



To the Dutch Minister for Medical Care

Opinion of the Director of the Office for Risk Assessment and Research

Date 23 July 2019

Re. **Opinion on possible health effects of the food additive titanium dioxide (E171)**

Office for Risk Assessment & Research

Catharijnesingel 59
3511 GG Utrecht
PO Box 43006
3540 AA Utrecht
The Netherlands
www.nvwa.nl

Contact

T +31 (0)88 223 3333
F +31 (0)88 223 3334
risicobeoordeling@nvwa.nl

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Background

Titanium dioxide (TiO₂) is a white colouring agent with many applications, including use in foods. As a food additive with number E171, titanium dioxide is permitted in the quantities required (*quantum satis*). The substance merely acts as a colouring agent and therefore partly determines the appearance of a food and has no nutritional value.

The International Agency for Research on Cancer (IARC) has designated titanium dioxide as possibly carcinogenic to humans after inhalation. In 2017, the European Chemicals Agency (ECHA) published an opinion which proposes that TiO₂ be classified as a category 2 carcinogen after inhalation. In 2016, the European Food Safety Authority (EFSA) published an opinion in which it was concluded that no health effects for humans were to be expected upon (present) oral exposure (i.e. by ingesting it). After publication of the EFSA opinion, a number of articles were published in scientific literature, reporting harmful effects of exposure to E171 in laboratory animals. On behalf of the European Commission, EFSA carried out an analysis in 2018 aimed at the question whether the newly published data were sufficient to amend the previously conducted risk assessment. EFSA concluded that the studies in question were not sufficient for this. EFSA did, however, recommend performing (or ordering) further research. Unaware of the fact that EFSA would carry out an evaluation, BuRO organised a workshop in parallel. One of the publications considered by EFSA concerns research conducted in Maastricht and published online in October 2016. The dissertation containing this article was published in November 2018. The conclusion was that, on the basis of insolubility and inertia, it is no longer valid to assume that E171 has no toxic effects. There are indications that E171 can promote intestinal cancer. This has not yet been demonstrated in humans but raises cause for concern. A recent ANSES report (ANSES, 2019) is in line with this notion.

Questions from BuRO

In response to signals in scientific literature about the harmful effects of the exposure of laboratory animals to E171 and the widespread use of the substance, the Office for Risk Assessment and Research (BuRO) asked the following questions.

1. Do studies with laboratory animals and *in vitro* studies reveal a toxicologically relevant risk after oral exposure to the food additive E171?
2. What are the strong and weak points of these studies?

3. Are the animal models used relevant to the situation regarding humans? Are the established effects relevant to humans?
4. Is the level of exposure used in these models relevant to humans?
5. Can the data from these studies be extrapolated to humans?
6. Are there any epidemiological data on effects in humans after oral exposure?
7. What are the gaps in the knowledge required to perform an adequate assessment of the risk to human health following oral exposure?
8. What follow-up actions can we recommend for the policy relating to public health?

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Approach

To answer the above questions, BuRO organised an international workshop, where researchers who study the effects of the food additive titanium dioxide (E171) were invited to present their work. Scientists who are not involved in such research, but are involved in risk assessments relating to chemicals and work for national institutes that carry out such risk assessments (ANSES, BfR, FHI, RIVM, DTU, FSA, EFSA), were also invited.

BuRO also carried out a literature study regarding exposure to and the effects of E171, to further substantiate the conclusions of the workshop.

BuRO answered the questions based on the report from the workshop and the literature study. The participants in the workshop are not responsible for these answers.

Findings

Please refer to the appendix of this opinion for more information and the workshop report. The findings from the workshop matched the conclusions drawn by EFSA in its opinion in 2018, namely that the studies not evaluated previously were not sufficient to revise the risk assessment performed by EFSA in 2016. However, one conclusion from the workshop participants was that the data recently published do give rise to concern and that further research is required to ascertain whether measures are required, and if so which ones.

Other findings:

1. Although E171 has been used in foods for about 50 years, the concentrations are unknown, as is whether its use is increasing. The latter is likely though.
2. E171 is widely used in confectionery, sauces and baked goods, as well as in toothpaste. It is merely used to improve the appearance of the food (colour, gloss) and has no nutritional value.
3. Children ingest relatively large amounts of E171 per kg body weight per day. However, the exact intake is unknown; estimates vary.
4. There are now indications from laboratory animal and *in vitro* studies that adverse health effects may occur in consumers and in particular in people with increased intestinal permeability, after ingestion of E171. It is unknown whether there is a threshold value for these effects.

Answers to the questions

1. Do studies with laboratory animals and in vitro studies reveal a toxicologically relevant risk after oral exposure to the food additive E171?

Yes. Although only a limited number of studies have been performed involving oral exposure of rats and mice to E171, they do indicate toxicologically relevant effects in the laboratory animals. These studies, which were performed by independent research groups, all point in the same direction, suggesting tumour promotion, while *in vitro* studies appear to confirm this effect.

2. What are the strong and weak points of these studies?

In a few studies, only neoplastic lesions were induced in rats. The studies revealing promotion of colon cancer in mice use models involving chemical induction of colon cancer, or using a transgenic tumour model where exposure to E171 promotes tumour formation. These models are mainly used as research models and dose-response relationships were not properly investigated. This is why it is not really possible to carry out a risk assessment based on these studies. A risk assessment based only on the *in vitro* studies would be inadequate too. On the other hand, the results of the studies carried out at independent research institutes do point in the same direction.

3. Are the animal models used relevant to the situation regarding humans? Are the established effects relevant to humans?

Colon cancer is a major public health issue; its incidence has been increasing over the past 20 years. It is unclear whether the effects observed in the laboratory animal studies are relevant to humans, since it is unknown whether the mechanisms that lead to these effects will also occur in humans. Although the laboratory animal models have their limitations, there is no reason to consider them toxicologically irrelevant.

4. Is the level of exposure used in these models relevant to humans?

The levels of exposure in the oral studies involving laboratory animals for which effects in the colons of rats were found are relevant to humans. Concentrations were used in the laboratory animal studies of 0.2 (no effect) and 5 and 10 mg/kg body weight per day (pre-neoplastic lesions), while 5 mg/kg was used in the studies that show the promotion of colon cancer. These doses are in the same order of magnitude as the 95th percentile of the long-term exposure of the Dutch population of 1.3 mg/kg body weight per day (P95) or the 95th percentile of 14.8 mg/kg body weight per day for children in Europe. It should be noted here that the physicochemical form of E171 as produced by various manufacturers may differ in terms of the particle size distribution. The studies involving mice and rats are possibly not representative for all forms of E171 available on the market. It is not clear whether the absorption of TiO₂ increases at higher intake levels.

5. Can the data from these studies be extrapolated to humans?

Animal testing is used within toxicology in order to extrapolate the data to humans. Differences between laboratory animals and humans and between individual humans are taken into account by using uncertainty factors. The data from laboratory animals for E171 can be extrapolated to humans to a limited degree. However, there are no reliable dose-response data in this case, while it is also unclear whether the mechanisms that form the basis for the effects observed in the laboratory animals will also occur in humans and whether they will occur to the same extent.

6. Are there any epidemiological data on effects in humans after oral exposure?

There are no epidemiological data on adverse effects of E171 in humans, more particularly on a possible association of exposure to E171 with colon cancer. It should be noted that such an association has not been previously studied either.

7. What are the gaps in the knowledge required to perform an adequate assessment of the risk to human health following oral exposure?

A full analysis of the presence of TiO₂ in various foods and a reliable characterisation of the various forms of E171 in foods in terms of particle size distribution are not available. The mechanisms along which the effects observed in laboratory animals occur are still largely unclear. In particular, it is still uncertain whether direct genotoxicity occurs in addition to tumour promotion in the laboratory animals. It is unknown whether the mechanisms of which we are

currently aware also occur in humans after oral exposure. The relevance to humans of the effects of E171 in laboratory animals and in *in vitro* studies is therefore still unclear.

Dose-response relationships on which a risk assessment can be based, such as for tumour formation or tumour promotion, are not available. More information about potentially sensitive groups will also be required. Examples are persons with increased intestinal permeability, as a result of which TiO₂ particles are more easily absorbed into the interstitial tissue of the intestinal tract, with potentially adverse effects.

It should be noted that the trade association of TiO₂ manufacturers, in consultation with EFSA, intends to carry out what is referred to as an 'Extended One-Generation Reproductive Toxicity Study'. This study will not include tumour promotion as an endpoint, but a limited number of intermediary endpoints will be addressed. Such research requires time.

8. What follow-up actions can we recommend for the policy relating to public health?

Research aimed at the relevance of the laboratory animal data to humans is required to ascertain whether the risk observed for exposure to E171 actually also puts consumers at risk. Experimental studies in humans (intervention studies) would appear to be the most appropriate method for this. It is important to focus on differences in intestinal permeability between humans.

