed. The Panel concluded that the additional studies called for in its 2016 opinion should provide a more robust basis for addressing the reported effects in reproductive organs in the studies used by Heringa et al. (2016).

4. Conclusions

Based on the evaluation of the four studies concerning the potential adverse health effects of titanium dioxide, the Panel considered that:

- the results of the Bettini et al. (2017) study did not provide enough justification for a new carcinogenicity study, but, should additional useful mechanistic information become available, this could be reconsidered in future;

- the new *in vitro* findings in the study by Proquin et al. (2017) did not modify the conclusion on the genotoxicity of TiO₂ as stated in the previous EFSA opinion (EFSA ANS Panel, 2016) on the safety of TiO₂ (E 171) when used as a food additive;
- the effects of engineered TiO₂ nanoparticles reported by the Guo et al. (2017) study were of uncertain biological significance and therefore of limited relevance for the risk assessment of the food additive TiO₂ (E 171);
- there was significant uncertainty in the risk assessment performed by Heringa et al. (2016), which did not include a weight of evidence analysis of the whole database;
- the four studies evaluated, highlighted some concerns but with uncertainties, therefore their relevance for the risk assessment was considered limited and further research would be needed to decrease the level of uncertainties.

Overall, three of the studies assessed in this opinion reported that TiO₂ was able to induce various effects in *in vitro* and *in vivo* models. These studies may be useful for hazard identification of TiO₂. The Panel considered that the limited relevance of the protocols of these studies to the use of E 171 under realistic conditions in food hampered the use of the data in the risk assessment of the food additive E 171. In the fourth study by Heringa et al. (2016), numerous assumptions were made, which resulted in large uncertainty in their conclusion.

More research exploring the possible effects observed in three of the four studies could address their applicability to the risk assessment of the food additive E 171 under realistic conditions of use.

Altogether, the Panel concluded that the outcome of the four studies did not merit re-opening the existing opinion of EFSA related to the safety of TiO₂ (E 171) as a food additive.

Recommendations

The Panel recommended that:

- in order to substantiate the observations in the Bettini et al. (2017), biomarkers for putative preneoplastic lesions in the colon as additional parameters should be examined in the extended one-generation reproductive toxicity study recommended by EFSA (EFSA ANS Panel, 2016);
- further studies on TiO₂ NP should include administration in a food matrix.

Documentation provided to EFSA

- 1) Answers to questions shared with the authors of the four publications (attending as hearing experts) to support the discussion at the ANS plenary meeting open to observers on 16 May 2018; submitted on 8 May 2018 by Prof Mahler (for the Guo et al., 2017; see Appendix D) and on 15 May 2018 by Dr Houdeau (for the Bettini et al., 2017; see Appendix B) and Dr Oomen (for the Heringa et al., 2016, see Appendix E).

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Abbreviations

ACF	aberrant crypt foci
ADI	acceptable daily intake
ANS	Food Additive and Nutrient sources added to Food
ANSES	French Food Safety Agency
BSA	bovine serum albumin
DC	dendritic cells
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMH	1,2-dimethylhydrazine
ESR	electron spin resonance
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
IAP	intestinal alkaline phosphatase
IFN- γ	interferon- γ
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
LOAELs	lowest observed adverse effect level
MoEe	Margins of external Exposure
MoEi	Margins of internal Exposure
MoS	Margins of safety
MP	microparticulate
NF- κ B	nuclear factor kappa B
NOAEL	no observed adverse effects level
NP	nanoparticles
PBS	phosphate-buffered saline
PD	Point of Departure
PP	Peyer's Patches
RIVM	National Institute for Public Health and the Environment
ROS	reactive oxygen species
SC	Scientific Committee
SEM	scanning electron microscopy
TiO ₂	titanium dioxide
TEM	transmission electron microscopy
TNF- α	tumour necrosis factor- α
WG	Working Group

Appendix A – ANSES Opinion 2017: English translation by the Panel of the conclusions from the French original

On the basis of this study, ANSES concluded that the group of experts acknowledges the scientific content of the publication and the data reported in spite of their limitations as discussed with the authors. This study adds new information to the EFSA opinion (2016) as regards a potential promoting effect of E 171.

