ANSES Opinion
Request No 2019-SA-0036

The Director General

Maisons-Alfort, 12 April 2019

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on the risks associated with ingestion of the food additive E171

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 12 April 2019 shall prevail.

On 28 February 2019, ANSES received a formal request from the Directorate General for Competition Policy, Consumer Affairs and Fraud Control, the Directorate General for Health, the Directorate General for Food and the Directorate General for Risk Prevention to provide scientific and technical support on the risks associated with ingestion of the food additive E171.

1. BACKGROUND AND PURPOSE OF THE REQUEST

E171 is a food additive used as a colouring. It is a mixture of titanium dioxide (TiO_2) particles in dispersed, aggregated or agglomerated form, whose size can vary from a few dozen to several hundred nanometres. Data from the literature indicate that the proportion of particles regarded as nanoparticles (i.e. with three dimensions less than or equal to 100 nm) within the food additive E171 is highly variable depending on the commercial batches. According to the literature data, this proportion can vary from 6 to 55% (25% on average, see Annex 2) by number and can reach up to 3.2% by mass (EFSA 2016).

According to the recommendation on the definition of a nanomaterial proposed by the European Commission[1], therefore, E171 is not regarded as a nanomaterial because the number of particles with one or more external dimensions in the size range between 1-100 nm accounts for less than 50% of the total particle population, for the majority of E171 batches on the market. However,

[1] According to the European Commission's recommendation on the definition of a nanomaterial dated 18 October 2011, "nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

according to the definitions reported in the INCO[2] and Novel Food[3] regulations, in these regulatory contexts, E171 is regarded as a nanomaterial.

In recent years, various studies have investigated TiO_2 at national and European level for different routes of exposure: inhalation (pulmonary) and ingestion (oral).

Regarding the pulmonary route, in 2006, the International Agency for Research on Cancer (IARC) classified TiO_2 in Group 2B as a substance that is "possibly carcinogenic to humans".

The occurrence of lung tumours in rats after inhalation or instillation of TiO_2 led ANSES, on 20 May 2015, to submit a proposal to the European Chemicals Agency (ECHA) to classify TiO_2 as a Category 1B carcinogen (a substance that is presumed to have carcinogenic potential for humans) by the pulmonary route, under the CLP Regulation. This proposal aimed to cover TiO_2 in all its crystalline phases and combinations of phases, for sizes below 10 µm and all particle morphologies. On the basis of this proposal, ECHA's Risk Assessment Committee adopted an opinion on 9 June 2017, in which it concluded that TiO_2 fulfils the criteria for classification in Category 2 as a suspected human carcinogen through the inhalation route[4].

As part of its national mission to develop health reference values, ANSES was asked to define a toxicity reference value (TRV) for TiO_2 in nanoparticle form. The Agency recommended a chronic TRV by inhalation for P25 TiO_2 in nanoparticle form of 0.12 $\mu g.m^{-3}$ (ANSES 2019).

Regarding the oral route, in 2016, the European Food Safety Authority (EFSA) published an opinion on the re-evaluation of E171 as a food additive (EFSA, 2016), based on a detailed review of the literature data on TiO_2 particles, and on data provided by industry on incorporated (or measured) levels of E171 for certain foodstuffs. This opinion concluded that current consumer exposure to the food uses of E171 was unlikely to pose a health risk.

In 2017, ANSES issued an opinion (2017-SA-0020) on a study (Bettini *et al.* 2017) in rats exposed orally to E171 or TiO_2 nanoparticles. In this study, the authors examined the passage and distribution of TiO_2 in certain target organs, the genotoxic and inflammatory effects of TiO_2 in the intestine, and the preneoplastic lesion initiating and promoting effects in the colon. The expert group concluded that this study revealed new information on the hazard characterisation of E171 (see Section 3.4.2) but did not call into question EFSA's 2016 conclusions regarding the risk assessment of E171.

In 2018, EFSA (EFSA 2018) reviewed four new studies on the hazard characterisation of the food additive E171 and TiO_2 nanoparticles, including the study by Bettini *et al.* (2017) that was examined in the above opinion. The EFSA expert group concluded that these studies could be useful for the hazard characterisation of TiO_2. Nevertheless, they considered that the uncertainties identified for each of these studies meant that they could not be used in a risk assessment. In this context, in view of the results of these studies, EFSA did not consider it justified to reopen the E171 evaluation dossier (EFSA 2016).

In view of the study recommendations issued by EFSA (2016) and ANSES (2017), and the latest publications on the hazard characterisation of the food additive E171, ANSES was asked to:

[2] Regulation (EC) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers

[3] Regulation (EC) No 258/97 concerning novel foods and novel food ingredients

[4] https://echa.europa.eu/fr/-/titanium-dioxide-proposed-to-be-classified-as-suspected-of-causing-cancer-when-inhaled

1) identify the oral toxicology studies carried out since publication of the INRA results in 2017 (Bettini *et al.* 2017).

2) on the basis of the results of these new studies, update as necessary the recommendations it had made in 2017 (ANSES 2017).

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

In view of the time available for carrying out the expert appraisal, ANSES decided to entrust it to an *ad hoc* "TiO_2" emergency collective expert appraisal group (GECU). The GECU met on 12 March, 25 March and 5 April 2019, to work on expert appraisal reports prepared by the GECU experts. The work was adopted by the GECU on 5 April 2019. In view of the timetable, the GECU's work was presented to the Expert Committee on "Assessment of physico-chemical risks in food" (CES ERCA) on 11 April 2019.

The experts' declarations of interests are made public via the ANSES website (www.anses.fr).

To respond to the request, the GECU conducted a literature search of publications on hazard characterisation of the food additive E171. With this in mind, the literature search was carried out in the Scopus and PubMed databases using the following keywords: ("E171" or "food grade TiO_2" or "food-grade TiO_2" or "food grade titanium dioxide" or "food-grade titanium dioxide" or "food grade TiO_2 nanomaterial" or "food-grade TiO_2 nanomaterial" or "food grade titanium dioxide nanomaterial" or "food-grade titanium dioxide nanomaterial" or "food grade TiO_2 nanoparticle" or "food-grade TiO_2 nanoparticle" or "food grade titanium dioxide nanoparticle" or "food-grade titanium dioxide nanoparticle") and (toxicity or toxicology or cytotoxicity or immunotoxicity or genotoxicity or toxic or neoplastic or preneoplastic or carcinogenesis).

Following this literature search, the GECU defined criteria for selecting studies relevant to the request. All the criteria below were applied:

- The substance used in the study systems was clearly identified as the food additive E171 or food-grade titanium dioxide;
- The E171 or food-grade titanium dioxide was characterised from a physico-chemical point of view;
- *In vivo* studies were conducted orally;
- The study was published between 2017 and 2019.

This therefore excluded studies for which:

- The substance used was not the food additive E171 or food-grade titanium dioxide;
- The exposure pathways studied were the respiratory or dermal routes;
- Publication was prior to 2017 (the year of publication of the most recent ANSES opinion on E171).

Application of these criteria led the GECU to select 25 publications (see bibliographic references at the end of this document).

Although the request related to the updating of data from toxicological studies, the GECU also considered articles on the characterisation and behaviour of E171 in biological and food simulants.

These data, which are complementary to the toxicological studies, may provide insights into the mechanisms of particle interaction or accumulation in certain biological compartments.

Lastly, in addition to updating the toxicological data, the GECU decided to include E171 occurrence data for certain food categories (Annex 1). Two types of data were considered: quantities of E171 incorporated in food formulations (data provided by industry), and analytical data on quantification of E171 within different food categories (data from the literature, NGOs and DGCCRF inspections).

3. ANALYSIS AND CONCLUSIONS OF THE GECU

3.1. The food additive E171

The food additive E171 consists of titanium dioxide particles (TiO_2, CAS number: 13463-67-7). TiO_2 is an inorganic substance present in two major crystalline forms (anatase and rutile) and with a molecular weight of 79.88 g/mol. E171 is a white powder that is insoluble in water and organic solvents but soluble in hydrofluoric acid and hot concentrated sulphuric acid. The data in the literature indicate great variability in the proportion of crystalline phases and the particle size distribution in the different commercial batches of E171 (Yang *et al.* 2014; EFSA 2016). The size of the particles can vary from a few dozen to several hundred nanometres, in dispersed, aggregated or agglomerated form. The percentage of particles regarded as nanoparticles (i.e. with three dimensions less than or equal to 100 nm) varies, depending on the literature data, from 6 to 55% by number (see Section 3.7) and from 0 to 3.2% by mass, according to the EFSA opinion (2016).
The food additive E171 is mainly used as a food colouring, with the maximum levels of incorporation being defined in Regulation (EC) No 1333/2008 on food additives. Thus, for 51 food categories, E171 can be used *quantum satis*[5].

3.2. Uses of the food additive E171

The food additive E171 is widely used as a colouring in different food categories. In France, E171 is used in the production of confectionery, desserts and ice cream, bakery and pastry products, biscuits, cakes, chocolate bars, refrigerated desserts, etc. (source GNPD[6]).
TiO_2 does not appear (since 2015) in the "food manufacturing" uses sector of the R-nano reporting tool used by ANSES within a regulatory framework on behalf of its supervisory ministries.

3.3. Regulations

Nanomaterials are regulated according to their uses. In 2018, the European Commission adopted the revision of several annexes regarding the registration dossiers for substances in nanoform. This revision clarifies the information and identification requirements for nanoparticle substances. The new rules come into force on 1 January 2020.
The European Commission is also working on a new definition of nanomaterials to replace the one of 18 October 2011[7].

[5] Article 3 (2) of Regulation (EC) No 1333/2008 states that "*quantum satis*" means that no maximum numerical level is specified. The substance may be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose.
[6] Global New Products Database.
[7] Commission Recommendation of 18 October 2011 on the definition of nanomaterial

The regulations on Cosmetics[8], Biocides[9] and food (INCO[10] and Novel Food[11]) stipulate the obligation to label products containing nanomaterials with the word [nano]. In the area of food, the INCO and Novel Food regulations offer the following definition for engineered nanomaterials: "*engineered nanomaterial: means any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale."*

3.4. Summaries of assessments of the food additive E171

3.4.1. Summary of EFSA's opinion on the re-evaluation of E171 (2016)

In 2016, the European Food Safety Authority published an opinion on the re-evaluation of the food additive E171 (EFSA, 2016), based on a detailed review of the literature data on TiO_2 particles. With regard to the available data on absorption, distribution and excretion, the EFSA expert group concluded that the absorption of orally-administered TiO_2 is extremely low and that a small amount of TiO_2 (no more than 0.1% of the administered dose) is absorbed by the gut-associated lymphoid tissue. Following its absorption, TiO_2 is distributed to various organs, each with variable accumulation and elimination rates. However, the vast majority of TiO_2 is eliminated in the faeces.
Numerous *in vivo* and *in vitro* genotoxicity studies based on tests of chromosomal aberration, mutagenic potential and DNA damage including base oxidation have been reported for different sizes and crystallinities of TiO_2 particles, and give contradictory results. Nevertheless, the EFSA expert panel pointed out that the reliability of the DNA damage test (Comet assay) has been questioned when applied with nanomaterials. Indeed, studies highlight the fact that DNA damage is caused by TiO_2 nanoparticles during the post-treatment stages and not during the treatment itself. The panel also questioned the reliability of some positive results observed for *in vivo* and *in vitro* studies, in which the variation in experimental parameters could affect the stability of particles, thus modifying their bioavailability and biological activity.
The EFSA panel (2016) concluded that “based on the available genotoxicity database and the Panel’s evaluation of the data on absorption, distribution and excretion of micro- and nanosized TiO_2 particles, orally ingested TiO_2 particles (micro- and nanosized) are unlikely to represent a genotoxic hazard in vivo”.