Laboratory animal studies with tumour formation as their endpoint or intermediary endpoints that may explain tumour promotion, that involve establishing dose-response relationships, may form the basis for a better risk assessment.

NVWA-BuRO recommendations

To the Dutch Minister for Medical Care

1. Engage in discussion with the food producers to reduce exposure to E171 and/or TiO₂.
2. Examine the contribution of E171 or titanium dioxide in other products, such as medicines, to consumer exposure, especially in people with increased intestinal permeability.
3. In view of the possible adverse health effects of ingesting E171 and in consultation with EFSA, the trade associations intend to conduct additional laboratory animal research – an Extended One-Generation Reproductive Toxicity Study (EORGTS) – in which cohorts are included for reprotoxicological and immunotoxicological tests. BuRO primarily recommends encouraging this research also include analyses of parameters that are important for the development of colon cancer, and for which indications were found in the aforementioned studies that they are influenced by the intake of E171.
4. Study the effects found in laboratory animals and in *in vitro* studies in humans. Even though it is not possible to study colon cancer as a direct result of exposure to E171 in intervention research in humans, it is possible to study intermediary parameters that are associated with colon cancer and for which it was proved in laboratory animals and *in vitro* that they are influenced by E171. The results of such a study will provide insight into the relevance of the laboratory animal and *in vitro* studies. A valid risk assessment will not be possible as long as these studies are not completed.

Yours sincerely,

Prof. dr Antoon Opperhuizen
Director of the Office for Risk Assessment and Research

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SUBSTANTIATION

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E171 (titanium dioxide, TiO₂)

TiO₂ is a colouring agent permitted by the European Union as food additive E171¹. There are various crystalline forms of TiO₂, with anatase and rutile being the most important ones. E171 is used in a wide range of foods, especially in confectionery and chewing gum (see Figure 1). It merely acts as a colouring agent and therefore partly determines the appearance of a product, but has no nutritional value.

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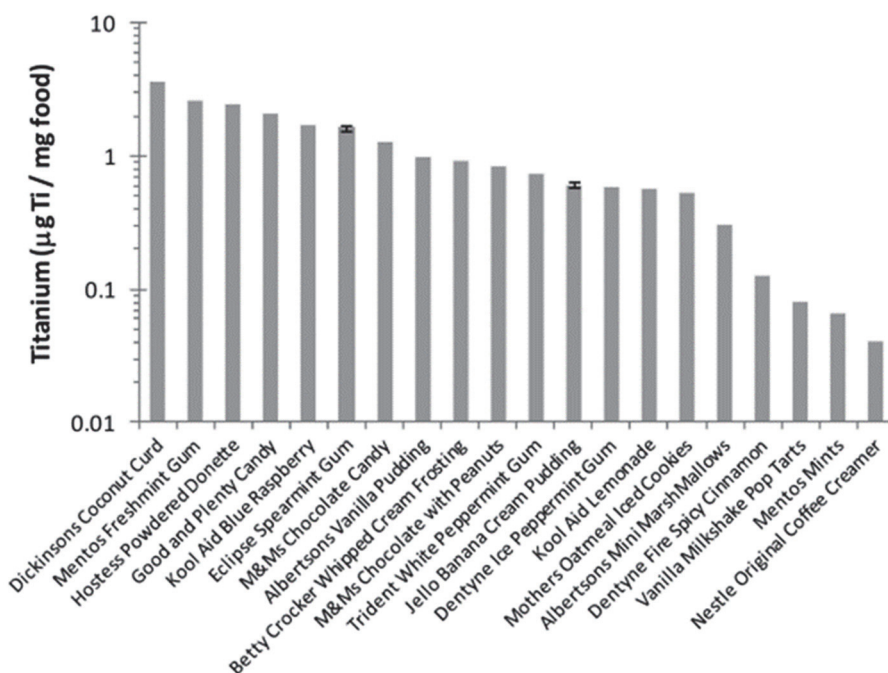


Figure 1. Concentrations titanium in foods (Weir et al., 2012).

Even though the food additive E171 is not considered to be a nanomaterial, because not more than 50% of the particles is larger than 100 nm (Regulation 2015/2283, Article 3(2f)), E171 does contain particles with these dimensions (EFSA, 2016). Peters et al. (2014) found that 10-15% of the particles in E171 were smaller than 100 nm; the primary particle size was between 60 and 300 nm.

Legislation

TiO₂ is registered under E171 as a food additive. The European Commission has classified E171 in Group II, Food colours authorised at *quantum satis*. The anatase form of E171 may be used in many groups of foodstuffs in the required quantities (*quantum satis*) (Food Additives Database² and Regulation (EC) No 1333/2008). There are a few exceptions. Rutile titanium dioxide has also been permitted since 2004 (see Directive 2008/128/EC and (EFSA, 2005)).

¹ Directive 2008/128/EC describes purity specifications currently included in Regulation (EC) No 1333/2008 and defined E171 as: Titanium dioxide consists essentially of pure anatase and/or rutile titanium dioxide which may be coated with small amounts of alumina and/or silica to improve the technological properties of the product.

² https:

//webgate.ec.europa.eu/foods_system/main/?event=substances.search&substances.pagination=1

In 1966 in the US, the Food and Drug Administration (FDA) approved the use of TiO₂ in foodstuffs up to a maximum concentration of 1% by weight³.

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Effects

TiO₂ was evaluated by the FAO/WHO Expert Committee of Food Additives (JECFA) in 1969 and by the Scientific Committee on Food (SCF) in 1975 and 1977. No 'Acceptable Daily Intake (ADI)' was established due to the low solubility, low absorption, absence of tissue accumulation and absence of toxic effects of titanium dioxide (JECFA, 1970). EFSA (EFSA, 2005) considered the rutile and anatase forms of titanium dioxide to be chemically identical, but different in their crystalline structure and light reflection. The EFSA panel decided that the toxicological database applied to both forms due to the identical bioavailability of both forms. The EFSA panel did not establish an ADI either.

The International Agency for Research on Cancer (IARC) has designated titanium dioxide as possibly carcinogenic to humans after inhalation (Group 2B). The IARC working group concluded that there was insufficient evidence in humans for carcinogenicity of titanium dioxide and that there was sufficient evidence in experimental animals for carcinogenicity (IARC, 2010).

The European Chemicals Agency (ECHA) concluded in 2017⁴ that TiO₂ meets the criteria for being potentially carcinogenic after inhalation. The ECHA pointed out that – in terms of the mechanism for the lung cancer observed in rats – this does not constitute classic intrinsic toxicity, since TiO₂ particles deposited in the lungs are deemed to be responsible for the observed toxicity rather than the dissolved form of TiO₂. The European Commission is currently in discussion with the EU Member States to assign a legal status to the hazard classification of the substance.

Intake

EFSA (EFSA ANS Panel, 2016) calculated the intake of the food additive TiO₂ based on maximum levels in foods as reported to the EFSA (defined as the 'maximum level exposure assessment scenario') and the 'use levels' reported to the EFSA by the industry and Member States (defined as the 'refined exposure assessment scenario'). In the 'maximum level exposure assessment scenario', the average exposure was between 0.4 mg/kg body weight per day for babies and the elderly, and 10.4 mg/kg body weight per day for children. The 95th percentile of the intake distribution was 1.2 mg/kg body weight per day for the elderly and 32.4 mg/kg body weight per day for children. In the 'refined estimated exposure scenario', the estimated average intake was between 0.2 (for babies and the elderly) and 5.5 mg/kg body weight per day (children) and the 95th percentile of the intake was between 0.5 (elderly) and 14.8 mg/kg body weight per day (children). As no brand loyalty is expected for the intake of TiO₂, EFSA argued that the latter scenario in which brand loyalty was not applied provided the most realistic estimate for the intake.

Christensen et al. (Christensen et al., 2015) estimated the intake of titanium dioxide at about 1 mg titanium dioxide/kg body weight per day for adults and at 2 mg titanium dioxide/kg body weight per day for children. Chewing gum had the highest concentration of titanium dioxide; the intake quantity of nano titanium dioxide per piece of chewing gum could be as high as 7.5 mg, depending on the brand of chewing gum (Christensen et al., 2015). Powell et al. (Powell et al., 2010) estimated the intake of titanium dioxide at 5 mg/dag in the United Kingdom. Weir et al. (Weir et al., 2012) estimated the average intake of the

³ <https://www.fda.gov/ForIndustry/ColorAdditives/ColorAdditiveInventories/ucm106626.htm>

⁴ <https://echa.europa.eu/nl/-/titanium-dioxide-proposed-to-be-classified-as-suspected-of-causing-cancer-when-inhaled>

British population at 2-3 mg/kg body weight per day for children under ten years of age and at about 1 mg/kg body weight per day for other age groups. For the United States this was respectively 1-2 and 0.2-0.7 mg/kg body weight per day (Weir et al., 2012). In view of the many possible applications of nano titanium dioxide, its use was expected to increase significantly in the future (Christensen et al., 2015).