The experts concluded that:

- the inflammatory effects reported in the *in vivo* studies appeared limited and their biological significance was not established. In addition, the statement about an inflammatory reaction was not adequately substantiated by the data reported;
- as regards the Comet assay in the Peyer Patches, in the absence of positive controls in the studies and of historical data, it is not possible to conclude on a possible genotoxic effect.

The data presented in this study do not question the risk assessment of E 171 performed by EFSA and cannot be used before having been confirmed by additional studies with E 171 administered to animals in the food matrix. The potential promoting effect of E 171 in the colon must be confirmed by longer term studies, which should evaluate more biomarkers. In these studies, an additional treated group with a sufficient number of animals is needed before being able to conclude on a possible initiating effect of E 171.

Appendix B – Questions and answers to the authors of the Bettini et al. (2017) study

- 1) On page 9 under 'Animals and experiments design', the three series of experiments are described. The Panel would like to know whether the rats were fasted in the first series of toxicological studies before treating them by gavage.

Answer: No, rats were not fasted before oral treatment with TiO₂, and whatever the study (one week through gastric gavage or 100 days in drinking water).

- 2) Could you please tell us if the potential agglomeration of ultrasonicated test material was checked before its administration to the animals?

Answer: Yes, the dispersion state was checked by DLS before treatment to rats (no change upon 48 h). Ultrasonicated TiO₂ suspension orally given to rats in water was thus renewed every 2 days to ensure stable dispersion state during experiments.

- 3) On page 2 under 'Results', it is mentioned that titanium dioxide particles were found in the Peyer's Patches (PP) of the small intestine, colonic mucosa and liver of rats administered TiO₂ (E 171) by gavage for 7 days, but not in the controls. In the same rats, Ti was detected in the gut lumen, PP, colon mucosa and liver. In this respect:

- The Panel is aware that in other publications, background levels of Ti have been reported in control animals in experimental studies on Ti absorption from nanoparticles. What is your explanation of the fact that in your study, no Ti was detected in control rats?

Answer: TiO₂ is rapidly absorbed: ≤ 6–8 h after a single dose, and does not accumulate significantly into the intestinal wall (unpublished); repeated exposure is needed (e.g. daily for one week or more) to observe particles into the gut mucosa and cells; our studies were performed with nano-SIMS and μXRF imaging, after application of a threshold to extract the Ti signal over the background noise: no Ti level measurements were performed in this study (e.g. ICP-MS).

- How much Ti was detected in the liver?

Answer: Not measured, but all organs were available for ICP-MS dosage (100-day study).

- Could it be a difference in absorption and distribution between a bolus administration and a chronic exposure in drinking water?

Answer: No difference observed between bolus and drinking water.

Appendix C – Questions and answers to the authors of the Proquin et al. (2017) study

- 1) On page 140 and 141 under 'Materials and methods', it is described that the Caco-2 cell lines (at passage 24–32) 'were cultured for three days to reach 80–100% confluency before exposing them to different concentrations of E 171, TiO₂ MP, or TiO₂ NP'. The Panel noted that, according to the literature, the differentiation of Caco-2 cells may require as much as three weeks in culture before being fully achieved. In this respect:
 - Could you please clarify the rationale for choosing a shorter period in culture (3 days) before the treatment with the test materials?
 - Would you consider that these experimental conditions could have affected the representativeness of this cell model to extrapolate to an *in vivo* situation?
- 2) The Panel considered your findings on ROS generation. On page 144 (figure 4), under cell-free conditions, only TiO₂ NP (at mid-dose) and E 171 (at the highest dose) induced a significant increase in ROS generation compared to the control. Conversely, it is stated that in Caco-2 cells only TiO₂ MP (at the mid concentration) produced a significant increase of ROS compared to the control. However, data in figure 5 (page 145), show higher signals of cellular radical formation for controls than MP-treated cells. Could you please explain how data on ROS formation in Caco-2 cells are interpreted to reach such conclusion? Moreover, how do you interpret the inconsistency in the findings on the ROS generation in cell-free and cellular conditions, and how do you consider this in reaching the final conclusion on this endpoint?
- 3) In figure 6 on page 145, showing the results of the Comet assay, you stated that the DNA damage in Caco-2 cells was dependent on the size of the particles. Could you please explain this statement in relation to the results presented in figure 6?