Regarding the reproductive toxicity studies, E171 does not appear to have adverse effects, unlike non-food-grade TiO_2, for which contradictory results have been reported, particularly on the change in hormone levels. However, shortcomings were identified in the methodology used for these studies. Due to the lack of robust data on reproductive toxicity, the EFSA expert group was unable to reach a conclusion on this effect or set a health-based guidance value.

[8] Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products
[9] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products
[10] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004
[11] Regulation (EU) No 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001

With regard to the immunotoxic effects of TiO_2, the EFSA panel considered that given the absence of clear characterisation of the material used in the various available studies, and in view of the difference in effects observed following various routes of administration and the diversity of effects reported, it could not reach a conclusion on the possible immunotoxic effects of the food additive E171.

Carcinogenesis studies carried out before 1979 (NTP 1979) were conducted using TiO_2 Unitane® (anatase form) in B6C3F1 mice (50 individuals/sex) at doses ranging from 0 to 6500 mg of TiO_2.kg $bw^{-1}.d^{-1}$ for males and from 0 to 8350 mg TiO_2.kg $bw^{-1}.d^{-1}$ for females for 103 weeks via diet. Histopathological examinations were performed on about thirty organs. The results indicated that TiO_2 administered orally at these doses is not carcinogenic in mice. Identical studies were conducted in rats (50 individuals/sex) at doses ranging from 0 to 2250 mg TiO_2.kg $bw^{-1}.d^{-1}$ for males and 0 to 2900 mg TiO_2.kg $bw^{-1}.d^{-1}$ for females for 103 weeks via diet. Histopathological examinations were also performed on about thirty organs. The results indicated that TiO_2 administered orally (via food) is not carcinogenic in rats at this dose range. Based on these carcinogenicity studies in rats and mice, the EFSA expert group set the no observable adverse effect level at 2250 mg.kg $bw^{-1}.d^{-1}$.

Regarding exposure data, the EFSA panel developed different exposure scenarios based on data provided by industry and Member States. The maximum exposure scenario took into consideration the maximum use (or measured) values reported by industry for foods (for which data are known) likely to contain E171. The average exposure value calculated for infants and the elderly was 0.4 mg.kg $bw^{-1}.d^{-1}$ (1.2 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) and 10.4 mg.kg $bw^{-1}.d^{-1}$ (32.4 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) for children. This scenario is regarded as the most protective since it considers lifelong exposure to maximum incorporated (or measured) levels of E171.
EFSA also determined two refined exposure scenarios. In the first scenario, which took food brand loyalty into account, it was assumed that the population is exposed in the long term to maximum incorporated (or measured) levels of food additives for one food category and to an average level for the other food categories. The average exposure value calculated for infants and the elderly was 0.4 mg.kg $bw^{-1}.d^{-1}$ (1.1 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) and 8.8 mg.kg $bw^{-1}.d^{-1}$ (30.2 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) for children.
The second scenario did not take brand loyalty into account and it was assumed that the population is exposed in the long term to average incorporated (or measured) levels of food additives for all food categories. The average value for infants and the elderly was 0.2 mg.kg $bw^{-1}.d^{-1}$ (0.5 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) and 5.5 mg.kg $bw^{-1}.d^{-1}$ (14.8 mg.kg $bw^{-1}.d^{-1}$ at the 95th percentile) for children.
The panel considered the scenario not taking brand loyalty into account as the most appropriate and realistic scenario for risk assessment because it was assumed that the population would be exposed in the long term to food additives found at average use levels in food.

The lowest margin of safety calculated from the no observable adverse effect level of 2250 mg.kg $bw^{-1}.d^{-1}$ was therefore 150. According to the EFSA expert panel, this margin of safety is considered not to be of concern (because it is higher than 100 for non-genotoxic and non-carcinogenic compounds under the guidance for submission of food additives, EFSA, 2012).

In its opinion, the EFSA expert group made the following recommendations:

- In order to establish a health-based guidance value for the food additive E171, an extended one-generation or multigeneration study, according to OECD guidelines, should be performed with the food additive E171 in compliance with EU specifications and in which the particle size distribution would first be characterised;
- The EU specifications for E171 should include a characterisation of TiO_2 particle size distribution using appropriate statistical descriptors (range, median, quartiles), as well as the

percentage (by number and mass) of nanoparticles used as a food additive. The measurement methods should comply with the EFSA guidance document[12];
- The maximum limits for the impurities of the toxic elements (arsenic, mercury, cadmium and lead) mentioned in the EU specification for TiO_2 should be revised to ensure that E171 is not a significant source of exposure to these elements.

The group concluded that once reproductive toxicity data were available, a health-based guidance value could be determined.

3.4.2. Summary of ANSES's opinion on the analysis of the publication by Bettini *et al.* (2017)

In the article by Bettini *et al.* (2017), groups of rats were exposed for seven days (by gavage) or 100 days (by administration in drinking water) to a batch of E171 (redispersed by ultrasonication in bovine serum albumin (BSA) solution) or to reference TiO_2 nanoparticles (NM-105, Joint Research Centre JRC), only used for the seven-day exposure, at doses similar to those observed for human exposure. The authors were interested in the crossing of the gut barrier, the distribution in some organs, the variation in immunological parameters, and the genotoxic and carcinogenic potential (initiation, promotion) of TiO_2.

The two materials (E171 and NM-105) underwent physico-chemical characterisation and, before use, were dispersed in BSA solution (0.05% m/v) by ultrasonication using a protocol derived from that described in the reports of the European Nanogenotox programme[13]. This preparation of E171 may seem unrepresentative of the food additive E171 as found in foods but enables certain hazards associated with uses of E171 to be identified.

The parameters and tools used for the physico-chemical characterisation appear relevant and this characterisation is very complete. Regarding size measurements, the NM-105 had a mean particle diameter of 22 ± 1 nm (range 10 to 45 nm) under the transmission electron microscope (TEM). The primary particle size distribution determined by TEM ranged from 20 to 340 nm (mean and standard deviation 118 ± 53 nm) and 44.7% of the particles had sizes less than 100 nm in diameter. The TEM images showed that the particles from the E171 batch were spherical in shape, and were partly isolated or in the form of small agglomerates when dispersed in water before oral administration to rats by gavage or via drinking water. The physico-chemical characteristics (crystalline form, size distribution, nanoscale fraction) of E171 selected by Bettini *et al.* were those mainly reported for other food-grade E171 batches and therefore appear to be representative of the market.

Following the E171 and NM-105 dispersion protocol, two oral exposure routes were used in this study. In the first, rats were exposed for seven days by intragastric gavage to a dose of E171 or NM-105 of 10 mg.kg $bw^{-1}.d^{-1}$. In the second, rats were exposed for 100 days via drinking water, renewed every two days, to E171 doses of 200 µg.kg $bw^{-1}.d^{-1}$ and 10 mg.kg $bw^{-1}.d^{-1}$.

Concerning the results relating to immunotoxicity, the authors focused on monitoring different parameters: the variation in the number of dendritic cells (DC) and lymphocytes (Tregs and Th), changes in the concentration of several cytokines (TNF-α, IL-10, IL-1β, IFN-γ, IL-17, IL-8, IL-6, IL-18), lymphocyte proliferation and cytotoxicity. Overall, the percentages of variation reported in this study, when statistically significant, remained relatively low and their biological significance still

[12] EFSA Scientific Committee, 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal 2011; 9(5): 2140, 36 pp. doi: 10.2903/j.efsa.2011.2140. Available online: www.efsa.europa.eu/efsajournal.htm
[13] https://www.anses.fr/fr/system/files/ANSES-Ft-Nanogenotox_FinalReport.pdf

needs to be confirmed. In addition, the interpretations given by the authors are possible working hypotheses but are not fully supported by the results. Therefore, although these results do highlight changes in some immune parameters, they are not sufficient to confirm any impairment of immune homeostasis. In conclusion, with regard to the inflammatory and immunomodulatory aspects, the assessments currently available for E171 used as a food additive are not called into question by the data from this study.
Genotoxicity was studied via a comet assay performed on cells of Peyer's patches after seven days of exposure to E171 or NM-105 at a dose of 10 mg.kg $bw^{-1}.d^{-1}$. The study authors did not observe any genotoxic effect on cells isolated from Peyer's patches from rats exposed to E171 or NM-105 for seven days. However, uncertainties related to the experimental protocols (absence of a positive control, number of doses tested, choice of cells) mean that the relevance of this study could not be assessed.

The studies of carcinogenic potential focused on the occurrence and development of aberrant crypt foci (ACF) in rats, whether or not induced by an initiator, exposed to E171 (previously redispersed in BSA and ultrasonicated) via drinking water for 100 days. These ACF are considered by the authors to be the earliest histopathological lesions related to colorectal cancer formation.
In order to study the impact of E171 on ACF promotion, adult male Wistar rats were pre-treated with an intraperitoneal injection of 1,2-dimethylhydrazine (DMH) at a dose of 180 mg.kg bw^{-1} to initiate the carcinogenesis process in the colon. Seven days after treatment, the rats were randomly assigned to three groups of 12 rats and then exposed to doses of 0 (control, water only), 200 µg E171.kg $bw^{-1}.d^{-1}$ or 10 mg E171.kg $bw^{-1}.d^{-1}$ in drinking water for 100 days. The authors did not observe any significant increase in the total number of ACF per colon at the two doses tested compared to the control. However, statistically significant increases in the number of aberrant crypts per colon and the number of "large ACF" per colon were observed exclusively in the rat group exposed to 10 mg E171.kg $bw^{-1}.d^{-1}$.
In order to study the mechanisms involved in the potential promoting effects observed, *in vitro* studies were conducted on the cytotoxicity of E171 in normal (unmutated Apc gene (Apc +/+)) or preneoplastic (mutated Apc gene (Apc min/+)) rat colon epithelial cells. According to the authors, exposure to NM-105 induces preferential cytotoxicity in preneoplastic cells. They therefore advanced the mechanistic hypothesis of preneoplastic cell selection in the early stages of the transformation process.
In order to investigate the ability of E171 to initiate the spontaneous onset of ACF, adult male Wistar rats were exposed to doses of 0 (control, water only) or 10 mg E171.kg $bw^{-1}.d^{-1}$ in drinking water for 100 days. Among the animals exposed to E171 at a dose of 10 mg.kg $bw^{-1}.d^{-1}$, four out of eleven rats had ACF in the colon, while none of the 12 rats in the control group had ACF, the difference between the two groups being statistically significant. For three of the four rats, the number of aberrant crypts per ACF was low (1 to 3). For the fourth rat, a focus of 12 aberrant crypts was observed, indicating a more severe lesion.

The 2017 members of the GECU considered that the methodology used by the authors had followed a well-established scientific model.
A potential promoting effect of E171 was observed in the colon. While the increase in the appearance of "large ACF" seemed moderate, it should be noted that ACF are presumed to be predictive of preneoplastic lesions. This promoting potential was also reported in mice after initiation (by azoxymethane-induced colitis), under conditions similar to the present study with tumour induction in the colon (Urrutia-Ortega et *al.* 2016). In the study by Bettini *et al.* (2017), the post-exposure follow-up time for animals was insufficient to assess the intensity of the E171 promoting effects on tumour incidence. This possible promoting effect cannot be attributed to the nanoscale fraction of E171 on the basis of *in vitro* tests.
With regard to the initiating potential of E171, the number of animals tested was too small to judge its significance. In addition, the study by Urrutia-Ortega *et al.* (2016) performed on mice did not report any initiating effect of E171.

In its opinion, ANSES made the following recommendations:

The potential promoting effect of E171 observed in the colon needs to be confirmed by experiments incorporating the use of additional biomarkers and/or performed over longer exposure periods, in order to assess tumour induction. An additional group containing a greater number of animals is needed to confirm or refute a possible initiating effect of E171.