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Rompelberg et al. estimated the intake of added titanium dioxide (additive E171) for various Dutch population groups using intake data from Dutch food consumption surveys and the concentration data from Peters et al. (Peters et al., 2014; Rompelberg et al., 2016). The titanium in milk and dairy products, for example, which probably comes from the environment or animal feed, was not included in the exposure calculation. For children aged 2-6 years, the estimated intake from toothpaste was included. The average long-term intake of titanium dioxide ranged between 0.67 mg/kg body weight per day for children aged 2-6 years, to 0.17 mg/kg body weight per day for people aged 7-69 years, and 0.06 mg/kg body weight per day for elderly persons aged 70 years and over. The respective P95 values were 1.29, 0.50 and 0.23 mg/kg body weight per day. The foods that contributed most to the titanium dioxide intake were toothpaste (only for children), confectionery, including chewing gum, coffee milk powder, fine pastry products and sauces.

Sprong et al. (Sprong et al., 2015) calculated a median long-term intake of titanium dioxide (E171) of 1.4, 0.7 and 0.5 mg/kg body weight per day for children aged 2-6 years, people aged 7-69 years and people aged 70 years and over, respectively, based on usage concentrations ('use levels') in foods in which E171 is used supplied by the industry in the Netherlands and the quantities of colouring agent that are processed. Use levels of 1603 foods were obtained, 133 of which contain E171. The highest concentrations were reported for flavoured, fermented milk and sauces. The use levels varied greatly within one and the same category of foods. The group of foods that contributed more than 10% to the overall intake were fine (decorated) pastry products, sauces and desserts (mainly custard/vanilla custard). The actual intake is probably lower because of the assumed situation in which all foods in a category were included rather than only those foods to which the colouring agent was actually added (e.g. all cakes rather than just cake with a white layer).

The intake levels estimated for the Netherlands are clearly lower than the intake levels estimated by EFSA.

In the Netherlands, duplicate food samples for about 125 persons are regularly collected each year. In 2014, food samples were collected for children aged 2-6 years. These food samples contain an average of 0.15 mg titanium/day (minimum of 0.02 mg/day and maximum of 4.17 mg/day). In 2011, duplicate food samples were collected for adults. These food samples contain an average of 2.2 mg titanium/day (minimum of 0.5 mg titanium/day and maximum of 8.1 mg titanium/day). These results show that there can be major differences in intake and that the intake levels estimated above are probably significant overestimations of the actual intake.

Absorption

Heringa et al. (Heringa et al., 2016) prepared an overview of all the studies into the absorption of TiO₂. These researchers concluded that it is highly unlikely that no oral absorption at all occurs. As various studies (including ones in humans) show an increase in blood or tissue concentrations, it can be concluded that TiO₂ particles and nanoparticles are absorbed, but only in very small percentages (0.02% (Geraets et al., 2014), 0.06% (Bachler et al., 2015)).

Even though most studies indicate that limited absorption of TiO₂ occurs in rats and humans (Cho et al., 2013; Geraets et al., 2014; Jones et al., 2015), clear absorption of TiO₂ nanoparticles was observed in laboratory animals via what are called 'microfold cells (M-cells)', which envelop the Peyer's patches (Brun et al., 2014; Janer et al., 2014). In humans, deposition of TiO₂ particles was also observed in the Peyer's patches, especially in humans suffering from inflammatory bowel disease (IBD) (Powell et al., 1996; Hummel et al., 2014). The study by Heringa et al. (Heringa et al., 2018) showed that the presence of TiO₂ does not remain limited to the intestinal tract, but that it also ends up in the human liver and spleen.

Toxicokinetics

Heringa et al. (Heringa et al., 2016) carried out a risk assessment of TiO₂ in which toxicokinetic information was included. They based their assessment on exposure estimates for the Dutch population (Rompelberg et al., 2016); toxicokinetic information reported by Geraets et al. (Geraets et al., 2014) and toxicological studies (NCI, 1979; Wang et al., 2013; Jia et al., 2014; Tassinari et al., 2014). Based on oral exposure estimated using external dosages, no risk of adverse effects was expected in humans, except for a potential effect on ovaries. However, if toxicokinetic information on TiO₂ in nanoform was included, effects in the liver, ovaries and testes could not be excluded. Christensen et al. (Christensen et al., 2015) estimated that about 20-40% of the particles of titanium dioxide (TiO₂, E171) used as a food additive are smaller than 100 nm, depending on the type of food.

Heringa et al. (Heringa et al., 2018) carried out measurements in the livers and spleens of deceased persons. They found both total titanium and nanosized TiO₂. The quantity was lower than what can be considered safe for laboratory animals, but partially higher than the levels that can be considered safe for humans after applying conventional safety factors.

Apart from uncertainty about exposure and toxicokinetics, there is also uncertainty about the harmful effects of TiO₂, and E171 in particular. Most studies of TiO₂ were not conducted using the food additive E171 and they are mainly inhalation studies.

Risk assessment

For an initial risk assessment of the food additive TiO₂, please refer to the EFSA opinion (EFSA ANS Panel, 2016). The conclusion in the latter is that the current application of TiO₂ entails no risk of adverse effects for consumers. EFSA based its conclusion on the limited absorption and bioavailability of TiO₂ after oral exposure and the fact that almost all TiO₂ leaves the body via the faeces in unaltered form. Only a small part is absorbed by lymph tissue in the intestines and then ends up in various organs. EFSA concluded that there were no indications of genotoxicity. EFSA did, however, report that there were indications of reprotoxic effects, although these were observed in studies that had not been conducted with E171, but with incompletely characterised TiO₂ as a test material. As no 90-day oral toxicity study, multi-generation study or extended one generation reproduction toxicity (EORGT) study with E171 is available, EFSA was unable to determine an ADI. Based on a carcinogenicity study of TiO₂ in mice and rats, EFSA set the No Observed Adverse Effect Level (NOAEL) at 2250 mg TiO₂/kg body weight per day, which was the highest dose administered in the relevant study (EFSA ANS Panel, 2016).

Tumour (promotion)

EFSA (EFSA ANS Panel, 2016) concluded that there is no risk of adverse effects in consumers for the current application of TiO₂. However, indications have by now been published in scientific literature that TiO₂ could affect tumour development. Apart from effects of TiO₂ found in the studies conducted by the NCI (NCI, 1979), Wang et al. (Wang et al., 2013), Jia et al. (Jia et al., 2014) and Tassinari et al. (Tassinari et al., 2014), a number of studies were also conducted with the food additive E171, which indicate potential tumour promotion. Urrutia-Ortega et al. (Urrutia-Ortega et al., 2016) found an increase in colon tumour numbers in mice that were exposed to 5 mg E171/kg body weight per day in combination with the tumour initiators azoxymethane (AOM) and dextran sulphate sodium (DSS). They also found that the expression of markers associated with tumour development, such as COX2, Ki67 and β -catenin, had increased. The risk assessment carried out by EFSA in 2016 referred to this study, but in view of the fact that this study was conducted with only a single dose and considering the uncertainty about the model's relevance to humans, this study was not included in the assessment.

In follow-up studies conducted by Proquin et al. (Proquin et al., 2018a), a transgenic Cre-LoxP mouse model was used, in which colon tumours developed spontaneously. The development of colon tumours was also stimulated in this model through oral exposure to 5 mg E171/kg body weight per day for nine weeks.

In both laboratory animal models, the effects of the exposure to E171 on gene expression were studied. In the model where colon tumours were induced by AOM and DSS and where the animals were exposed to E171 prior to the development of tumours, the expression of genes associated with the immune system was inhibited. In addition, effects were found on the expression of genes that are potentially involved in the production of antioxidants. Furthermore, modulation of genes associated with the identification of (colorectal) cancer was observed during the period of exposure, but also before tumours formed. These data obtained by means of transcriptomics represented early biological effects that were the result of exposure to E171 and preceded tumour development in this AOM/DSS mouse model (Proquin et al., 2017; Proquin et al., 2018b). The transgenic Cre-LoxP model confirmed these effects of E171 on gene expression, with the biological processes affected by E171 being followed by tumour development (Proquin et al., 2018a). Although any causality has yet to be proven, it would seem obvious that these effects resulted in tumour promotion.

Bettini et al. (Bettini et al., 2017) studied tissue distribution and immunotoxicological effects of E171 in rats that were orally exposed to 10 mg/kg body weight per day for seven days. TiO₂ was found in the Peyer's patches, whilst the frequency of dendritic cells involved in antigen presentation had increased and the frequency of regulatory T cells involved in the suppression of immune responses had decreased. This effect was still observed hundred days after ending the exposure. Systemic effects were also found, in particular an increased ratio of Th1/Th17 cells. Even though no inflammatory lesions were found in the intestines, promotion of microinflammation was observed, as well as initiation of preneoplastic lesions⁵. Promotion of aberrant crypt foci in the colon was also observed in the model, which involved simultaneous exposure to the carcinogen dimethylhydrazine.