Written answers to these questions were not provided, but these were answered during the discussion at the technical hearing at the ANS Plenary open to observers¹.

Appendix D – Questions and answers to the authors of the Guo et al. (2017) study

- 1) It is noted that the daily intake of titanium dioxide nanoparticles has been reported to be 10^{12} – 10^{14} nanoparticles per day. In this respect, could you please provide more information on how these doses were converted to nanoparticles per meal, and particle per mL?

Answer: The doses used in this study were based on the published TiO₂ consumption estimates made by Lomer et al. (2004). We took these estimates (in mass), converted them to a number of 30 nm nanoparticles, and divided that number of nanoparticles by the surface area of the small intestine. This density of exposure was then matched in an *in vitro* model.

- 2) Under 2.6 'Acute and chronic exposure to NPs', it is noted that the 'NP solutions were made fresh every day'. Could you please explain whether doses expressed as particle/ml were also daily measured?

Answer: The chronic exposure solutions were made fresh daily. This was because when we measured these solutions after they had been stored for 24+ h there was a great deal of particle aggregation. The measurements on nanoparticle characteristics were performed on solutions that were made freshly using the same methods as we used to make our exposure solutions. The zeta potential, etc. was not measured before every exposure experiment.

- 3) Under 2.1 'Nanoparticles dose calculations' the following it is stated: 'Chronic doses were three times (3X) the acute dose'. Could you please clarify the regimen of the administration of the doses for the chronic exposure (e.g. one single dose, repeated doses, etc.)?

Answer: For the chronic exposure experiments, the medium containing a known amount of nanoparticles was replaced every 24 h for five days.

- 4) The Panel noted that the cell surface structure (e.g. glycocalyx amount and composition, which is of importance in regulating the possible interactions of cells with particles), is dependent on the culture conditions. In this study, the cells were cultured for 16 days instead of 21 as usually done (Frey et al., 1996; Ramaker et al., 2016). It is however acknowledged that co-culture with HT29-MTX cells favour the constitution/composition of glycocalyx. No TEM analysis of the cell cultures was performed (only scanning) and there was no direct observation of the glycocalyx. In addition, the cells were rinsed in PBS before exposing them to titanium dioxide nanoparticles. In this respect, could you please discuss what could be the consequence of an altered glycocalyx on the interactions of Caco 2 cells with titanium dioxide nanoparticles?

Answer: We have been developing assays that assess the mucus layer following exposure to various food additives. We do not yet have reliable data.

- 5) Could you please clarify why cell cultures treated with TiO₂ but not exposed to Fe or Zn isotopes were not used when looking at ROS generation and gene expression?

Answer: Cells exposed to Fe and Zn were used for gene expression studies.

- 6) In this study, no cytotoxicity tests were performed on the Caco 2 cells treated with TiO₂; the doses applied were considered to be non-cytotoxic based on data from the literature. However, culture conditions (time, number of passages, co-culture,) are known to influence cell morphology/physiology, which, as a result may modify the response of cells to a cytotoxic compound. In addition, in this study, it is reported a marked decrease in the Trans Epithelial Resistance (TER) in the cells that had received chronic doses of TiO₂, but not an acute dose. Could you please discuss the consequence of these observations as regards interaction of TiO₂ with the cell surface and the resulting reported effects? Alkaline phosphatase activity is expressed per mg of cell protein. What were the changes in the cell protein content between treated and untreated cultures?