ANSES also reiterated:

- *The need to carry out the studies required for fully characterising the hazard associated with E171, in accordance with procedures and a timetable to be defined;*
- *That the studies recommended in ANSES's opinion (2017), as well as those previously mentioned by EFSA, should be carried out with a sufficient number of animals per dose group and in an environment that ensures that experiments are conducted in accordance with good laboratory practice. It will be essential in the longer-term to acquire additional data useful for reaching a decision on the observed signals, in a context of broad and quantum satis use of a food additive without an ADI;*
- *The need to develop relevant toxicological study protocols to assess the health risks of products containing nanomaterials (effective physico-chemical characterisation, detailed and reproducible protocols, participation of the human and social sciences, etc.);*
- *Whenever hazards are identified for human health or the environment, the Agency recommends weighing up the value for the consumer or the community of placing on the market such products containing nanomaterials, for which the benefits should be clearly demonstrated;*
- *Its recommendation to limit the exposure of employees, consumers and the environment as part of a gradual approach, in particular by promoting safe products that do not contain nanomaterials and are equivalent in terms of function, effectiveness and cost;*
- *The challenges of strengthening the traceability of consumer products containing nanomaterials, essential for risk assessment work. In this context, ANSES emphasises the challenges associated with improving the reporting process implemented as part of the national R-nano portal, in order to ensure a better description of the nanomaterials placed on the market, their precise uses and the associated population exposure.*

3.4.3. Summary of the EFSA opinion (2018) on the evaluation of four new publications not considered during its re-evaluation of E171 in 2016

In 2018, the European Commission requested that EFSA evaluate four new studies (Heringa *et al.* 2016, Bettini *et al.* 2017, Guo *et al.* 2017 and Proquin *et al.* 2017) on the toxicity of TiO_2 used as a food additive, and indicate whether these studies would merit reopening the 2016 evaluation dossier for E171.

In the study by Guo *et al.* (2017), Caco-2 and HT29-MT model cells (mimicking intestinal epithelial cells) were exposed to TiO_2 nanoparticles (20-40 nm). The authors suggested that at physiologically relevant concentrations (reflecting human exposure levels), TiO_2 nanoparticles may promote the generation of reactive oxygen species (ROS) in an *in vitro* intestinal model, thus inducing pro-inflammatory signals that could affect the proper functioning of intestinal epithelial cells. The EFSA expert group concluded that the TiO_2 nanoparticles used in this study were not representative of the food additive E171 and that the results observed in this *in vitro* intestinal model were difficult to transpose to humans.

For the 2017 study by Bettini *et al.* (details of which are reported in Section 3.4.2), the EFSA expert group believed that: (i) administration of E171 by drinking water or gavage was not representative of the use of E171 in food matrices; (ii) variations in immunological biomarkers were not biologically significant; (iii) the use of aberrant crypt foci (ACF) as the only biomarker was not sufficient for the study of preneoplastic lesions in the colon; (iv) the initiation potential of E171 was observed in a small number of animals and has not been confirmed in other studies (Urrutia-Ortega *et al.* 2016).

In the study by Proquin *et al.* (2017), the authors evaluated TiO_2 cytotoxicity, ROS generation and *in vitro* genotoxicity on two cell lines (Caco-2 and HCT116). For this purpose, the authors used three forms of TiO_2: E171, microparticulate and nanoparticle. All three materials induced cytotoxicity at doses of 1000 µg/mL, and from 100 µg/mL for E171. Nano-TiO_2 and E171 generated ROS, but this ROS production was inhibited in the presence of BSA in the medium. According to the authors, this inhibition seemed to be related to the formation of corona[14] on the surface of the particles. The authors also showed that the three materials tested caused DNA damage and that E171 may interact with chromosomes and the mitotic apparatus. The EFSA expert group considered that this study was useful for hazard identification but not suitable for risk assessment, and did not challenge the conclusions of EFSA (2016) regarding the genotoxic effects of TiO_2. It also believed that cellular uptake of TiO_2 particles may decrease under *in vivo* conditions due to the presence of mucus on the surface of the epithelial cells. Lastly, the EFSA expert group mentioned similar studies (Vila *et al.* 2018) conducted on differentiated Caco-2 cells showing contradictory results to those presented in the study by Proquin *et al.* (2017) (on undifferentiated Caco-2 cells).

In the study by Heringa *et al.* (2016), the authors carried out a risk assessment exercise on E171 for humans and highlighted the gaps in knowledge, particularly concerning the concentration of nano-TiO_2 in different organs, effects on reproductive organs, reproductive toxicity and endocrine disruption. This point was taken into account by EFSA (2018), which recommended conducting an extended one-generation reproductive toxicity study of E171 (EOGRTS, OECD guideline 443), as well as a better assessment of E171 and TiO_2 nanoparticles taking into account interactions with food matrices. The EFSA expert group considered that the uncertainties mentioned by the authors do not allow a risk assessment of E171 to be carried out.

The EFSA expert group concluded that these four studies revealed new *in vitro* and *in vivo* effects that may be useful for hazard identification of E171. However, according to EFSA, the uncertainties inherent in each of the studies do not allow these data to be used for the risk assessment of E171 and do not merit reopening the evaluation dossier for the food additive E171.

GECU comment on the EFSA opinion (2018)

According to the EFSA expert group, the results of Bettini *et al.* (2017) do not justify the establishment of a carcinogenicity study. EFSA supported its conclusions by referring to the study by Wijnands *et al.* (2004). In this study, the authors suggested that the size and number of ACF is not a predictive indicator of the preneoplastic condition or the number of colorectal tumours.
For the GECU, this argument is questionable because the study by Wijnands *et al.* suffered from methodological and conceptual flaws: there was no description of the enumeration or characterisation (surface area, location of colon segments) of ACF. The GECU therefore considers that this study cannot be used to draw any conclusions about the non-predictive nature of ACF with respect to a preneoplastic condition or the number of colorectal tumours.

[14] The corona is the layer of biomolecules adsorbed on the surface of particles

3.5. Analysis of publications on the toxicology of the food additive E171

The publications identified and selected in accordance with the procedures specified in Section 2 are listed in the "References" annex at the end of this opinion. With regard to the scientific reviews resulting from the literature search, the GECU did not appraise each of the publications mentioned in these reviews but focused only on the review authors' conclusions.
As a preamble to the analysis of the publications relating to the toxicology of E171, the GECU would like to point out that the vast majority of the studies selected were conducted with samples of E171 or food-grade TiO_2 previously dispersed in BSA solution and then sonicated. This protocol, as recommended in the Nanogenotox[15] and NanoREG[16] programmes, aims to harmonise sample preparation conditions in order to obtain theoretically comparable suspensions between different laboratories in terms of particle size distribution. The GECU believes that the sonication step may have the effect of redispersing particles organised into aggregate or agglomerate structures, which is not fully representative of E171 as found in foodstuffs. Nevertheless, the GECU considers that these conditions help identify certain hazards associated with E171.

3.5.1. Analysis of literature reviews

Two literature reviews published by Winkler *et al.* (2018) and Sohal *et al.* (2018a) investigated the toxicity of different forms of TiO_2. These two studies summarised the progress made in identifying the TiO_2 hazard and also highlighted missing data and uncertainties limiting the implementation of a suitable risk assessment for nanomaterials. Most of the studies reported in these two reviews examined nanoscale anatase and/or rutile forms of TiO_2, very few concerned E171 or "food-grade" TiO_2. The GECU focused on studies where the substance used in the test systems was the food additive E171 or a form of food-grade TiO_2. The analysis of the oral *in vivo* experimental studies therefore highlighted the following points:

- The very low but rapid systemic absorption of TiO_2 particles larger than 100 nm (primary particles). Kreyling *et al.* (2017) showed, using radiolabelled particles, that 0.6% (by mass) of 70 nm anatase particles passed the intestinal barrier one hour after administration and about 0.05% (by weight) were internalised after seven days. Two studies in volunteers conducted by Pele *et al.* (2015) and Böckmann *et al.* (2000), on the absorption of gelatine capsules containing 50 mg of food-grade TiO_2, confirmed rapid absorption in humans (2 h after ingestion) of TiO_2 particles (maximum TiO_2 blood concentrations were observed 6 h after oral administration), with the absence of lesions in the intestinal mucosa having been verified in parallel (Pele *et al.* 2015).

- The absence of acute toxicity of TiO_2 particles of different sizes (25, 80 and 155 nm) administered at high doses (5 mg.kg bw^{-1}, in a single dose by gavage according to OECD Guideline 420) in mice (Wang *et al.* 2007). The study of the biodistribution of the titanium (Ti) element by inductively coupled plasma mass spectrometry (ICP-MS) showed that the Ti element was found in all organs (liver, spleen, kidneys, lungs, brain) regardless of particle size. Ti levels in the brain were significantly higher when mice were exposed to 155 nm TiO_2 particles compared to 25 nm nanoparticles. The concentrations in the spleen were equivalent for all three types of particles. These results indicate that Ti particles can be transported to other tissues/organs after intestinal absorption.

[15] https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf
[16] http://www.nanoreg.eu/

- The low reproductive toxicity of different forms of pigment (153 nm anatase; 195 and 213 nm rutile) and nanoscale (42 nm anatase, 47 nm rutile, 43 nm anatase (89%) and rutile (11%) mixture) TiO_2 studied according to OECD Guideline 414. However, the assessment did not go beyond the foetal stage (structural abnormalities and foetal growth), and the functional deficits that represent an important aspect of development are not addressed in this guideline. Nevertheless, two studies using nanoscale forms of TiO_2 have shown for one, the presence of Ti in the hippocampus and impaired memory processes in rats (Mohammadipour *et al.* 2014, 2016); and for the other, histological alterations in the thyroid and changes in testosterone levels in male and female Sprague Dawley rats treated orally for five days with anatase (20-60 nm) at doses of 1 and 2 mg.kg $bw^{-1}.d^{-1}$ (Tassinari *et al.* 2014).

- The lack of developmental studies, and on neurodevelopment in particular, is a weakness in the assessment of the toxicity of E171.

- The inflammatory response in the intestines of mice treated with TiO_2 (anatase) in 260 nm microparticulate form (food-grade) or 66 nm nanoparticle form, administered in suspension in water by gavage once a day for 10 days (100 mg.kg $bw^{-1}.d^{-1}$); the response is reflected in increased pro-inflammatory cytokines, particularly those of the Th1 and Th17 pathways, and in histological changes in the intestinal mucosa in mice treated with TiO_2 nano- and microparticles, changes that were not observed in controls. Hypertrophy and hyperplasia were observed in the three regions of the small intestine (duodenum, jejunum, ileum) during treatment with nanoparticles, while the ileum was the only one affected during treatment with TiO_2 microparticles (Nogueira *et al.* 2012).
- The sensitivity of young rodents to TiO_2 nanoparticles revealed in two experimental studies, and suspected effects on spermatogenesis.

- The carcinogenicity of TiO_2 considered negative by the oral route by EFSA (2016), based on two-year studies in rats and mice (NTP 1979) exposed to Unitane® 0-220; Winkler *et al.* (2018) identified uncertainties related to the lack of Unitane® characterisation and the analysis of the results.

In conclusion, the reviews by Winkler *et al.* (2018) and Sohal *et al.* (2018a) highlighted the following points:

- Rapid but very low systemic absorption of TiO_2 particles in animals, and confirmed but not quantified absorption in humans (in volunteers);
- The absence of acute toxicity in adult animals, and of effects on reproduction (fertility and foetal development);
- The lack of developmental studies, and on neurodevelopment in particular, with food-grade TiO_2, which is a weakness in the assessment of the toxicity of E171;
- An inflammatory response in the intestine;
- The suspected sensitivity of young animals;
- Acute toxicity studies do not show any toxic effects of TiO_2 nanoparticles, unlike chronic toxicity studies at realistic doses (based on daily dietary intake) and over the long term.