⁵ The development of primary tumors is often preceded, both in humans and experimental animals (mainly rodents), by the appearance of lesions referred to as preneoplastic. These consist of genetically and phenotypically altered cells exhibiting a higher risk of malignant evolution than normal cells. These lesions generally lack one of the principal characteristics of neoplastic lesions: the capacity to grow autonomously after cessation of the stimuli that induced the lesion (Encyclopedia of Cancer; Schwab, 2017).

Even though the studies by Urrutia-Ortega et al. (Urrutia-Ortega et al., 2016; Proquin, 2018c) and Bettini et al. (Bettini et al., 2017) were performed using different laboratory animal models, the results of the studies all point in the same direction, i.e. that E171 ultimately causes the formation of colon tumours by means of oxidative stress, effects on the immune system, effects on the signalling and induction of histopathological effects such as microinflammation and preneoplastic lesions in the colon.

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The findings are supported by *in vitro* studies. *In vitro* studies carried out by Proquin et al. (Proquin et al., 2017) demonstrated that E171 was capable of producing reactive types of oxygen, whilst chromosomal damage was observed in Caco-2 cells and HCT116 intestinal epithelial cells. Dorier et al. (Dorier et al., 2017) conducted experiments with Caco-2 cells and with a co-culture of Caco-2 cells and mucus-producing HT29-MTX cells. E171 induced toxicity in these models through the accumulation of reactive types of oxygen, accompanied by a decreased expression of the antioxidant enzymes catalase, superoxide dismutase and glutathione reductase. DNA damage was found in these cells, suggesting that E171 induces oxidative stress, which leads to DNA damage.

On 15 April 2019, ANSES, the French National Authority for Food Safety, the Environment and Employment, issued an opinion on risks related to the intake of the food additive E171 (ANSES, 2019), in which they re-evaluated potential carcinogenic effects of E171 in the context of publications that have been published since the article by Bettini et al. (Bettini et al., 2017). The report includes the following observations.

In *in vitro* studies with human intestinal cell cultures, E171 particles appear to accumulate in the mucus secreted by the cells. However, the role of barrier and facilitation (due to accumulation) of this mucus is not yet clearly elucidated. In systems mimicking digestive conditions E171 is biodegradable and dissolves very slightly. Physicochemical conditions vary throughout the digestive tract which leads to dynamic agglomeration processes. Translocation mechanisms and reactivity in the intestinal barrier may likely generate lymphocytic inflammation. Given the persistence of TiO₂ and tissue accumulation of internalized particles (half-life: 650 days), even if the absorption rate is low, there may be a long-term toxic potential of E171.

A number of *in vitro* studies have been performed to assess potential genotoxic effects of TiO₂ particles. A genotoxic effect was reported in 60% of studies (among which only one study that was conducted with E171). The majority of publications reviewed showed that the genotoxic effect was caused by a secondary mechanism via oxidative stress. One *in vivo* study was performed with E171 since 2017, but the ANSES report concluded that the study protocol applied was not relevant (doses of E171 used were not representative of potential exposure data in humans and were administered only once a week for ten weeks. In addition, DNA damage was not evaluated in the gastrointestinal tract. Moreover, primary alterations to DNA were measured 24 hours after the last administration which allowed the repair phenomena to take place. There was a weak correlation between the length of telomeres and doubling time. And finally, data expressed were pooled and expressed in relative terms, while no positive controls were used. Hence, although there are no reliable studies showing a direct interaction of TiO₂ particles with DNA and/or the mitotic apparatus, ANSES considered that direct effects of TiO₂ on genetic material or other molecules interacting with the genetic material cannot be excluded.

In addition, ANSES considered that the carcinogenesis studies (NCI, 1979) were carried out without any prior characterization of Unitane® (anatase, unspecified

size distribution) which was assumed to be food grade. EFSA (EFSA ANS Panel, 2016) concluded, based on the NTP studies, that E171 is not carcinogenic. However, there is no information on similarities or differences between the physicochemical characteristics of Unitane® and E171. The tumour promoter-like potential of E171 was experimentally observed by Urrutia-Ortega et al. (Urrutia-Ortega et al., 2016) and Bettini et al. (Bettini et al., 2017) and studies by Proquin et al. (Proquin et al., 2018a; Proquin et al., 2018b; Proquin, 2018c) corroborate the concern raised by ANSES (ANSES, 2019) during its analysis of the study of Bettini et al. (Bettini et al., 2017). But also these studies had shortcomings, for which reasons ANSES considers that these data need to be confirmed by setting up new studies including the use of several biomarkers relevant to carcinogenesis.

In summary, although ANSES indicates that the results of the INRA study (Bettini et al., 2017) and a number of recently published studies are not sufficient to call into question EFSA's assessment of E171, ANSES underlines the need to carry out studies to fully characterise the hazard related to E171, with a methodology and timeframe to be defined. ANSES also argues that their review indicates effects of E171 that have not yet been identified, in particular possible promoting effects on carcinogenesis. These possible effects of E171, which have been observed in relation to the colon, need to be confirmed by additional studies. In addition, ANSES points to other ongoing studies in France, which will be published shortly and which describe other potential effects of TiO₂. These studies relate in particular to the ability of TiO₂ to cross the blood-brain barrier. ANSES wants these results to be examined by EFSA as part of its assessment of food additives. ANSES also indicates that TiO₂ is present in many foods, as well as in various medicines.

Ongoing and future research

Even though a lot of research is being conducted regarding TiO₂, especially as a nanomaterial, and mainly after inhalation exposure, only a limited amount of research is conducted into the effects of oral exposure. RIVM and RIKILT are active in the Netherlands in the field of (analytical) methods for measuring and estimating exposure. As indicated above, the titanium dioxide manufacturers intend to conduct an Extended One-Generation Reproductive Toxicity Study (EOGRTS) of E171 in consultation with EFSA. More mechanistically oriented research into the effects of exposure to E171 is being performed in France (Toulouse, Toxalim) and Grenoble (Université Grenoble Alpes), as well as at Maastricht University. In Zurich (Universität Zürich), clinical research is being conducted in patients, mainly aimed at the effects in the bowels of patients with IBD. Maastricht University has plans to conduct intervention research to study whether mechanisms observed in laboratory animals and in *in vitro* studies also apply to humans as a result of exposure to E171.

Further information on the current EFSA activities on titanium dioxide (E171) can be found in the latest EFSA statement published on 13 May 2019 on the review of the risk related to the exposure to E171 performed by ANSES⁶. In this statement, in addition to EFSA's opinion on the new scientific evidence assessed by ANSES, useful information on the ongoing toxicological studies being performed by interested business operators and their timeline can be found (on page 10). Regarding the ongoing assessment by EFSA of the data submitted by interested business operators on the particle size distribution of titanium dioxide (E171), information is publicly available in the EFSA Register of Questions⁷. This latter

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⁶ <https://www.efsa.europa.eu/en/efsajournal/pub/5714>

⁷ <http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2018-00625>

assessment is ongoing with a deadline for its finalisation at the latest by 15 September 2019.

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Conclusions and discussion

In 2016, EFSA concluded that no harmful effects are to be expected for the current levels of exposure to E171. Even though effects of E171 have by now been observed, these studies are currently not yet sufficient to conduct a well-substantiated risk assessment. The studies conducted in mice and rats that provide an indication of tumour promotion by E171 in the intestinal tract were not conducted in accordance with OECD guidelines and should be considered exploratory. This conclusion is in line with EFSA's evaluation in 2018.

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Despite this, the publications published since 2016 do give rise to concern, also in light of the increased incidence of colon cancer among the Dutch population. In 1990, the Netherlands Comprehensive Cancer Organisation reported a colon cancer incidence of 4621 cases per 100,000 persons per year. In 2018, the incidence was 9585 per 100,000 persons per year. This involves a total of 53,934 people in the Netherlands (prevalence in 2018)⁸. Even though no direct connection can be made with exposure to TiO₂, the results of the described studies provide grounds to investigate this further with realistic intake doses.

This conclusion is in line with the conclusions drawn by the workshop participants (see appendix). In consultation with EFSA, the trade association intends to conduct an EORGTS, mainly aimed at the question whether a risk assessment can be performed based on reproductive effects. With regard to the potential tumour-promoting effect of E171, it is doubtful whether the performance of an EORGTS or chronic exposure test would be suitable for detecting the promotion of colon cancer. Colon tumours are rare in laboratory animals (Johnson & Fleet, 2013) and regular mice and rats are therefore possibly not sensitive enough to detect such effects. Whereas regular wild-type mice and rats are a good model for healthy individuals, the question is whether regular animals are also a good model for individuals with increased intestinal permeability, such as individuals who have IBD (inflammatory bowel disease) or Crohn's disease.