Answer: We have done more studies on TiO₂ effects on gut permeability with an *in vivo* (drosophila) model. Based on that data (currently under review) I do not think that TiO₂ has a significant effect on gut permeability. I can look at the cell protein raw data to see if there were dramatic changes in cell protein content. I do not believe that there were, and I

cannot confidently say that changes in cell protein are the result of TiO₂ exposure or inefficiencies in the sonication method we used to disrupt the cell membrane. As we have continued to perform this assay we have found that to fully release cell protein into solution we need to scrape the well and then sonicate the solution a second time.

- 7) The study reported some variations in the expression of proinflammatory genes after treatment with 'chronic doses' of TiO₂ only, not after exposure to 'acute doses'. These variations were limited, not dose-related, variable; e.g. for TNF-, there was a significant increase for Zn (at one dose), and a significant decrease (at two doses) for Fe. As regards NFKB1, there was a significant decrease at one dose for Fe and a significant increase at the same dose for Zn. Could you please discuss the biological significance of these changes? Do you have any information about other modifications of Caco 2 cells that might be associated with these variations in the expression of proinflammatory genes; e.g. production/secretion of cytokines, changes in the expression of membrane antigens, etc.?

Answer: No, we did not measure the secretion of additional cytokines.

- 8) Could you please provide your opinion on how this *in vitro* model is representative of an *in vivo* situation in human?
- 9) Why do you assume that exposure to particles is occurring at the wall of the gastrointestinal tract rather than with the contents in the lumen and ignore that *in vivo* exposure is more likely to result in interactions with the luminal contents (food, digestive enzymes, chime, microflora). These interactions are likely to modify the nanoparticulate material so how relevant is their administration in a medium intended to maintain them as nanoparticles on the cell surface to the *in vivo* situation?

Answer to Questions 8 and 9: This study was the first that our lab published on nano-TiO₂ exposure. We have since made efforts to make the *in vitro* model more realistic by including bacteria (<https://doi.org/10.1080/17435390.2018.1457189>) and studying the food additives in a food matrix (under review). Based on those results I would say that a more complicated model, or *in vivo* studies, may be needed to fully assess the effects of TiO₂ nanoparticles on gut function, although a simplified cell culture model may be useful for deciding on which materials to test or directing resources toward more complex studies. When we added a beneficial bacteria to our cell culture model, for example, the effects of TiO₂ nanoparticle exposure were remediated. We have also performed studies *in vivo* to look at the functional effects of TiO₂ ingestion and changes to the microbiota, but that data has not been peer reviewed or published yet.

Appendix E – Questions and answers to the authors of the Heringa et al. (2016) study

- 1) It was noted that from the NCI (1979) study, you identified a LOAEL based on the non-neoplastic effects observed (e.g. hyperplasia of bile ducts), whereas in the EFSA Scientific Opinion a NOAEL was identified from this same study based on the absence of carcinogenicity. Could you please explain the rationale behind this choice?

Answer: The NCI (1979) study was a study focused on potential carcinogenicity and reports a NOAEL at the highest tested dose of 50,000 ppm (corresponding to 2,500 mg/kg bw per day in rat). In this study, no significant increase in tumours was found. However, in the non-neoplastic analyses in this study, as given in the study annexes, a number of effects were reported.

In Appendix C, on non-neoplastic lesions in the rat, Table C1 (for male rats) shows the following incidences with a clear dose–response relationship or a clear effect of TiO₂ dosage (in italics):

Lesion	Incidence in control group (n = 49)	Incidence in low-dose group (1,250 mg/kg bw per day in rat) (n = 50)	Incidence in high-dose group (2,500 mg/kg bw per day in rat) (n = 50)
Lung: Congestion	0	6 (12%)	13 (27%)
<i>Lung: haemorrhage</i>	0	5 (10%)	6 (12%)
Heart: fibrosis	1 (2%)	8 (16%)	12 (24%)
Liver: bile duct hyperplasia	0	21 (42%)	27 (54%)
Seminal vesicle: atrophy	0	6 (12%)	10 (20%)