Winkler *et al.* (2018) concluded that the gaps and uncertainties in the toxicological studies of TiO_2 concerned the following points:

- The long-term toxic potential of TiO_2 given the persistence and tissue accumulation of internalised particles (half-life time: 650 days), even if the absorption rate is low;
- The translocation mechanisms and the reactivity on the intestinal barrier that could generate lymphocyte inflammation.

Winkler *et al.* (2018) and Sohal *et al.* (2018a) recommend assessing:

- Endocrine disrupting potential, chronic effects on different lymphoid tissues (especially the intestinal barrier, liver, spleen) and systemic absorption;
- Effects on development and neurodevelopment;

in order to assess the health risks including for the most sensitive population components.

For the members of the GECU, these recommendations for TiO_2 in general apply even more so to E171 due to certain weaknesses in the toxicological dossier noted above.

3.5.2. Characterisation of E171 in biological media or food simulants

Due to the great diversity of E171 batches on the market, the physico-chemical characterisation of this food additive is an essential prerequisite for its risk assessment[17,18]. In addition to the identification process, the physico-chemical characterisation of E171 in biological media or food simulants helps provide information for understanding certain biological mechanisms (biomolecule adsorption, particle diffusion, digestion and absorption, etc.) observed in *in vivo* and *in vitro* study systems.

The study by Yusoff *et al.* (2018b) compared the behaviour of food-grade TiO_2 and P25 (reference nanoscale material). The physico-chemical characteristics of these two materials were studied in deionised water, BSA solution (1 and 20 mg/mL), sucrose solution (1 and 100 mg/mL), or mixed BSA/sucrose solution (high BSA - low sucrose or low BSA - high sucrose). This article followed an initial study (Yusoff *et al.,* 2018a), which demonstrated the decrease in size of food-grade TiO_2 agglomerates in the presence of BSA and sucrose, and the stability of the respective corona.
In their second study (Yusoff *et al.* 2018b), the authors showed, in solution, a behaviour of P25 nanoparticles (83% anatase, 17% rutile, mean size 22 ± 4 nm, specific surface area 54 m^2/g) that differed from that of E171 particles (anatase, size: 60-360 nm of which 30% (by number) of particles < 100 nm, specific surface area 8 m^2/g):

- P25 agglomerated significantly more than E171 in high sucrose concentrations, and then had a positive surface charge;
- Corona formation on the surface of P25 particles, in the presence of BSA and sucrose, was promoted compared to E171 particles;
- P25 sedimentation in water was slower than that of E171;
- Unlike P25, E171 systematically had a negative surface charge, regardless of the dispersion medium. The presence of sucrose and BSA increased the negative surface charge of the E171: the higher the sucrose/BSA ratio, the more this surface charge was negative.

This study showed the relative stability of the E171 suspensions, which were always negatively charged regardless of the base solution: sucrose, BSA or a mixture. The presence of BSA reduced the average hydrodynamic size of E171 particles from 366 nm to between 200 and 300 nm. The authors concluded that P25 cannot be used as an alternative to E171 in toxicity tests. Indeed, P25, although a homogeneous material used as a reference, does not behave in the same way as E171 in food simulants consisting of BSA and sucrose.

In the publication by Zhang *et al.* (2019), the authors investigated the impact of the food matrix on the fate and toxicity of E171 in the gastrointestinal tract. The authors developed a food matrix model

[17] https://www.anses.fr/fr/system/files/ANSES-Ft-Nanogenotox_FinalReport.pdf
[18] https://www.efsa.europa.eu/en/efsajournal/pub/5327

based on the American diet[19]. This model was in the form of an oil-in-water emulsion, consisting mainly of oil droplets that are likely to aggregate (depletion phenomenon). This food model, in fluid or powder form, has been characterised in *in vitro* systems mimicking the digestion conditions of the mouth, stomach and intestine. In particular, the authors demonstrated that flocculation of the oil droplets was promoted under stomach conditions due to a more acidic pH that modified the surface charge of these droplets. The authors then investigated the cytotoxicity of E171 in a model of intestinal epithelial cells (triculture of Caco-2, HT29-MTX, Raji B). For this purpose, E171 was dispersed in a buffer or food model and incubated successively in media mimicking the conditions of the mouth, stomach and intestine. Lastly, the digestate was placed in contact with the intestinal epithelial cells and the release of lactate dehydrogenase (marker of altered cell plasma membrane) was quantified. The authors showed that the E171 dispersed in a food model was less cytotoxic than the same E171 dispersed in a buffer, regardless of the concentration of TiO_2 particles (0.75 and 1.5% m/m). For the highest concentration of TiO_2 particles, cytotoxicity was reduced by a factor of five when the particles were dispersed in the food model. The authors suggested that the adsorption of nutrients on the surface of the particles modifies the state of charge and aggregation of TiO_2 particles, thus modifying their mobility within the mucus and their absorption by epithelial cells.
The GECU points out that the characterisation of TiO_2 particles after dispersion in food simulants and then in the study system mimicking the intestine could have provided additional information for understanding absorption phenomena by epithelial cells.

In the publication by Sohal *et al.* (2018b) the authors studied the dissolution kinetics and behaviour of TiO_2 (A200 classified by the supplier as food-grade) in salivary, gastric and intestinal simulants and then in a model mimicking the sequential oral-gastric-intestinal digestion cascade. The objective of this study was to characterise the digestive environment in terms of changes in size, size distribution, morphology and pH modification.
In the simulated salivary fluid, no significant changes in particle size distribution, agglomerate size, total particle concentration and ion concentration were observed for TiO_2.
In the simulated gastric fluid, the concentration of TiO_2 particles in the 120-140 nm size range decreased by 82%. The agglomerate size increased only slightly for TiO_2 during dissolution tests but no significant release of ions was observed.
In the simulated intestinal fluid, examination of the particle size distribution and changes in particle concentration after eight hours of incubation showed a 40% increase in the 150-250 nm size range. The total particle number concentration also increased by 65% in the simulated intestinal fluid, but no significant release of ions was recorded for TiO_2.
Concerning dissolution in a physiologically relevant digestion cascade, no significant release of Ti^{4+} ions was recorded for TiO_2. The size of the agglomerates increased from about 330 nm initially to about 600 nm at the end of the gastric phase. The size of the 500 nm agglomerates of TiO_2 particles then decreased during transfer to the intestinal phase and increased (~900 nm) at the end of this phase. The maximum dissolution of TiO_2 was 0.42% at the beginning of the intestinal phase. The particle number concentration and size of TiO_2 agglomerates, as well as the adsorption of organic components on the particle surface and the corona effects, probably lead to such dissolution behaviour, a conclusion that is reinforced by TEM observations.
In conclusion, TiO_2 is biodurable[20] and persistent under oral and gastric digestion conditions. In the intestinal fluid, TiO_2 was dissolved to a maximum of 0.2% and the size of the agglomerate increased from 330 nm to 570 nm (average value over the entire intestine). Examination of all the results showed that E171 was biodurable in each of the digestive fluids studied and during the oral-gastric-intestinal digestion cascade with maximum TiO_2 dissolution of 0.42% at the beginning of the intestinal phase.

[19] "What We Eat in America" NHANES 2013–2014

[20] Biodurable is the ability to resist chemical/biochemical alterations. It contributes significantly to biopersistence

In this study, the authors used anatase-type TiO_2 referenced under the code A200. According to the supplier, this substance has properties similar to food-grade TiO_2. However, the lack of information on the identification of A200, in particular on size distribution or specific surface area, as well as the lack of information on the uses of this substance, meant that the GECU was unable to confirm whether it is similar to E171 in terms of physico-chemical characteristics, behaviour and health effects.

- **GECU conclusions on the characterisation of E171 in biological media or food simulants**

The GECU points out that the physico-chemical characterisation of E171 batches has become almost systematic and that the data generated are of good quality due to the choice of appropriate analytical techniques and the measurement of relevant parameters.

Physico-chemical characterisations in complex environments have shown the influence of food simulants on the behaviour and fate of E171 in test systems. These observations highlight the importance of the sample preparation stage as a prelude to toxicological studies.

These studies also showed that the behaviour of the model nanoparticles of TiO_2 (P25) may differ from that observed with E171 and that their use as an alternative is not desirable when characterising the hazard of the food additive E171.

The studies by Sohal *et al.* (2018b) in systems mimicking oral-gastric-intestinal digestion conditions showed that E171 is biodurable and dissolves only very slightly (up to 0.42%). The variable physico-chemical conditions throughout the digestive tract lead to dynamic agglomeration processes (increase and decrease in agglomerate size), which may explain the low levels of oral absorption of TiO_2 particles.
This point supports the hypothesis of toxicological concern following acute and/or chronic exposure to E171 mainly regarding the integrity of the gastrointestinal barrier, without it being possible to rule out adverse effects at a systemic level.
Between 2017 and March 2019, three publications on E171 studied the interactions of TiO_2 with bacterial strains and the impacts of TiO_2 exposure on the quantity and quality of intestinal mucus. Summaries of these publications are provided below.

3.5.3. Studies on the effects of E171 on the microbiota and intestinal barrier

The studies reported in this section were not conducted on animal microbiota but in *in vitro* test systems on a few bacterial strains reflecting neither the exhaustiveness nor the complexity of the human intestinal microbiota.

The objectives of the study by Radziwill *et al.* (2018) were to examine the interactions between bacteria (from commensal intestinal flora or foodborne) and TiO_2 (food-grade compared to a reference nano-TiO_2) and assess their impact on bacterial growth.
This first study was carried out on isolated cultures of eight bacterial strains belonging to the species *Escherichia coli*, *Lactobacillus rhamnosus*, *Lactococcus lactis* (subsp. *lactis* and *cremoris*), *Streptococcus thermophilus* and *Lactobacillus sakei*. After adding different concentrations of TiO_2 ranging from 32 to 320 µg/mL to the culture medium prior to bacterial growth, each strain's tolerance to TiO_2 was determined by measuring the duration of the lag (latency) phase, the maximum specific growth rate, and the final absorbance (measured every 15 min for 24 hours). Bacterial viability was assessed by spreading TiO_2-exposed (group exposed to 320 µg/mL) and non-exposed bacteria onto plates and counting the number of colony-forming units (CFU) after 24 hours of incubation.

The elemental composition (the recorded ions were $^{12}C_{14}N$, ^{32}S, $^{31}P_{16}O_2$, $^{46}Ti_{16}O$ and $^{48}Ti_{16}O$) of two bacteria – *E. coli* and *L. lactis* – exposed to TiO_2 was mapped. Lastly, to answer the question about TiO_2/bacteria interactions and possible associated toxicity, imaging techniques were used to study the internalisation of TiO_2 in two model bacteria (*E. coli* K12 and *L. lactis*) exposed to 320 µg/mL of TiO_2.
The results showed that bacterial growth was inhibited by E171 for all the selected bacteria.
Regarding internalisation, deep ultraviolet (DUV) fluorescence imaging revealed a co-localisation of TiO_2/bacteria which, in some cases, caused morphological and structural damage to *E. coli* and *L. lactis*. One part of the bacterial population interacted strongly with E171 and, to a lesser extent, with nano-TiO_2. The results showed that around 7% of exposed *E. coli* bacteria trapped E171 while no internalisation was observed for *L. lactis*. These results appear to support other toxicity results observed in bacteria exposed to E171 showing that alterations in growth kinetics and reduced viability are greater for *E. coli*.
By scanning electron microscopy (SEM), a small number of bacteria of the genus *L. lactis* exposed to TiO_2 showed extensive cell wall damage with leakage of intracellular components. For *E. coli*, bacteria treated with E171 were twisted and rougher with surface rifts (wider and shorter cells, or even completely deformed).
In conclusion, morphological and ultrastructural changes limited to a small number of bacterial cells were noted. E171 particles were tightly associated with the surface of the bacteria, causing some deformation at the contact zone. TiO_2 can also be partially trapped by bacteria to induce moderate bacterial toxicity, with the most significant effects observed in *E. coli*.