In the proposed follow-up research of laboratory animals (the EORGT study in particular), the cohort for detecting immunotoxicological effects is also important in addition to the cohort for reprotoxicological effects, in view of the findings relating to immunotoxicological effects in the literature. Furthermore, these studies should also include analyses that are important for the development of colon cancer, for which indications were found in the aforementioned studies that this is influenced by exposure to E171.

So far, research of E171 has been limited to laboratory animal studies and *in vitro* studies, but further research in humans is required to establish the relevance of these findings, such as in the form of intervention research. Whereas it is not possible to study colon cancer as a direct result of exposure to E171 in intervention research, it is possible to study intermediary parameters that are associated with the development of colon cancer and for which it was proved in laboratory animals and *in vitro* that they are influenced by the intake of E171.

A well-substantiated risk assessment will not be possible as long as these studies are not completed. The concern to which the aforementioned studies give rise is supported by the results of the workshop.

⁸ Source: Nederlandse Kankerregistratie (NKR), IKNL (Netherlands Comprehensive Cancer Organisation); <https://www.cijfersoverkanker.nl/>

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List of abbreviations

ADI	Acceptable Daily Intake
ANSES	Agence nationale de sécurité sanitaire, de l'alimentation, de l'environnement et du travail
AOM	azoxymethaan
BfR	Bundesinstitut für Risikobewertung
DSS	Dextran Sulfate Sodium
DTU	Technical University of Denmark
ECHA	European Chemical Agency
EFSA	European Food Safety Authority
EOGRT(S)	Extended one generation reproduction toxicity (study)
IBD	Inflammatory Bowel Disease(s)
FHI	Folkeshelse Instituttet
FSA	Food Standards Agency
JECFA	FAO/WHO Expert Committee of Food Additives
NOAEL	No observed adverse effect level
OECD	Organization for Economic Cooperation and Development
SCF	Scientific Committee on Food

APPENDIX

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Report of the Workshop on Possible adverse effects of food additive E171 (titanium dioxide), organized by the Netherlands Food and Consumer Product Safety Authority (NVWA)
Amsterdam, July 5-6, 2018

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Chairmen's preamble

Titanium dioxide (TiO₂) is extracted from ilmenite mineral and it has three crystalline forms: rutile, anatase, and brookite. Rutile is the most stable form, while anatase and brookite convert to rutile at temperatures above 550°C and 750°C, respectively. Initially it was predominantly used as a white pigment in oil paints and started to be massively produced in the beginning of the 20th century. TiO₂ has a high refractive index and is resistant to UV radiation, which is the reason why it is also used in sun blockers to protect the skin from sunburn. In addition to the widespread use of TiO₂ as white pigment in many products including paints and coatings, it is also applied in personal care products and a wide range of processed foods. The market value of TiO₂ was around € 3 billion in 2017.

In food, the rutile and anatase forms are allowed for use as an additive under the name of E171. E171 is a white pigment and the peak of the size distribution is around 200-300 nm as these particles cause the white colour. E171 contains a fraction (10 to 40% by number) of particles <100 nm. The main commercial reason for its use is that consumers are more likely to buy and eat products that are brighter or more vibrant in colour because they look fresher. E171 delivers whiteness, opacity, and creamy effects. The number of companies in the food industry using E171 as an ingredient is large with 289,000 companies worldwide.

The effects of TiO₂ on human health have been mostly studied after exposure via inhalation and were found to be tumorigenic for lungs in rats. A causal relationship has been established between TiO₂ and an increase of both malignant and benign lung tumours reported in inhalation or instillation studies. Although the detailed mode of action is still unclear, an inflammatory process and indirect genotoxic effect by reactive oxygen species (ROS) production seems to be the major mechanism to explain the effects induced by TiO₂. It is considered that this mode of action is principally coupled to the biopersistence and poor solubility of TiO₂ particles. TiO₂ was reported to be observed in the cell nucleus in various *in vitro* and *in vivo* studies. Even if this finding is still debated, a genotoxic effect by direct interaction with DNA cannot be excluded. Subsequent to these inhalation studies in animals, in 2010 the International Agency of Research on Cancer (IARC) has classified TiO₂ as a possible carcinogen to humans after inhalation. The RAC committee by European Chemicals Agency (ECHA) has proposed to classify TiO₂ as Carc. Cat 2 for the inhalation route only, following a proposal by France.

In addition to inhalatory exposure, humans are exposed to TiO₂ by oral ingestion and dermal application. In Europe, manufacturers are allowed to use TiO₂ at *quantum satis* in foods since 1969, after an assessment made by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The evaluation was performed based on earlier studies assessing TiO₂ absorption and toxicity in animals and humans after oral administration. Ingestion of TiO₂ occurs every day through the high number of products to which it is added. The quantities ingested by humans were estimated in several studies in the UK, USA and the Netherlands. In the Netherlands, exposure was estimated to reach an average of around 0.7 mg TiO₂/kg bw/day for young children at the age of 2-6, and around 0.2 mg TiO₂/kg bw/day at ages from 7 to 69. Elderly, 70 years and older were estimated to ingest around 0.1 mg TiO₂/kg bw/day. More recently, the European Food Safety

Authority (EFSA) performed an estimation of the average daily exposure to TiO₂ from its use as a food additive across 33 dietary surveys carried out in 19 EU countries. This estimation was based on a non-brand-loyal scenario which assumed that the population is exposed to the mean reported use/analytical levels of TiO₂ in 14 food categories for a long period of time. In this scenario validated by EFSA, which was the most appropriate and realistic scenario for risk characterization, it was estimated that the mean exposures ranged from 0.2 mg/kg bw/day for infants (from 12 weeks to 11 months) to 5.5 mg/kg bw/day for children (3 to 9 years old). At the 95th percentile, EFSA estimated the exposure could reach 14.8 mg/kg bw/day for children. Whereas TiO₂ was originally considered to be unable to translocate from the gut lumen into the interstitial tissues, in rodents TiO₂ translocation to the blood stream and accumulation in other organs has recently been shown, even if the bioavailability is low. Yet, after ingestion, TiO₂ nanoparticles have been found in intestinal mucosa, colon epithelial cells, Peyer's patches, liver, spleen, brain, testis, ovaries, thyroid, lungs as well as blood. This implies that body cells can become in contact with the particles and adverse effects may ensue from this interaction. Poor elimination was observed, which suggests that the TiO₂ particles may accumulate in time. Hence, assessment of potential toxic effects such as inflammation, DNA damage, effects on the immune system and cancer is relevant. Indeed, such effects have been described to occur in experimental studies whereas in other studies such effects were not observed. Considering the signals on potential toxicity in scientific literature and the wide-spread use of E171, further assessment of the robustness of the available information and their value for risk assessment and regulatory purposes is needed.

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Aim and organization of the workshop

The Netherlands Food and Consumer Product Safety Authority organized a workshop to evaluate the data on potential adverse effects of oral ingestion of the food additive E171.

The specific aims were:

1. to present an overview of studies on the biological effects of E171 and the potential health effects of food grade TiO₂, and in particular;
2. to appraise the quality of *in vivo* studies available in terms of the conclusions made;
3. to conclude whether these studies point to mechanisms that may underlie effects of E171;
4. to evaluate whether the data provide information on a dose-effect relationship;
5. to conclude whether the studies give rise to a concern for safety in the population consuming E171;
6. to decide if the current data would be sufficient for a re-assessment of the risk of E171; and
7. if not, to identify data gaps, in particular what is missing for an appropriate risk assessment for regulatory purposes.

Participants to the workshop were:

- Prof. Yolanda Chirino, Universidad Nacional Autónoma de México, Mexico
- Dr Hubert Dirven, Norwegian Institute of Public Health, Norway
- Dr David Gott, Food Standards Agency, UK
- Dr Minne Heringa, RIVM, The Netherlands
- Dr Eric Houdeau, INRA, France
- Prof. Theo de Kok, Maastricht University, The Netherlands
- Dr Federica Lodi, EFSA
- Dr Hubert Noteborn, NVWA/EFSA Scientific Committee, The Netherlands
- Dr Agnes Oomen, RIVM, The Netherlands
- Dr Morten Poulsen, DTU, Denmark

- Prof. Gerhard Rogler, Klinik für Gastroenterologie und Hepatologie, Zürich, Switzerland
- Dr Holger Sieg, BfR, Germany
- Dr Bruno Teste, ANSES, France
- Prof. Ruud Woutersen, The Netherlands

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The workshop was organized on behalf of the Netherlands Food and Consumer Safety Authority by:

- Dr Jacqueline Castenmiller
- Prof. Antoon Opperhuizen
- Dr Dick Sijm
- Dr Dirk van Aken
- Prof. Henk van Loveren

Background documents, agenda and lectures presented

Background documents are listed in Annex I of this report.

The full agenda is presented in Annex II.

The lectures presented in the workshop are listed in Annex III.