Table C2 (for female rats) shows the following incidences with a clear effect of TiO₂ dosage (in italics):

Lesion	Incidence in control group (n = 50)	Incidence in low-dose group (1,250 mg/kg bw per day in rat) (n = 50)	Incidence in high-dose group (2,500 mg/kg bw per day in rat) (n = 49)
<i>Lung: Congestion</i>	0	12 (24%)	10 (20%)
<i>Lung: haemorrhage</i>	1 (2%)	8 (16%)	9 (18%)
Heart: fibrosis	0	10 (20%)	5 (10%)
Liver: bile duct hyperplasia	0	14 (28%)	14 (29%)
<i>Mammary gland: galactocele</i>	2 (4%)	14 (28%)	14 (29%)

In Appendix D, on non-neoplastic lesions in the mouse, Table D1 (for male mice) shows the following incidences with a clear dose–response relationship or a clear effect of TiO₂ dosage (in italics):

Lesion	Incidence in control group (n = 50)	Incidence in low-dose group (1,250 mg/kg bw per day in rat) (n = 50)	Incidence in high-dose group (2,500 mg/kg bw per day in rat) (n = 49)
Mesenteric lymph node: lymphangiectasis	1 (2%)	12 (25%)	15 (31%)
<i>Liver: necrosis</i>	0	0	8 (16%)

Table C2 (for female mice) shows the following incidences with a clear effect of TiO₂ dosage (in italics):

Lesion	Incidence in control group (n = 49)	Incidence in low-dose group (1,250 mg/kg bw per day in rat) (n = 50)	Incidence in high-dose group (2,500 mg/kg bw per day in rat) (n = 50)
<i>Uterus: hyperplasia, cystic</i>	17 (35%)	42 (86%)	38 (78%)
<i>Ovary: follicular cyst</i>	1 (2%)	11 (23%)	8 (17%)

We have asked for historic control values for these rats at this institute, but these were not available according to the institute. Thus, perhaps the increased incidences in the exposed groups lie within the historic control range and were therefore not seen as significant by the study directors. However, we considered that the chance of seeing a > 10% increase in incidence (i.e. > 5 additional animals showing the effect, of the 50 animals in the group) precisely in the exposed groups only, and this being due just to background variation, should be quite small, even if such incidences had been found sometimes in the past in a control group. We can thus not fully exclude that the increases are a natural variation in these cases, but it is more likely that the increase in incidence is then caused by the exposure. In addition, we see a large correspondence between the male and female rats in these increased incidences, making it even more likely that the effects seen in rats are not caused by natural variation, but by the exposure. The fact that the higher dose often does not give a much higher response than the lower dose, may be explained by a potential limit in the absorption capacity in the gut at these very high doses. Finally, some of the organs affected correspond to organs where other key studies, with TiO₂ nanoparticles specifically, have reported effects as well: liver (Wang et al., 2013) and ovary (Tassinari et al., 2014). Seminal vesicles were not analysed by Jia et al. (2014) or others as far as we are aware, while Jia et al. (2014) did report other effects on the male reproductive organs. The mammary gland has not been analysed by any oral TiO₂ study either, as far as we know, the uterus only in a 5-day study, in which hyperplasia can hardly form.

Altogether, we found that these effects cannot be dismissed, but should be taken along in our risk assessment, together with other study results. We therefore suggest that a LOAEL may be set at the lowest tested dose: 25,000 ppm (or 1250 mg/kg bw d in rats).

- 2) Could you please provide your opinion whether the results from the dietary Extended One-Generation Reproductive Toxicity study, which was commissioned by the industry following a request from the European Commission in response to the Panel recommendations further to the re-evaluation of titanium dioxide (E 171) as a food additive, would be sufficient to address the effects discussed in your publication? If not, what additional study would you recommend?

Answer: According to the website of the European Commission, the dietary Extended One-Generation Reproductive Toxicity study would include developmental neurotoxicity, developmental immunotoxicity and various endocrine parameters. It was recommended in Heringa et al. (2016) to obtain more clarity on endocrine effects and effects on fertility, which should be addressed in the study requested by the EC.