The *in vitro* study by Dudefoi *et al.* (2017) used a defined anaerobic intestinal bacterial community containing 33 bacterial strains (MET-1) and exposed for 48 h at 37°C in the dark with agitation to 100 and 250 mg/kg of E171, considered by the authors as low and high exposures respectively, equivalent to those observed in the intestine after ingestion of pieces of chewing gum or confectionery. An E171/porcine pancreas α-amylase mixture was also studied to model the additional presence of a digestive enzyme. Physiological, biochemical and genomic DNA analyses were carried out following the treatments.
Levels of CO_2 and N_2 and total gas volumes for the treated cultures were similar to the control cultures, indicating that E171 had no significant influence on bacterial metabolism. In terms of biochemical analysis, E171 had little impact on the overall fatty acid composition, with slight variations in saturated fatty acid composition for the two E171 concentrations tested
Genomic DNA analysis revealed similar patterns between MET-1 treated and not treated with E171. No differences in phylogenetic distributions were noted between the groups exposed to E171 and control groups. When the taxonomic distribution was normalised with respect to total fatty acids, there was no negative impact on MET-1 viability after E171 treatments.
The addition of E171 resulted in slight increases in the relative proportions of *Acidaminococcus intestini*, *Eubacterium ventriosum* and *Eubacterium rectale* strains.
In conclusion, the addition of relevant concentrations of E171 (100 to 250 mg/L) had little impact on bacterial respiration, fatty acid profile or phylogenetic composition. These results suggest that food-grade TiO_2 particles do not significantly alter the human intestinal microbiota.

Talbot *et al.* (2018) used *in vitro* and *in vivo* test systems. *In vitro*, differentiated HT29-MTX cells (mucus-secreting) were treated with E171 at 40 µg/mL. *In vivo*, groups of male rats were exposed orally for seven days to 10 mg/kg bw/day or for 60 days to doses of 0.1 and 10 mg/kg bw/day of E171 (and NM-105). According to the study authors, these dose levels are similar to the estimates calculated by EFSA (2016) with, for the most protective scenario (i.e. lifetime exposure to maximum levels of E171), average exposure estimates ranging from 0.4 mg/kg bw/day for infants and the elderly to 10.4 mg/kg bw/day for children.

The use of confocal laser scanning microscopy on HT29-MTX cells exposed to E171 demonstrated the penetration and heterogeneous accumulation of TiO_2 particles in mucus, probably due to the non-uniform amount of mucus covering the cells.
Analysis of the caecal content of rats at the end of short-term or subchronic treatment showed little or no impact on the composition of short-chain fatty acids compared to controls, regardless of the type of TiO_2. The authors also noted the absence of modification of mucin O-glycosylation in the small intestine or of O-glycans in the colon of rats exposed orally for seven or 60 days to TiO_2, regardless of the type of TiO_2. These results indicate that the cohesive properties and protective function of the mucus do not appear to be impaired.
In conclusion, *in vitro*, E171 particles accumulated in the mucus secreted by HT29-MTX cells. *In vivo*, exposure to E171 had little or no impact on the overall short-chain fatty acid composition of the caecum, probably indicating the absence of substantial quantitative changes in mucus. Regarding mucus quality, no impairment of mucin O-glycosylation in the small intestine or distal colon of rats treated for seven or 60 days was observed with E171 compared to untreated animals, indicating that it had little impact on the population of the intestinal microbiota with "mucophilic" potential.

- **GECU conclusions on the effects of E171 on the microbiota and intestinal barrier**

The three publications reported in this section studied TiO_2 interactions on bacterial strains and intestinal mucus.
On bacterial cultures grown and exposed to E171 in isolation, morphological and ultrastructural changes limited to a small number of bacterial cells were noted. TiO_2 can also be partially trapped by bacteria and induce moderate bacterial toxicity. On the other hand, the addition of relevant concentrations of E171 (reflecting exposure levels in humans) had little impact on bacterial respiration, fatty acid profile or phylogenetic composition.
In vitro, on cultures of human intestinal cells, E171 particles appeared to accumulate in the mucus secreted by the cells. The role of this mucus as a barrier and translocation facilitator (due to accumulation) is not clearly understood.
In rats, exposure to E171 had little or no impact on the overall short-chain fatty acid composition of the caecum, probably indicating the absence of substantial quantitative changes in mucus. Regarding mucus quality, no impairment of mucin O-glycosylation in the small intestine or distal colon of rats treated for up 60 days was observed with E171 compared to untreated animals.
In conclusion, there is insufficient evidence to conclude that E171 significantly alters the intestinal microbiota and intestinal mucus following oral exposure.

3.5.4. Studies on inflammation

Riedle *et al.* (2017) investigated the adjuvant properties of TiO_2 and the consequences on IL-1β and TNF-α secretion and pro-inflammatory effects. Secretion of IL-1β can be triggered by pathogen-associated molecular patterns such as lipopolysaccharides (LPS) and bacterial peptidoglycans. In this study, the authors considered the influence of genotype on pro-inflammatory effects using mice with a mutation of the NOD2 gene. This mutation promotes secretion of IL-1β and therefore inflammation. The authors isolated macrophages from the bone marrow of mice with the NOD2 gene mutation (NOD2 mice) and mice without the mutation (WT mice). These macrophages were pre-stimulated with LPS at a concentration of 10 ng/mL for three hours and then exposed to food-grade TiO_2 (average particle size 119 nm with 40% less than 100 nm) at concentrations ranging from 5 to 100 µg/mL with or without muramyl dipeptide (MDP, a synthetic peptidoglycan) or bacterial peptidoglycan (PG) at doses of 0 or 10 µg/mL.

The authors showed a decrease in cell viability when TiO_2 concentration increased for both NOD2 and WT genotypes. The addition of MDP and PG to TiO_2 increased the effect very slightly compared to TiO_2 alone. The quantities of TiO_2 particles internalised by the cells increased in a dose-dependent manner for both NOD2 and WT genotypes; the presence of PG or MDP did not influence this internalisation.
In the absence of TiO_2, IL-1β secretion was not triggered on macrophages previously stimulated with LPS and in the presence of MDP. Secretion was stimulated very slightly in the presence of PG. On the other hand, there was a dose-dependent stimulation of IL-1β secretion in the presence of TiO_2 (between 5 and 100 µg/mL). According to the authors, these results confirm that LPS stimulates pro-IL-1β but does not trigger activation of the inflammasome. The latter, on the other hand, was activated by the presence of TiO_2 for both genotypes. After co-exposure of TiO_2 and MPD or PG, IL-1β secretion was further stimulated for the NOD2 genotype. TNF-α secretion was observed solely with macrophage prestimulation by LPS; this secretion was more pronounced for the NOD2 genotype. The presence of TiO_2, MDP and PG further stimulated TNF-α secretion, however no additive effects were observed between TiO_2 and bacterial fragments.

- **GECU conclusions on the inflammation studies**

The studies reported by Riedle *et al.* (2017) were conducted *in vitro* from bone marrow-derived macrophages from mice. The protocol in place (particularly concerning the collection of macrophages from bone marrow) was not able to mimic conditions that would make it possible to report on the potential effects associated with local inflammation.

3.5.5. Studies relating to genotoxic effects

In the publication by Jensen *et al.* (2019), the authors assessed the effect of E171 (99.8% anatase and 0.2% rutile) on intestinal barrier integrity *in vivo*, as well as its genotoxicity.
After dispersion of E171 in sterile water with 2% foetal calf serum added, production of reactive oxygen species (ROS) was measured on undifferentiated Caco-2 intestinal cells after three hours of exposure to doses from 0.125 to 125 µg/mL (i.e. 0.078 to 78 µg/cm^2). E171 did not induce the production of ROS in Caco-2 cells after three hours of treatment. However, potential interference during the test was not assessed.
The authors subsequently exposed female rats to 50 and 500 mg/kg bw/day intragastrically (gavage) once a week for 10 weeks. The rats were sacrificed 24 hours after the final administration. The classical and modified comet assays (used to detect oxidative lesions using DNA repair enzymes: formamidopyrimidine DNA glycosylase (Fpg) and human oxoguanine glycosylase 1 (hOGG1)) were performed using frozen organs (liver and lungs).
The comet assay, normal or modified, showed no DNA damage in the liver and lung. However, these studies did not have positive controls for DNA damage. The positive control used in the study means that it is not possible to confirm whether the test was conducted properly or to verify enzyme activity.
The authors did not observe any change in haematological parameters in the serum of rats treated with E171. At the highest dose, E171 caused a reduction in the gene expression of a tight junction protein (TJP1) in the colon. The translocation of particles or other compounds and the systemic effects observed could therefore have been caused by the loss of integrity of the intestinal barrier. No effect on the expression level of another tight junction protein (occludin) was observed.
No effect on telomere length was observed in the liver and spleen of rats exposed to E171. A decrease in telomere length, a marker of cell senescence and age-related diseases such as cancer, was reported in the lung with the highest dose of E171. For this study, the response variability was very high (percentage decrease with a 95% confidence interval from -0.2 to -34.4%). By exposing

A549 lung cells *in vitro* to the plasma pool of rats exposed to E171, a decrease in telomere length was observed but only with the lowest dose of E171.
Lastly, THP1 cells (human monocyte model) previously exposed or not to an agent causing oxidative damage ($KBrO_3$) were incubated for 30 min with protein extracts from the lungs of rats treated orally with E171 before exposure to Fpg. The repair of oxidative DNA damage caused by $KBrO_3$ was not affected by the lung extract.
The GECU considers that the doses used in the *in vivo* study (especially the highest) appear to be unrepresentative of the data on potential human exposure and were administered according to a protocol that is far removed (once every week for 10 weeks) from the one recommended in OECD guideline 489 for the comet assay. In addition, the DNA damage was not assessed on primary exposed organs (gastrointestinal tract). Moreover, the effects, particularly the primary DNA alterations and oxidative stress, were measured 24 hours after the last administration, enabling repair phenomena to occur. Lastly, the choices made for certain protocols (absence of positive controls, use of extract pools, non-specific immunolabelling) and the expression of certain data (results expressed in relative terms, low correlation between telomere length and doubling time) do not allow the relevance of the study results to be assessed.

The publication by Gea *et al.* (2019) investigated the *in vitro* cytotoxic and genotoxic effects of different shaped TiO_2 nanoparticles, P25 and food-grade TiO_2 particles (anatase with an average size of 150 ± 50 nm) on human lung cells (BEAS-2B). The particles were dispersed in water containing 1% dimethylsulfoxide (DMSO) before homogenisation by ultrasonication. The concentrations used ranged from 5 to 80 µg/mL (1.3 to 20.7 µg/cm^2) for the cytotoxicity measurements and from 20 to 160 µg/mL (5.2 to 41.6 µg/cm^2) for the genotoxicity studies. Cytotoxic effects were assessed following measurements of mitochondrial activity and alteration of the plasma membrane of the cells. Genotoxic effects were determined by measuring DNA damage through two comet assays, the first (alkaline comet assay) determined direct DNA damage, the second (Fpg-modified comet assay) followed the same protocol but in the presence of the DNA repair enzyme (Fpg) and enabled both direct and indirect DNA damage to be determined.
Concerning the cytotoxicity studies, the authors reported that P25 caused a slight decrease in cell viability from 50 µg/mL in the presence of light. This decrease in cell viability was less marked in the absence of light and appeared from 80 µg/mL. No cytotoxic effects were observed for the other forms of TiO_2 at the highest tested dose of 80 µg/mL after 24 hours of exposure in the presence or absence of light.
Regarding genotoxic effects in the presence of light, the authors observed that P25 was not genotoxic in the absence of Fpg but caused dose-dependent DNA damage in the presence of Fpg. Oxidative DNA damage was observed from 50 µg/mL for P25. In the case of food-grade TiO_2, dose-dependent DNA damage (from 50 µg/mL) was observed in the presence or absence of the Fpg enzyme, with oxidative damage considerably greater than direct damage. In the absence of light, the genotoxic effects of P25 were only ever observed in the presence of Fpg but with a lower intensity compared to the conditions under light. For food-grade TiO_2, in the absence of light, genotoxic effects were observed with or without Fpg, and oxidative damage was observed from the highest concentration (160 µg/mL). Lastly, Raman spectroscopic imaging showed the presence of TiO_2 particles (P25 and food-grade TiO_2) in lung cells.
The GECU noted several limitations in this study, such as the lack of information concerning the physico-chemical characterisation of food-grade TiO_2 and questionable methodological choices for the genotoxicity and cytotoxicity protocols (the presence of DMSO possibly affecting the oxidative response, the 24-hour exposure time allowing repair mechanisms to be established, different treatment conditions between the cytotoxicity and genotoxicity studies, interference (test responses that could be affected by the presence of particles) not investigated, choice of cells studied, absence of cytotoxicity data at the highest doses tested in genotoxicity studies).