Summary of the presentations

Dick Sijm welcomed the participants of the workshop and introduced the Netherlands Food and Consumer Product Safety Authority (NVWA) and in particular the Office of Risk Assessment & Research (BuRO). One of the tasks of BuRO is to advise NVWA as well as the ministries of Health, Welfare and Sport and of Agriculture, Nature and Food Quality on food safety issues. A key element of assessing risk is combining the information on toxicology (hazard) and exposure. In the case of E171, there is information on exposure and it is clear that consumers are exposed to E171 via food. It is also no longer disputed that following inhalation, titanium dioxide (TiO₂) is a carcinogen in rats. The concern that is currently arising is whether E171, which consists of TiO₂, may play a role in cancer formation following oral intake. This concern prompted the initiative taken by BuRO to invite leading experts in this area for discussions at the present meeting.

Henk van Loveren explained the aim of the workshop. The focus is on E171, and not on TiO₂ as used in non-food applications. An alert for health effects of E171 was the work done by Prof. Yolanda Chirino, together with Maastricht University, who observed signs of tumour promotion in a mouse model. In addition, comparable results were found in studies of other institutions (INRA and University of Zürich). In the same period when BuRO picked up the idea to organize a workshop, EFSA had to appraise some of the research mentioned in order to agree on the need for re-evaluating the EFSA opinion on E171 published in 2016. The focus of the current workshop is to discuss, on a scientific level, the status of different studies on E171.

Minne Heringa presented three papers on Dutch oral intake studies and the subsequent risk assessment carried out. The P95 of chronic oral intake of TiO₂ from foods, food supplements and toothpaste in the Dutch population (2-6 years, 7-69 years, 70+ years,) is 1.67 mg/kg bw/day. For children, toothpaste is assumed to be an important route of intake. Risk assessment was first based on conventional methodology, providing a margin of exposure with the critical toxicological endpoint. They also used a toxicokinetic model for experimental animals and humans translating oral intake levels to internal organ levels. The subsequent risk assessment based on tissue concentrations yielded that the margin of (internal) exposure was too limited for liver (only 36-122 for one key study). Thus, health effects could not be excluded.

This risk assessment was further refined by measurement of human liver and spleen levels. Post-mortem human liver and spleen samples from 15 individuals were analysed for TiO₂ nanoparticles and elemental Ti. In liver and spleen almost all TiO₂ was present as particles, of which at least 24% was in the nano range (<100 nm). Similar ranges of nanoparticles were found in the spleen and liver. Again, the margin of (internal) exposure was too limited for liver (only 9-40), thus health effects could not be excluded.

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Uncertainties were discussed. One uncertainty refers to the size ranges of the nanoparticles, and the assumption that toxicity may only stem from nanoparticles. Another uncertainty is the duration of exposure, which in humans may be lifelong, in contrast to the toxicity studies used for the risk assessment. Another uncertainty pertains to the type and completeness of the parameters included in the toxicity testing performed. Criticism was expressed on applying an intraspecies factor of 10 in the assessment including toxicokinetics, as the WHO has subdivided this factor into a factor of 3.16 for toxicokinetic differences and 3.16 for dynamic differences. It was indicated that it is to be assessed by e.g. EFSA if the present margin of internal exposure (factor 9-40 based on present data) is sufficient. In addition, some humans show a severely impaired gastrointestinal barrier function, and the absorption rate used for the risk assessment could be highly underestimated. It is unclear if this is sufficiently covered by the safety factors. Another uncertainty mentioned regarded the fact that the medical history of the persons from whom the post-mortem samples were obtained, was unknown. The subjects could have been poly-medicated patients. TiO₂ is a main ingredient in many drugs.

With regard to potential genotoxicity and tumour formation in the intestine, uncertainties also pertain to: 1) rate of absorption in the colon of healthy individuals, 2) genotoxic mechanisms, and 3) immunotoxic mechanisms (tumour progression). It was proposed to use Adverse Outcome Pathways (AOPs) to organize all effects found into a weight-of-evidence approach and to use the recently published Nanomaterial Genotoxicity Testing Roadmap of an ILSI-HESI working group to filter the conflicting genotoxicity outcomes.

Gerhard Rogler discussed inflammatory responses induced by TiO₂ in the gut. In Switzerland there are 12,000-15,000 adults with inflammatory bowel disease (IBD) per year. It is estimated that although these conditions are partially genetically determined (30%), environmental and lifestyle factors play a prominent role (70%). In China, where an increasing proportion of the population is adapting more western-style diets, the incidence of IBD is increasing in cities, while there are no IBD cases in the villages.

One potential mechanism that may underlie these conditions is activation of the inflammasome (via NLRP3) by inorganic particles. Food grade TiO₂ particles were found to affect colon epithelial cells in mice that showed a decreased mucous layer that was treatment-related. In *in vitro* models TiO₂ is shown to be taken up by the colon. At the same concentrations, the rutile form is taken up more readily than the anatase form. In these cells, TiO₂ can trigger the production of reactive oxygen species (ROS). Immune-stimulating effects, as evidenced by interleukin release, are also seen. It should be mentioned that these effects are not specific for TiO₂, as aluminium silicate particles also show the same type of effect and may therefore depend on the particle nature rather than the chemical nature of the material. It is estimated that around 1% of the population has a decreased mucous layer. Patients with IBD present (slightly) elevated serum levels of titanium, while highly elevated levels were found in ulcerative colitis (UC) active patients and patients with Crohn's disease (CD). Also, higher levels were observed in other locations of inflammation (joints, liver),

which are often coupled with IBD. The implication of this finding is that it may be clinically relevant to reduce TiO₂ intake in patients with IBD.

Eric Houdeau discussed the biology of the gastrointestinal tract (GIT) and the impact of E171 in the context of one week versus 100 days of oral exposure through drinking water in rats. Accumulation of TiO₂ is found in the Peyer's patches (organized lymphoid follicles), that serve as immune sensors of the gut. Microscopically, Peyer's patches appear as oval or round lymphoid follicles - similar to lymph nodes - that are located in the submucosal layer of the ileum and extend into the mucosa. There are no Peyer's patches in the colon. Of note, TiO₂ accumulation has been also reported in human Peyer's patches. The TiO₂ particles are not found in the M-cells of the Peyer's patches, but in the immune cells. The level of TiO₂ increased with age from adolescence to adulthood. This indicates a progressive accumulation of TiO₂ particles of dietary origin.

In vitro, cytotoxicity of T-cells isolated from Peyer's patches is observed after exposure to TiO₂, possibly explaining the observed fall in T-cell populations in the Peyer's patches from E171-exposed rats. An effect on the T-cells (total and T-regulatory cells) may lead to local immunosuppression in the gut, whereas a potent recruitment of Th1/Th17 immune cells in the spleen indicated E171 favouring a proinflammatory status at the systemic level. It is remarkable that the effect of food-grade E171 on T-cells is stronger than that of TiO₂ nanoparticles of 25 nm, and still observed after 100 days of E171 treatment.

The thickness of the mucous layer is sex-dependent and so is the permeability for different agents including TiO₂ nanoparticles that accumulate in the mucous layer (mucosa), possibly indicating a gender effect of TiO₂. Also, the mucous layer is thicker in the colon than in the small intestine. In addition to direct effects of TiO₂ on the gut epithelium, TiO₂ also affects the growth of bacteria in the gut, with delayed bacterial growth with increasing levels of TiO₂. This may cause indirect health effects in the host.

Exposure of rats to 10 mg TiO₂/kg bw/day for 100 days showed only low-grade inflammation in the colon mucosa, despite local translocation of TiO₂, and no inflammasome activation. However, initiation of preneoplastic lesions and an increased number of preneoplastic lesions were observed after 100 days of exposure of rats to 10 mg/kg bw/day, but not at a 200 µg/kg bw/day, suggesting the NOAEL for colorectal cancer promotion ranging between these two dose levels in rodents.

Yolanda Chirino mentioned that tortillas contain E171. Tortillas are main food items in Mexico with a consumption of at least 1 kg per week per capita. The TiO₂ content, however, is not known nor is there information on E171 in other food products in Mexico. Evidence from the literature on patients with Crohn's disease or ulcerative colitis that had high levels of titanium, led her group to hypothesize that E171 could enhance inflammatory-dependent tumour formation in the colon. In a mouse model, azoxymethane (AOM), a potent carcinogen, in combination with dextran sodium sulphate (DSS), an intestinal epithelial irritant, were used to induce colorectal cancer. E171 alone decreased the content of goblet cells (cells that secrete mucin) without tumour formation. In addition, increased tumour formation was observed when E171 was co-administered with AOM and DSS. *Ex vivo* cultivated colon cells exposed to E171 accumulated particles inside the cells and after cell division, cells were able to retain the particles. The exposure in these experiments was by gavage, but currently the effects of E171 in liquid foods (yoghurt, toothpaste, etc.) and solid foods (candy, etc.) are studied and compared with exposure through gavage. In addition, studies are carried out in young and adult mice. Preliminary data indicate that E171 in solid foods caused some

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inflammation in the colon, but that E171 in a liquid matrix caused more severe inflammation. In addition, preliminary data also showed some effects on haematological parameters, including decreased haematocrit values and decreased numbers of lymphocytic and phagocytic cells that remarkably were more pronounced in adult than in young mice.