Furthermore, it is indicated that tissue and urine levels of titanium will be monitored. As we consider it is important that the risk assessment of TiO₂ should be based on internal concentrations, we highly appreciate that tissue levels of titanium will be determined. However, it is unclear which tissues will be analysed and at which time points. In line with the EFSA draft guidance, in particular information on the absorption/bioavailability, distribution pattern and clearance is considered relevant. To that end, tissues should be analysed after multiple time points. Also the addition of a satellite group to investigate the elimination of Ti is recommended. The tissues that are considered most relevant to analyse are, to our opinion: liver, spleen, lymph nodes, gut tissue, gonads and possibly brain and bone marrow.

Also in line with the EFSA draft guidance, analysis of the presence of TiO₂ particles (rather than the element Ti) and their size in these tissues would be encouraged, although this may be technically challenging. Our recent study (Heringa et al., 2018) suggests that all Ti found in tissues was present as TiO₂ particles.

We also highly recommend investigating markers of liver damage (e.g. ALT, AST, ALP, bilirubin) and liver (including bile duct) pathology. This can be performed in the animals in the Reproductive study or in separate subchronic or chronic oral toxicity study. Based on other studies (Bettini et al., 2017), assessment of effects on the intestine should also be considered. Furthermore, we recommend applying the benchmark dose approach, using more dose groups with smaller numbers of animals (hence, using the same total number of animals). In this way, it is possible to get a good dose–response curve, which can also be expressed based on internal tissue concentration levels. High doses of particles may lead to agglomeration of the particles which may affect the absorption. Due to this phenomenon, the fraction absorption may be smaller than at lower (more realistic) concentrations. The inclusion of lower dose groups that are more representative for human exposure should therefore be considered in addition to higher dose groups.

Finally, it should be noted that TiO₂ in E 171 can be present in different ‘forms’ due to differences in crystal structure (anatase, rutile or a mixture), uncoated or coated with e.g. silica or alumina, or with different size distributions. It is known that such differences can affect toxicokinetic behaviour and hazard and thereby risk, but at present there is no understanding of how these physicochemical differences affect toxicokinetics and hazard and neither on which TiO₂ form would be worst case in terms of risk. Hence, it is considered relevant to test multiple forms and to develop a scientifically substantiated read-across argument to cover all forms of TiO₂ applied in food.

In summary, we are of the opinion that the study will provide valuable information. Nevertheless, we have a number of recommendations that we think are important to include:

- Perform the tissue monitoring in such a way that information on the absorption/bioavailability, distribution pattern and clearance is obtained.
- Investigate markers of liver damage (e.g. ALT, AST, ALP, bilirubin) and liver (including bile duct) pathology. Also consider effects on the intestine.
- Apply a benchmark dose approach to get a good dose–response curve which includes both lower dose groups that are more representative for human exposure as well as higher dose groups.

Consider that TiO₂ in E 171 can be present in different ‘forms’ and that the risk assessment of E 171 should cover all.

- 3) It was noted that in your risk assessment, the oral intake of titanium dioxide calculated by Rompelberg et al. (2016) was considered. In that publication, the oral intake of titanium dioxide was calculated from the exposure to food, food supplements and toothpaste. Could you please explain how the use of toothpaste could contribute to the oral intake of titanium dioxide from dietary sources?

Answer: We considered the oral intake of TiO₂ rather than the intake from dietary sources. As toothpaste is ingested to some extent by small children, this was taken into consideration. In Rompelberg et al. (2016) it was therefore assumed that children aged 2–6 years all toothpaste applied on a brush is swallowed. Swallowing the toothpaste for this age group is also advised to achieve a sufficient daily fluoride intake to prevent caries. The self-estimated portion of toothpaste per brushing event was 1.15 ± 0.14 g. When considering lifelong exposure, the contribution of toothpaste is limited.