In the study by Dorier *et al.* (2018), the authors examined the effects of E171 (primary particles 118 ± 53 nm) and two nanoparticle forms of TiO_2: NM-105 (23 nm) and A12 (12 nm) on human intestinal cells (undifferentiated Caco-2 and HT29 MTX) under *in vitro* conditions. Cytotoxicity, DNA damage, oxidative stress and endoplasmic reticulum stress were studied.
The TiO_2 particles were sonicated in ultrapure sterile water immediately before exposure of the cells. The hydrodynamic diameter and polydispersity index values for E171 were published in a previous article. After dilution in culture medium containing foetal calf serum, the authors indicated an increase in the hydrodynamic diameter of E171 particles from 415 ± 69 nm to 739 ± 355 nm and an increase in the polydispersity of the particles. After particle dispersion, the cells were exposed to doses of 20, 50, 100 and 200 µg/mL for the three forms of particles tested.
The authors observed no cytotoxic effects after six and 48 hours of exposure to E171 and NM-105 (results obtained with A12 not presented) at doses of up to 200 µg/mL.
All forms of TiO_2 led to an increase in ROS production regardless of the exposure time (between six and 48 hours), with a dose-dependent increase in the case of E171. The expression of genes encoding proteins involved in antioxidant defence mechanisms was measured. Only expression of the genes of the antioxidant enzyme superoxide dismutase 1 was reduced after 48 hours of exposure to E171 at 50 µg/mL. The authors observed no DNA damage, no increase in the level of oxidised guanine, no effect on the level of 53BP1 foci (marker of double-strand breaks), and no modification in the expression of certain repair genes and genes involved in endoplasmic reticulum stress. The authors concluded that E171 causes only minor effects on the intestinal epithelium.
The results of this study confirm other findings obtained from a similar study conducted by the same team (Dorier *et al.* 2017). In this publication, the authors also showed that E171 was moderately toxic on the same cell types, Caco-2 and HT29 MTX. The genotoxic effects (DNA damage via oxidative stress) of E171 in these cellular models were more pronounced after repeated exposure (three times per week for three weeks) to E171 compared to cells exposed for six, 24 or 48 hours.
The GECU noted several limitations in this study: concentrations were expressed in µg/mL and not $\mu g/cm^2$, and treatment conditions (well surface, volume) were not clearly established (except for the viability and comet assays), which does not facilitate conversion from one unit to another. Moreover, given these uncertainties, the concentrations tested do not appear to be similar for all the parameters studied. No data were provided on the stability of TiO_2 particles over time in the medium. Lastly, the use of a co-culture of undifferentiated Caco-2 and HT29-MTX limited the conclusion regarding the effect on the intestinal epithelium.

In the publication by Proquin *et al.* (2017), the authors investigated the *in vitro* cytotoxic and genotoxic effects of E171 (39% nanoparticles) on human intestinal cells (Caco-2 or HCT116) and compared them to those observed for TiO_2 nanoparticles (size ranging from 10 to 30 nm) and microparticles (mean size 535 nm).
E171 and TiO_2 nano- and microparticles were dispersed in 0.05% bovine serum albumin (BSA) by sonication for 30 min. In the case of the study of chromosome damage, demonstrated by a micronucleus assay, E171 was dispersed in a medium containing 10% foetal calf serum to avoid the appearance of false positives.
For the cytotoxicity study, Caco-2 cells were exposed to all three forms of TiO_2 at concentrations ranging from 0.001 to 1 mg/mL (0.143 to 143 $\mu g/cm^2$) for 24 hours. For HTC116 cells, only E171 was tested at concentrations ranging from 0 to 1 mg/mL (100 $\mu g/cm^2$). E171 produced a cytotoxic effect on Caco-2 cells at concentrations of 14.3 $\mu g/cm^2$ (0.1 mg/mL) and 143 $\mu g/cm^2$ resulting in a 27 and 73% decrease in cell viability after 24 hours of exposure, respectively. TiO_2 nanoparticles and microparticles produced a cytotoxic effect on Caco-2 cells at a maximum dose of 143 $\mu g/cm^2$ resulting in a 33 and 48% decrease in cell viability, respectively. In the case of HCT116 cells, no evidence of cytotoxicity was observed up to the maximum dose of 100 $\mu g/cm^2$.
The authors then focused on ROS generation in an acellular and cellular system (on Caco-2 cells) in the absence or presence of H_2O_2 (which can mimic an inflammatory environment). In the acellular system, in the presence of BSA, no ROS formation was observed with the three forms of TiO_2 for concentrations ranging from 0.143 to 143 $\mu g/cm^2$ in TiO_2 particles. The authors explained these

results by the presence of protein corona that may inhibit this production. In the same system, in the absence of BSA, ROS were generated in the presence of E171 and TiO_2 nanoparticles, with the quantities of ROS produced not statistically significant with or without H_2O_2. In the cellular system, in the absence of BSA, E171 and TiO_2 nanoparticles did not produce ROS up to the maximum tested dose of 1.43 µg/cm^2 with or without H_2O_2, only microparticles generated ROS from the lowest tested concentration of 0.14 µg/cm^2 in the presence of H_2O_2.
Primary DNA damage was assessed by a comet assay on Caco-2 cells with all three forms of TiO_2 at a concentration of 0.14 µg/cm^2 (and up to 1.43 µg/cm^2 for nanoparticles) with or without azoxymethane (AOM, genotoxic agent). E171, nano- and microparticles induced DNA breaks in Caco-2 cells with or without co-treatment with AOM.
Chromosome aberrations were assessed with a micronucleus assay, exposing HTC116 cells to E171 at concentrations ranging from 0 to 100 µg/cm^2. E171 caused an increase in micronuclei in HCT116 cells from 5 µg/cm^2. The authors also observed that E171 seemed to interact with the centromere region of kinetochore poles during mitosis and suggested that E171 was attached to the DNA of mitosis cells.
The GECU noted several limitations in this study. There was no indication of the stability of the particles over time or the impact of the different media used on this stability. No indication was provided as to how the controls were treated in terms of sonication.
Concerning the micronucleus assay, some of the recommendations of OECD guideline 487 were not followed (choice of cell line, measurement of cytotoxicity in the presence of cytochalasin B). Very few binucleated cells are observed in the photos, suggesting a problem performing the micronucleus assay on HCT116 cells, and no raw data were presented. In addition, the photos of the kinetochores proposed in the publication are inconclusive.
Lastly, not all results were obtained on the same cell line (micronucleus only on HCT116 cells and the rest of the studies on Caco-2 cells);
The GECU points out that in the EFSA evaluation (2018), some of these limitations had already been reported.

- **GECU conclusion on genotoxicity**

Many studies have been performed on the genotoxic effects of TiO_2 particles. In the review by Charles *et al.* (2018), the authors analysed 36 publications including *in vitro* genotoxicity data. A genotoxic effect was reported in 60% of the studies (only one study conducted with E171 was selected and indicated an *in vitro* genotoxic effect), although it was not possible to associate a type of TiO_2 (crystallinity, size, coating) with a specific response. Most publications analysed in this review showed that the genotoxic effect was caused by a secondary mechanism via oxidative stress.

The new studies analysed by the GECU do not call into question the conclusions of Charles *et al.* (2018). The generation of oxidative stress *in vitro* appears to be one of the mechanisms by which food-grade TiO_2 causes DNA damage. Only one *in vivo* study with E171 since 2017 was identified. Although the GECU noted a lack of genotoxic activity and oxidative stress generation, the study protocol followed for this study does not seem relevant.
Despite there being no studies showing a direct interaction of TiO_2 particles with DNA and/or the mitotic apparatus, the direct effect of TiO_2 on genetic material or other molecules interacting with genetic material cannot be excluded.

3.5.6. Studies on carcinogenic effects

The three publications by Proquin *et al.* (2018 a, b, c) were based on the results of an *in vivo* study that included a transcriptomic analysis of the distal colon of mice after repeated administration of E171 with or without co-treatment with azoxymethane (AOM) and dextran sodium sulphate (DSS) to mimic an initiation step (genotoxic effect of AOM) coupled with irritation (effect of DSS). It followed another study conducted by the same team (Urrutia-Ortega *et al.* 2016), which concluded that there was an increase in the number of tumours observed in the distal colon of mice exposed to E171 by ingestion for ten weeks and previously treated with AOM and DSS. The studies conducted by Proquin *et al.* sought to determine the molecular changes associated with the increased number of tumours, however, treatment was shortened to three weeks because physiological changes had already been observed after four weeks of treatment in the previous study (Urrutia-Ortega *et al.* 2016).
Initially, E171 (5 mg/kg bw/day, 39% nanoparticles by number) was administered by gavage to mice five times a week for three weeks. Four animals, i.e. two males and two females in each group (control and treated) were sacrificed after two, seven, 14 and 21 days.
Following treatment with E171, gene changes were observed on 417, 971, 1512 and 229 genes after two, seven, 14 and 21 days respectively. Among these gene changes, only 32 deregulated genes, 23 of which have known biological functions, were common to three of the four treatment durations. The observed modulations were more pronounced after seven and 14 days. After two days of treatment, three groups of processes were primarily affected: i) signalling (mainly genes involved in olfactory as well as G protein-coupled receptors) ii) immune response and iii) cancer signalling. After seven days of treatment, cell signalling was greatly affected and had a far higher number of deregulated genes than after two days. Cancer signalling was also affected and several new groups appeared: i) cell cycle, ii) oxidative stress, iii) neural response and iv) metabolism.
Using another approach, mRNA processing and membrane transport of small molecules also seemed to be processes that were affected. After two and seven days, signalling (olfactory and G protein-coupled receptors) was not affected but after 14 days, cancer signalling was modulated through several potential pathways. Effects were also observed on the immune response and cell cycle. Lastly, modulations affecting a smaller number of genes were noted with respect to oxidative stress, bone development and neural response. The second approach used for the analysis indicated effects on metabolism, in particular protein metabolism and the metabolism of certain diseases. After 21 days, only three groups of processes were affected: i) olfactory and G-protein signalling as at two and seven days, ii) oxidative stress and iii) metabolism. However, it should be noted that for the last two processes, the modulations and the number of genes involved remained low.
The authors concluded that E171 acts on the colon through different mechanisms (immune response, inflammation, olfactory and G-protein receptors, cell cycle, DNA repair, metabolism, serotonin receptors, cancer-related genes). Some of these mechanisms could explain how E171 may affect the development of colorectal cancer.