Theo de Kok presented mechanistic studies using gene expression profiling after exposure of mice to E171. In normal mice exposed to E171, gene expression changes were found that were related to DNA repair, GPCR and olfactory receptors, cancer signalling and development of colon cancer. In mice treated with azoxymethane (AOM) and dextran sodium sulphate (DSS), gene expression changes were studied before the formation of colon tumours. Expression of larger numbers of genes was observed than in mice treated with E171 only. The time course showed that the responses changed from increased signalling (2 days) to various responses at metabolism indicators and downregulation of the immune system (7 and 14 days), to initiation of tumour formation (21 days).

A murine model of colon cancer comprised a transgenic mouse with a specific knock-out in the colon resulting in spontaneous development of colorectal tumours. Exposure to E171 increased the number of mice with tumours as well as the number of tumours per mouse. The dose response was unclear, which may be caused by aggregation of the particles at the higher concentrations. The concentration of titanium in the tissues was not measured. There was a strong overlap among the biological processes reflected by the E171-induced gene expression profiles in the various animal models.

In earlier studies, E171 had already shown to induce ROS and genotoxicity in an *in vitro* model with the human colon-derived Caco-2 cell line. Gene expression profiling in these cells after E171 exposure showed that E171 modulated relevant molecular pathways related to cancer development: oxidative stress, inflammation, immune response and cancer signalling. Even if the genes affected in the different models were not identical, the processes in the different models were remarkably similar. The discussion of these results focused on the relevance of gene expression changes to reflect adversity. Altered gene expression does not equal tumour formation, but may reflect initial processes that precede tumour formation, in particular in time course studies. It was concluded that this evidence, as all evidence, is only limited in its ability to predict the actual risk of TiO₂ but may bridge the gap between *in vitro* data and *in vivo* data in animals and form another piece of the puzzle. More clear relationships between the gene expression changes and the tissue level effects ("phenotypic anchoring") was stated to be necessary to gain more confidence in the predictivity of gene expression changes. Furthermore, in analogy with the animal studies, establishing gene expression changes in humans may be valuable as these parameters could easily be assessed in human intervention studies and may demonstrate the potential activation of crucial processes related to tumorigenesis under relevant human exposure conditions.

David Gott presented the risk assessment of food grade titanium dioxide (E171) by EFSA as originally performed by EFSA in 2016, and the recent EFSA evaluation in 2018 of four selected studies on E171 exposure. The conclusion of the 2016 opinion was that based on the database available at that time and at the current exposure levels, the safety of E171 as a food additive was considered of no concern in the European population. It was noted that the information on particle size distribution in E171 is limited. In addition, the data set available to EFSA was found to lack data on reproductive toxicity. For this reason, EFSA has proposed that the industry carries out an Extended One Generation Reproduction Toxicity Study (EOGRTS). The workshop notes that investigation of preneoplastic lesions in

the gut should be added to the EOGRTS study request. The evaluation of the recent papers led to the conclusion that taken individually, each of these studies highlight some reason for concern, but that none was sufficient to form a basis for overturning the conclusion of the previous risk assessment and to reopen the EFSA opinion of 2016. It should be mentioned that these studies were designed as hazard identification and to evaluate possible mechanisms but were not designed to form a basis for a full risk assessment. Whereas in general terms the latter EFSA conclusion was supported by the discussions in the workshop, an uncertainty that was mentioned was the potentially higher susceptibility to TiO₂ effects in 1% of the population that has increased gut permeability due to health problems with their gut. This issue was not considered extensively in the EFSA evaluations given that the focus of the EFSA assessments is on the general population.

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Federica Lodi presented EFSA's activities with respect to the food additive E171. For the re-evaluation of E171, calls for data were launched from the end of 2006 and data were received in the following years until 2016. These data were considered in the EFSA opinion of 2016. As a follow-up activity of the scientific opinion of 2016 and in order to gather the missing information, in January 2017 a new call for data was launched by the European Commission, followed by registration of contact details of business operators (step 1) and confirmation of data submission, deadlines and milestones (step 2). The industry using TiO₂ delivered data on particle size and particle size distribution by June 30, 2018. The business operators of E171 will deliver results of a dietary Extended One Generation Reproductive Toxicity Study (EOGRTS) with 3 cohorts (for F2 generation, neurotoxicity and immunotoxicity), with E171 food-grade TiO₂ before August 2019. Furthermore, data on the impurities (of metals) in E171 will be delivered as well as data on the actual use of aluminium in E171 formulations.

ANSES was asked to assess the impact of one of the more recent studies (Bettini et al., 2017), on the safety of TiO₂ on consumer's health. EFSA followed closely the ANSES assessment (based on article 30 of the General Food Law, i.e. Regulation (EC) No. 178/2002). ANSES and EFSA jointly concluded that there was no need to reopen the EFSA opinion of 2016.

It was mentioned that ECHA classified TiO₂ as substance suspected of causing cancer via inhalation (category 2). Moreover, ANSES is the Member State carrying out the assessment of TiO₂ in the Community Rolling Action Plan (CoRAP) under the European Reach Regulation for TiO₂ in March 2019.

A new mandate from the EC was sent to EFSA in March 2018, requesting to assess four studies (as already mentioned above by David Gott) published after the publication of the 2016 EFSA opinion, and to indicate whether these studies would merit re-opening the existing opinion. This mandate was triggered by a note from the French authorities requesting interim measures to address the uncertainties with respect to the impact of TiO₂ on human health.

The workshop concluded that these plans are helpful in further advancing the database necessary for a full risk assessment of TiO₂. Especially inclusion of the cohort pertaining to the immune system seems very relevant in the context of the findings discussed during the workshop. However, it was also mentioned that the EORGTS may not necessarily address all the issues that raise concern for adverse effects of TiO₂.

Outcome of the discussions

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General discussion on the lectures

An estimated one percent of the population is affected by IBD or other disease resulting in an increased gut permeability. This prevalence is relevant for the discussion on which population to protect. Stress, alcohol and mycotoxins may increase permeability too. Also, people with food allergy may have increased permeability. So, the issues raised in the general discussions were what populations need to be protected and what is the substance of concern? The Scientific Committee for Consumer Safety (SCCS) concluded that TiO₂ nanomaterial is a genotoxic substance. The reasons for concern regarding the nanomaterial under specific consideration seems to be valid.

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Discussions in the break out groups

The discussion in the break out groups were structured along a number of questions relevant to achieve the aims of the workshop. All three break out groups addressed the same questions, that were subsequently discussed plenary.

Question 1. Is the exposure used in the studies relevant and comparable to human exposure?

There is a fair understanding of TiO₂ exposure in the population. However, there are many uncertainties. It is unknown to which extent the E171 that is on the market varies in composition. The size distribution and crystal structure may vary among different companies. These data have been provided by the industry recently but are not publicly available.

It is not known what the actual TiO₂ content of all food categories or some specific individual foods is. There is also intake of TiO₂ via medicines and food supplements that should be considered. To study the effects of E171, care should be taken that at least E171 employed in the toxicity studies is representative of the additive as it is on the market, and in compliance with EU specifications. As the crystal structure as well as the particle size distribution of TiO₂ is important for its possible effect after ingestion, studies, be it in *in vitro* systems, in animals or in humans, should be carried out with well-characterized TiO₂ (E171) as food additive. While in Europe most likely mainly anatase is used, it should be clear whether or not the test agent is a mixture of anatase and rutile TiO₂. Dietary studies in animals should apply realistic exposure regimens, i.e. not in powder form or as sonicated suspension and not via gavage but via diet. Only a few *in vivo* studies on E171 have been performed, and not all adhered to these notions.

Whereas there is only a modest number of oral exposure studies available with E171, more studies are performed on effects after inhalation or *in vitro*, and with specific nanosized TiO₂ particles. *In vitro* studies can be valuable in order to help understanding mechanisms along which E171 may cause its effects, and especially to delineate what fraction of E171 is most active in causing adverse health effects. Comparison between particles in native form, in a food matrix, or with coatings, may also advance the knowledge data base. But even such studies may eventually be better performed if exposure groups are included in which exposure to well described E171 is applied.

In general, the exposure levels in the *in vivo* oral studies finding effects on the colon in healthy animals are in a relevant range: 0.2 (no effects) and 5 or 10 mg/kg bw/day (preneoplastic lesions) vs. intakes of 1.7 and 14.8 mg/kg bw/day at the 95th percentile of the intake distribution of the Dutch population and children in Europe, respectively.

Question 2. Are animal and *in vitro* studies demonstrating a toxicological hazard of TiO₂ following oral exposure?

- What are the strengths and weaknesses of these studies?
- Are the models used adequate? Are the effects relevant?