In the publication by Proquin *et al.* (2018c), the authors wanted to test the hypothesis that after ingestion, E171 could induce gene expression changes associated with inflammation, disruption of cancer-related genes and impairment of the immune system before detectable tumours appear. The authors followed the previous experimental design but with a treatment including a single administration of AOM at 12.5 mg/kg by intraperitoneal injection one week before the experiment, as well as one administration of 2% DSS via drinking water during the first five days of the experiment.
The number of genes modulated after exposure to E171 preceded by AOM and DSS treatment was much higher than with E171 alone: 411 genes after two days, 3506 genes after seven days, 2553 genes after 14 days and 1178 genes after 21 days. Very few genes (only 27, of which eight have no known function) were common to three of the four treatment durations. After two days, only one biological function was modulated, that of olfactory and G-protein signalling. The modulation levels

were relatively high, and far higher than those observed during treatment with E171 alone. Overall, deregulation of expression levels was observed after seven days for genes involved in molecular transport, metabolism, signalling, xenobiotic metabolism, the extracellular matrix and immune response. After 14 days, 40 different pathways for eight biological functions were modified. The genes involved in the development of colon cancer and its signalling, modulated after 14 days, were genes for xenobiotic metabolism. Neural response and molecular transport were also processes affected by the treatment. Twenty-one days after the start of treatment, no changes in genes associated with xenobiotic metabolism or metabolism were detected. The modified pathways were signal transduction, immune response, extracellular matrix organisation and neuronal response, predominantly with decreased expression.
For all four treatment durations, modulations of expression were observed regarding signalling and the immune system. From seven days to 21 days after the start of treatment, the extracellular matrix and neuronal response were modulated. At seven and 14 days, especially marked effects were observed on signalling, metabolism, neuronal system and xenobiotic metabolism.
By comparing the results of the three publications, it appears that common processes are altered by E171 with or without prior treatment with AOM and DSS, such as olfactory receptor and G-protein signalling, as well as the immune and neuronal response. However, some processes are only affected in the presence of AOM and DSS, such as xenobiotic metabolism, molecular transport, haemostasis and extracellular matrix organisation.

The authors concluded that E171 affects biological mechanisms that can facilitate the development of cancer and that these are promoted with AOM/DSS treatment.

- **GECU conclusions on the carcinogenicity studies**

The GECU would like to point out that the carcinogenicity studies (NTP 1979) selected by EFSA during the evaluation of E171 were carried out without any prior characterisation of the Unitane® (anatase, size distribution not specified), which was assumed to be food grade. In its 2016 opinion, EFSA concluded that E171 was not carcinogenic based on study results obtained with Unitane®. However, there is no available information that would guarantee similarities in terms of physico-chemical characterisation between Unitane® and E171. The potential tumour-promoting-type effects of E171 demonstrated experimentally by Urrutia-Ortega *et al.* (2016) and Bettini *et al.* (2017) need to be confirmed by the implementation of new studies, in particular including the use of several biomarkers (see the GECU's recommendations in Section 3.7).

In the work conducted by Proquin *et al.* (2018, a, b, c), the authors concluded that E171 affects biological mechanisms that can facilitate the development of cancer and that these are promoted with AOM/DSS treatment. In addition, the GECU noted that the level of histone expression after seven days of treatment could be the basis for the epigenetic changes
The GECU also noted that the often minor responses with E171 alone may be related to the study protocol, which was conducted over only three weeks of treatment, with colons sampled two to three days after the last administration (except for the first two days of treatment); the choice of this duration is not supported by the results presented in the study by Urrutia-Ortega *et al.* (2016). In addition, two rats per sex were analysed per treatment duration, which could have increased the variability of the results compared to the use of only one sex, as the study by Urrutia-Ortega et *al.* was only conducted on male mice. Lastly, the conclusions regarding the increase in proliferation were unconvincing due to the quality of the immunolabelling of sections.

The evidence reported in the studies by Proquin *et al.* (2018 a, b, c) supported the questions about E171 raised by ANSES (2017) during its analysis of the study by Bettini *et al.* (2017), in particular relating to the promoting effects.

3.5.7. Studies on developmental defects

The study by Jovanovic *et al.* (2018) was carried out over twenty generations of *Drosophila* and reported measurements, after exposure to E171, of progeny viability (from egg to adult) in each generation, fertility, development, morphology, accumulation of the Ti element, genotoxicity in the third larval stage in the F1, F10 and F20 generations, and the amount of protein granules in trophocytes (nutritive cells).
This study followed a preliminary study (Jovanovic *et al.* 2016) conducted in *Drosophila melanogaster* over one generation that highlighted an increase in pupation time, the down-regulation of expression of genes involved in oxidative stress and the appearance of aberrant phenotypes following exposure to E171.
The authors found that the concentration of E171 in the feeding medium of *Drosophila* corresponded to human oral exposure of 20 mg/kg bw/day. The multigenerational study indicated that exposure to E171 significantly altered the development and reproductive dynamics of *Drosophila*, reduced fertility and increased genotoxicity. The appearance of aberrant phenotypes (< 0.1%) manifested as morphological anomalies (missing wing, deformed thorax), observed solely in insects exposed to E171, should also be noted. These phenotypic aberrations were not transmitted to the progeny (verified by crossing over the next five generations) and are believed to result from developmental defects.

In the study by Savic *et al.* (2018), the authors examined the effects of a sedimentary environment contaminated with E171 on the lifecycle and development of chironomid larvae (mudworms). The chironomid is a dipterous insect whose larval cycle takes place in sediment before emergence. The results showed that contamination of the sediment with E171 at concentrations above 1000 mg/kg of sandy sediment is toxic to chironomids (increase in mortality ratio and decrease in emergence ratio). Stress induced by TiO_2 at low concentrations (2.5, 25 and 250 mg TiO_2/kg of sediment) that had no effect on viability, resulted in morphometric changes in the head capsule and wings (significant variations in the size of the mentum (elongation, shape and presence of teeth), mandibles and wings) and malformations.

In the study by Ma *et al.* (2019), the authors compared the toxicological effects of non-nanoscale (non-food-grade) TiO_2, food-grade TiO_2 and P25 in the nematode, a model organism widely used in biology. Phototoxicity was measured after 24 hours of exposure, including three hours under UV light, in the nematode at doses ranging from 1 to 10 mg/L for all three forms of TiO_2. The results indicated that P25 is the most phototoxic form while non-nanoscale TiO_2 is the least phototoxic form. In the absence of UV, the authors observed a decrease in nematode longevity for all three forms of TiO_2, with a greater decrease for P25: up to 27%.
The authors also showed that, at comparable levels, all three forms of TiO_2 resulted in dose-dependent effects on reduced reproduction and shortened lifespan in the dose range tested (1 to 10 mg/L). Lastly, the results indicated vulval defects following exposure of nematodes to the three forms of TiO_2, with the greatest effects being observed with P25.

- **GECU conclusions on the developmental studies**

The work appraised in this section cannot be considered in a context of risk assessment for humans due to the study systems used and the difficulty of transposing the doses involved (studies by Ma *et al.* (2019) and Savic *et al.* (2018)) to exposure levels via ingestion in humans. However, this work does highlight some warnings about developmental defects.

Jovanovic *et al.* (2018) conducted a multi-generational study on *Drosophila*, a model for studying differentiation mechanisms. The main feature of this study on *Drosophila* was the appearance of developmental defects (of non-genetic origin) following ingestion of E171 at doses representative of

human exposure. The study by Savic *et al.* (2018) on the larval stages and emergence of the chironomid was consistent with that of Jovanovic *et al.* (2018) on *Drosophila*, i.e. the appearance of developmental defects induced by exposure to E171.

3.5.8. Studies on the effects of E171 on the cardiovascular system

In the study by Jensen *et al.* (2018a), the authors investigated the cardiovascular effects of E171 in *ex vivo* (aorta rings) and *in vivo* (rats, 1 gavage per week for 10 weeks, at exposure doses of 50 and 500 mg/kg bw) models. The study showed that oral exposure to E171 caused alterations in the vasomotor response of the coronary arteries, with no evidence of oxidative stress. E171 (at doses of 500 mg/kg) increased acetylcholine-induced vasorelaxation and 5HT-induced vasocontraction (5 hydroxytryptamine or serotonin).
The same responses were obtained *ex vivo* after direct exposure of the aorta rings. No significant differences in blood parameters indicating oxidative stress (ascorbate, malondialdehyde, tetrahydrobiopterin as a marker of eNOS pathway activation and dimethylarginine) were observed between the control and treated groups.

The same authors (Jensen *et al.* 2018b) verified these results *ex vivo* using human subcutaneous artery segments (obtained from surgical tissue samples from the abdominal belt) exposed to E171 at doses of 14 and 140 µg/mL. On the basis of the EFSA evaluation, the concentration of 140 µg/mL corresponds to the maximum average daily intake of 10.4 mg.kg $bw^{-1}.d^{-1}$ in children, and that of 14 µg/mL to one-tenth of this intake. *Ex vivo* exposure of 30 minutes and 18 hours (to 14 and 140 µg/mL) increased arterial vasoconstriction, while vasorelaxation was not significantly altered after 18 hours. Gene expression of endothelial cells (5-HT, 5-HT2A receptors; ICAM-1 and VCAM - intercellular and vascular cell adhesion molecules) was unchanged.

The objectives of the study by Freyre *et al.* (2018) were to examine the influence of the shape of different TiO_2 particles (E171 and two types of nano-TiO_2 anatase) but also of the dispersion medium (foetal bovine serum (FBS) or saline solution (SS)) on the response observed in the microvessel network, angiogenesis gene modulation and femoral ossification, in an embryonic model of the Leghorn hen *(Gallus gallus domesticus L.*). Seven days after fertilisation, a single dose of 10 µg/egg was injected via the blood flow of the chorioallantoic membrane (CAM) for each TiO_2 suspension. After a post-exposure recovery period of seven days, CAMs and embryo femurs were recovered to determine the impact of TiO_2 exposure on the microvessel network (on CAM), angiogenesis gene expression and femoral ossification (length and width).
The authors observed that depending on the medium used to suspend E171, there were significant differences in hydrodynamic size (measured by dynamic light scattering) and zeta potential, with values of 90.57 ± 4.6 nm / -21.1 ± 0.5mV and 695.5 ± 141.6 nm / -38.36 ± 1.57mV in FBS and SS, respectively.
Measurements of CAM microvascular density showed that all three forms of TiO_2 led to a decrease in average branch length (-8.8 µm for E171) when the TiO_2 was dispersed in SS, but no effect was observed in the case of FBS. On the other hand, the average maximum branch length was unchanged regardless of the types of TiO_2 and the medium used. No differences were observed in the expression of genes involved in angiogenesis regulation after treatment with TiO_2 (only a major general effect was observed when FBS or SS was used to disperse nanoparticles).
Concerning ossification, the femurs of eggs exposed to FBS alone showed alterations in mineralisation; these alterations were also observed in the femurs of eggs exposed to the different types of TiO_2 tested. None of the TiO_2 dispersed in SS induced alterations in femoral ossification.

As there is no consensus on the choice of dispersion medium to be used for toxicological studies of nanoparticles and for intravenous injection with *in vivo* models, the GECU considers that this study mainly demonstrates the importance of the medium used for nanoparticle dispersion, which differs in physical form and modifies the bio-nano interface. For E171, the only effect observed was in the microvascular density of CAM when dispersed in SS. No differences in the expression of genes involved in angiogenesis regulation were observed after treatment with different types of TiO_2. Lastly, no TiO_2-specific alteration of femoral ossification was noted.

- **GECU conclusions on the studies on the effects of E171 on the cardiovascular system**

In summary, the vascular effects identified in the first study (Jensen *et al.* 2018a) were significant, but remained low given the high level of exposure.
The authors concluded that exposure to E171 may lead to an increase in arterial tone, blood pressure, hypertension and heart failure.