In experimental settings, TiO₂ nanoparticles can promote and probably initiate putative preneoplastic lesions (aberrant crypts). It should be noted that the presence of aberrant crypt abnormalities not necessarily leads to tumour formation. However, such lesions raise a health concern. In addition, there are studies performed using mouse models in which colon cancer is either induced chemically or genetically, and in which tumour formation is enhanced by oral exposure to E171. The suitability of the models used was discussed. Usually carcinogenicity is studied in chronic exposure studies in rats and transgenic animals. Animals in which tumours are induced chemically are usually not used for risk assessment. Yet in view of the notion that the development of colon cancer in untreated control rats is highly unusual, it was postulated that the traditional model may actually be insufficient to cover sensitive population groups. It has been observed that IBD patients may have increases in flares due to the intake of TiO₂. This may be due to a deficient barrier function. Whether these patients also display an increased prevalence of colon cancer is not known. Normal rats may not be a good model for such patients. As the food additive E171 is used unrestricted in the entire population, the entire population should also be protected from adverse effects. The conclusion based on the available studies is that there is a hazard of TiO₂ which raises concern. Whether this would lead to a health risk is still unknown.

Question 3. Can the information from these studies be extrapolated to humans?

In the presentations on the first day of the workshop, as well as in the literature, convincing data are available that indicate a hazard from exposure to TiO₂ or E171. However, in line with EFSA (EFSA 2018), the available information is not yet sufficient to draw solid conclusions on human health risks. Uncertainties comprise insufficient characterization of the test materials used in experimental studies and hence insufficient dosimetry, a lack of information on dose-response relationships, differences in exposure duration between experimental studies and actual exposure in humans, a lack of mechanistic and kinetic understanding, a lack of knowledge on age dependency and possible differences between humans and animals.

Question 4. Are there any epidemiological data on an association between oral exposure and adverse effects of TiO₂?

The incidence of colon cancer in humans is around 6%. The incidence is rising, especially in the industrialized world. There is concern that the increase may be related to the diet, but there are currently no epidemiological data to show that there is a clear relationship of colon cancer with high intakes of E171. Studies that may address this would be comparisons of populations with and without colon cancer, and determination of current and past exposure to E171. Special attention should be given to people with specific conditions, such as reduced barrier function, e.g. people with IBD or diabetes, arthritis patients taking medicines for a long time, or stress-related colitis such as occurs in children. It is evident that such well-powered studies can only be performed if exposure to E171 and in particular the different fractions of E171 that may be the causative agent, can be assessed accurately.

Question 5. What are knowledge gaps to conclude on a potential hazard? What information is required to perform a risk assessment?

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There is still uncertainty on genotoxicity, with conflicting results so far. No carcinogenicity has been observed in healthy animals yet. And the studies showing preneoplastic lesions only tested one dose. There is still a lack of mechanistic understanding of TiO₂ effects. Adverse outcome pathways (AOPs) of colon cancer, including key events such as oxidative stress-related conditions, or inflammation in the GI tract have not been determined yet, while AOPs with more general relationships between inflammation and cancer have now emerged. Such AOP's might be helpful for the design of new studies to be undertaken, as well as the evaluation of their results and of already available information. A question that has not been answered is whether TiO₂ after ingestion merely acts as a tumour promoter or also as a tumour initiator. The information on especially the latter is not conclusive yet. For tumour promotion, suppression of the immune system may be of importance. Some of the studies point to the immune system as a possible determinant of the adverse effects, but the information on immune effects is scant. One issue that has so far gained little attention is the role of the microbiota. It is possible that TiO₂ after ingestion interacts with the microbiota, which may have consequences for gut health.

An important current knowledge gap is the kinetics within the gut and its excretion mechanism. How well is TiO₂ absorbed into the gut tissue and where does it go? Which cells are targeted? Where does it accumulate? How is it excreted? What are differences between humans and experimental animals? What is the internal dose-response relationship? As indicated earlier, the causative fraction of E171 has not yet been identified. Even if it is considered that the nanosized fraction is the dominant factor, also other fractions may have effects.

Question 6. Do we have enough information to advise risk managers to take further actions? Which actions?

It can be concluded that there are various signs suggesting a hazard, but a real risk assessment is not yet possible. Risk managers are therefore advised to support further research that will be able to robustly assess the risk associated with the intake of E171.

Risk managers have already asked industry to follow-up on the data gaps identified during the re-evaluation of TiO₂ as a food additive and have given timelines for their completion. Studies that will be undertaken include an EORGTS (deadline mid 2019). Whereas this study is not oriented to detect carcinogenicity, inclusion of the cohort to identify potential immunotoxicity is a benefit and preneoplastic lesions should be assessed in the gut as an additional parameter. Chronic carcinogenicity studies may be fit to confirm tumour formation, but such studies are expensive and require large numbers of animals, hence it is more ethical to first carry out specific, smaller studies, such as the EORGTS in which several of the endpoints or intermediate actions as identified in AOPs, are included. In addition, the sensitivity of the standard carcinogenicity study for colon cancer is an issue; more animals might be needed than usual.

Alternative to research in animals, intervention studies in humans could be of value. These studies should then be designed in a way that exposure is very well controlled and provides groups of high and groups of low or virtually no exposure. Outcome parameters should be those that have been identified in the experimental studies so far or are part of AOPs of adverse conditions in the GI tract. The studies should be of sufficient duration.

Whereas currently industries are performing relevant studies, further in-depth studies to address the health concerns are warranted, and the industry and governments share a responsibility to prevent adverse effects in the population.

Recommendations

Further delineation of the cascade of adverse events leading to colon cancer and other adverse conditions should be stimulated, to also link the found gene expression changes with these adverse outcomes. This goes beyond the direct aim of TiO₂ research but may eventually help to better design investigations on the mechanisms of effects of TiO₂ as well as *in vivo* studies directly oriented at risk assessment.

Further *in vivo* studies in rodents should be stimulated to confirm effects as they have been identified so far, i.e. oriented at tumorigenesis and the possible underlying mechanism of immune suppression. Gut associated lymphoid tissues as well as the intestinal microbiota should be considered as possible causal factors for (pre)tumour formation. Such studies should also provide information on internal dose response relationships, starting from the relevant levels of the general population. Whereas long term carcinogenesis studies are considered the golden standard, they require many animals to obtain sufficient sensitivity for colon cancer detection. It may be questioned if such studies are the most adequate for this purpose as healthy animals may not be the best model for populations with a compromised barrier function. Studies to be carried out should include parameters that have been shown to be affected by TiO₂ or that are important early markers of tumour formation.

So far, *in vivo* studies have been performed in rodents. To substantiate the relevance of the effects, testing in another species, better mimicking humans, would be advisable. A candidate could be the mini pig. Even though testing possibilities in humans are more limited, as the proof of the pudding is in the eating, testing in humans would be preferential. It is recommended that a human intervention study is undertaken that assesses key parameters as identified in the *in vitro* and animal studies, and in which subjects are exposed to E171 in concentrations that are sufficiently different to allow effects on the outcome parameters to occur.

It is recommended that studies carried out, should be performed in close collaboration with risk assessors and risk managers.

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Annex I. Background documents

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Annex II. Programme of the Workshop on Possible adverse effects of food additive E171 (titanium dioxide)

Office for Risk Assessment & Research

Thursday, 5 July 2018
Chair: Henk van Loveren

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- 09:30 – 09:40 Dick Sijm: Welcome and Mission of the Office for Risk Assessment & Research of the Netherlands Food and Consumer Safety Authority (NVWA)
- 09:40 – 09:55 Tour de table of participants
- 09:55 – 10:05 Henk van Loveren: Aim of the workshop
- 10:05 – 10:45 Minne Heringa: Occurrence of titanium dioxide in food, and toxicokinetic considerations
- 10:45 – 11:15 Coffee
- 11:15 – 11:55 Gerhard Rogler: Inflammatory responses induced by titanium dioxide in the gut
- 11:55 – 12:35 Eric Houdeau: Fate and long-term effects of E171 in the GI tract under normal conditions
- 12:35 - 13:35 Lunch
- 13:35 – 14:15 Yolanda Chirino: Promotion of colon cancer by E171
- 14:15 – 14:55 Theo de Kok: Insight in the mechanisms of E171 induced adverse reactions in the gut
- 14:55 – 15:25 Tea
- 15:25 – 16:05 David Gott: Risk assessment of food grade titanium dioxide by EFSA
- 16:05 – 16:35 Federica Lodi: Titanium dioxide (E171): update on EFSA's activities
- 16:35 – 17:00 General discussion
- 18:00 – 22:00 Dinner in Amsterdam

Friday, 6 July 2018
Chair: Dick Sijm

- 09:00 - 10:30 Breaking up in three groups to discuss questions posed
- 10:30 - 11:00 Coffee
- 11:00 - 11.30 Reporting by breakout groups
- 11.30 - 12.30 General discussion, conclusions, follow up actions, wrapping up
- 12:30 Adjourn and lunch

Annex III. Lectures presented in the workshop

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