The GECU notes that these results support the impairment of vascular microcirculation and cardiac effects reported in rodents after inhalation of TiO_2 nanoparticles (Nurkiewicz *et al.* 2008; Kan *et al.* 2012 and 2014) or after intratracheal administration (Savi *et al.* 2014).

3.6. GECU conclusions and recommendations

Since ANSES's opinion (2017-SA-0020) on the oral toxicity of E171, several studies on hazard identification of E171 have, despite certain methodological limitations, highlighted new signals (changes in histone regulation or developmental effects) or reported previously published effects (e.g. genotoxic effects observed *in vitro* and mediated by oxidative stress).

Physico-chemical characterisation studies in food and biological simulants show that TiO_2 is biodurable within the oral-gastric-intestinal tract and that corona formation, due to the adsorption of food matrix components, modifies the surface properties of TiO_2 particles and can influence their fate *in vivo*.

In studies on interactions between TiO_2 particles and certain bacterial strains *in vitro*, there is no indication that E171 can significantly alter the intestinal microbiota following oral exposure. The GECU would like to point out that the studies reported in this opinion were conducted exclusively *in vitro*, and therefore do not reflect the complexity or exhaustiveness of the intestinal microbiota. *In vivo*, exposure to E171 probably does not induce substantial qualitative or quantitative changes in mucus.

Studies in *Drosophila* have shown developmental defects of non-genetic origin after ingestion of E171 at doses equivalent to the levels of exposure observed in humans.

Studies conducted *ex vivo* (on segments of human subcutaneous arteries) and in rats have shown that high doses of exposure to E171 can cause cardiovascular effects that may lead to hypertension and heart failure.

Recent *in vitro* genotoxicity studies provide no new evidence but confirm that DNA damage is generated by oxidative stress induced by TiO_2. However, the latest studies do not rule out the possibility of a direct effect of TiO_2 on DNA and/or on the mitotic apparatus that could be causing the damage.

Carcinogenicity studies report changes in gene expression in an initiation/inflammation model as well as epigenetic effects suggested mainly by changes in histone expression, which may be consistent with the potential promoting effect discussed in the studies by Ortega *et al.* (2016) and Bettini *et al.* (2017). In addition, the NTP (1979) conclusions stated that "*TiO_2 was not carcinogenic by the oral route for mice, but that no firm conclusion can be reached about the possible carcinogenicity of this compound to rats*".

Based on the analysis of the publications reported in this opinion, the GECU is making the following recommendations.

In its 2017 opinion, ANSES reiterated the need to implement protocols for toxicological studies and physico-chemical characterisation adapted to nanomaterials. The GECU points out that the characterisation of E171, and of all substances containing a nanoscale fraction, is an essential first step for all hazard identification and exposure calculation studies. The GECU notes that the characterisation of substances containing a nanoscale fraction, E171 in particular, has become more systematic and robust through the use of appropriate analytical techniques and the measurement of relevant parameters.

In view of the results provided in the studies by Proquin *et al.* (2018 a, b, c), the GECU reiterates its recommendations (ANSES 2017) to confirm the potential promoting effect of E171 observed in the colon via experiments integrating the use of several biomarkers (aberrant crypt foci, mucin depleted foci, beta catenin accumulated crypt) and/or over longer exposure periods in order to evaluate tumour induction. An additional group with a larger number of animals (compared to the study by Bettini *et al.* (2017)) is needed to confirm or refute a potential initiating effect of E171.

Studies in 2018 (Jovanovic *et al.*, Savic *et al.*) identified developmental defects in invertebrates following exposure to E171. The GECU therefore recommends exploring the toxic potential of E171 on mammalian development. This recommendation is supported by the lack of knowledge on reproductive effects and endocrine disruption highlighted by Heringa *et al.* (2016).

In 2018, EFSA recommended conducting an extended one-generation reproductive toxicity study of E171 (EOGRTS, OECD guideline 443), as well as a better assessment of E171 taking into account interactions with food matrices. The GECU emphasises that in the case of E171, the full version of the EOGRTS should be performed (2018) including the study of neurodevelopmental toxicity effects, developmental immunotoxicity and endocrine parameters, especially sex and thyroid hormones.

4. ANSES'S CONCLUSIONS AND RECOMMENDATIONS

ANSES endorses the conclusions and recommendations of the GECU.

Based on the literature review conducted by the GECU, 25 new studies on the oral toxicity of E171 published since 2017 were identified. Some of these studies revealed new signals, such as changes in histone regulation or developmental defects in invertebrates, as well as *in vitro* genotoxic effects via oxidative stress (effects identified for different forms of nanoparticle TiO_2, including E171). However, none of these new studies were able to confirm or refute the potential carcinogenesis-promoting effect of E171 reported in the study by Bettini *et al.* (2017).

In view of these points, which are unable to resolve the uncertainties regarding the safety of the additive E171, ANSES reiterates its 2017 conclusions (opinion 2017-SA-0020). It recommends:

- Precisely characterising E171, on a physico-chemical level. Due to the great heterogeneity of E171 batches produced and placed on the market, the absence of a precise physico-chemical characterisation of this food additive has now become an obstacle to assessing the risks associated with its consumption;

- Better characterising the possible E171 hazard, which requires the rapid acquisition of additional data that can be used to reach a decision on the various observed signals. These data mainly concern reprotoxicity studies, also mentioned by EFSA in 2016, and *in vivo* genotoxicity studies specific to E171, which should be conducted in light of the results obtained *in vitro*. In a context of widespread use of the food additive E171, authorised in 51 food categories and according to the principle of *quantum satis* (no maximum limit is set, the substance does not have an ADI), manufacturers will be expected to make this information available;

- As part of the process for obtaining marketing authorisation for the additive, assessing the justification for its use for the consumer, which must be based on clearly established benefits (technological value, substitution impossible, value to the consumer or community).

Moreover, and pending a better characterisation of the hazard and the risks of E171, ANSES restates its previous general conclusions on nanomaterials aimed at limiting the exposure of workers, consumers and the environment as part of a gradual approach, in particular by promoting safe products that are equivalent in terms of function and effectiveness, and do not contain nanomaterials.

signed, Dr Roger GENET

KEYWORDS

E171, Dioxyde de titane, Additif alimentaire

KEYWORDS

E171, Titanium dioxide, Food additive

ANNEX

Annex 1: Presentation of the participants

PREAMBLE: The expert members of the Expert Committees and Working Groups or designated rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organisation.

Members of the GECU
Ms Valérie FESSARD – Head of Unit – ANSES – Expertise in toxicology
Mr Fabrice NESSLANY – Laboratory Director – Expertise in toxicology
Ms Paule VASSEUR – Professor Emeritus – Expertise in toxicology

Scientific coordination
Mr Bruno TESTE – Scientific Project Leader – ANSES
Ms Eleni ANASTASI – EU-FORA Fellow (State General Laboratory of Cyprus/ANSES)

Annex 2: Data on E171 occurrence in certain food categories

In order to identify the concentration levels of E171 within different food categories, the GECU listed the data on occurrence of this food additive that were available in the literature. Additional information was obtained from European (EFSA) and national (RIVM) agencies, NGOs (*Agir pour l'Environnement*)) and the DGCCRF, in the context of monitoring nanomaterial labelling.

According to EC Regulation No 1333/2008 [1], TiO_2 is authorised in Europe as a food additive (E171) at *quantum satis* in 51 food categories defined according to the regulations [21].

To this end, two approaches were implemented:

- The use of data corresponding to usage levels reported by manufacturers. For these data, the GECU referred to the information reported in the EFSA opinion (EFSA 2016) and the RIVM report (2016). Concentration data (minimum, maximum, mean) were calculated for each food category where data were available. For the calculation of the mean, a weighting approach was implemented to combine data from different sources (in particular for the EFSA and RIVM data).

- The use of analytical data providing information on E171 concentration levels in different food products. Information on products and analytical methods was collected to facilitate the categorisation of food products and assess the robustness of the measurements. This led to 292 items of data from the analysis of food products being considered, with each food product being categorised according to the FCS approach (food categorisation system, as stated in Regulation (EC) No 1333/2008). Concentrations (minimum, maximum and mean) of E171 were calculated for each of the food categories.

The concentrations of E171 in the different food categories calculated from the usage data reported by industry and the analytical data are presented in Table 1. Occurrence data have been provided for 25 of the 51 food categories.

According to the review carried out, the highest concentrations of E171 are found in a few food categories; in particular, confectionery, dairy analogues, fine bakery products, sauces and also in food supplements in which the maximum concentration was measured (26,950 mg/kg).

Table 1. Concentrations of the food additive E171 in mg/kg or mg/L per food category (according to the FCS categorisation). Min., Mean and Max. correspond respectively to the minimum, mean and maximum concentrations. The data highlighted in grey at the bottom of the table correspond to the food categories (1.1; 1.2; 1.2; 1.7.2; 4.2.1; 4.2.6; 5.1; 9.1; 11.4) for which the incorporation of E171 is not permitted.

FCS number	Food category	Quantity of data	Usage levels (industry) or analytical data (mg/kg or mg/L)			Type of data	Sources
			Min.	Mean	Max.		
01.4	Flavoured fermented milk products including heat-treated products	1		48		Industry	[3]
01.6.3	Other creams	1		1.2		Analytical data	[6]
01.7.1	Unripened cheese, excluding products falling in category 16	5	0.3	0.9	1.5	Analytical data	[5, 6]
01.7.5	Processed cheeses	8	0.4	2.3	11.5	Analytical data	[6]
01.8	Dairy analogues, including beverage whiteners	2	125	2563	5000	Industry	[2, 3]
		11	10	1590	7820	Analytical data	[5, 6, 11, 14, 15]
03	Ice-cream	23	1	398	1902	Industry	[2, 3]
		2	0.1	0.2	0.4	Analytical data	[11]
04.2.4.1	Fruit and vegetable preparations, excluding compote	2	0.3	0.3	0.3	Analytical data	[11]
05.2	Other confectionery including breath-refreshening microsweets	15	9.5	911	4500	Industry	[2, 3]
		51	0.3	985	21940	Analytical data	[5-8, 13, 15, 17, 19]
05.3	Chewing gum	56	100	3563	16000	Industry	[2, 3]
		37	0.3	2475	12100	Analytical data	[4-11, 17, 20]
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	27.0	0.1	728	20000	Industry	[2, 3]
		7	1.1	2291	5988	Analytical data	[5, 6, 14, 17]
06.3	Breakfast cereals	2	2.0	2.1	2.2	Analytical data	[6]
06.7	Pre-cooked or processed cereals	1		3.5		Analytical data	[6]
07.2	Fine bakery products	5	76	754	2338	Industry	[2, 3]
		22	0.3	608	4037	Analytical data	[5, 6, 11, 15, 19, 20]
08.3.3	Casings and coatings and decorations for meat	2		18	35	Industry	[2]

09.2	Processed fish and fishery products, including molluscs and crustaceans	40	0.4	6.8	135	Analytical data	[12, 18]
12.4	Mustard	1		0.3		Analytical data	[5]
12.5	Soups and broths	1		193		Industry	[2]
12.6	Sauces	24	500	1514	4000	Industry	[2, 3]
		16	0.3	1360	7270	Analytical data	[5, 6, 11, 17, 20]
12.7	Salads and savoury-based sandwich spreads	7	83	83	83	Analytical data	[8]
		1		2500	3000	Industry	[2]
12.9	Protein products, excluding products covered in category 1.8	5	1700	3040	5000	Industry	[3]
14.1.4	Flavoured drinks	6		28	70	Industry	[2]
		29	0.1	877	8320	Analytical data	[6, 8, 11, 15]
15.1	Potato-, cereal-, flour- or starch-based snacks	1		2.0		Analytical data	[6]
15.2	Processed nuts	7	500	2398	7000	Industry	[2, 3]
		2	1920	2460	3000	Analytical data	[15]
16	Desserts, excluding products covered in categories 1, 3 and 4	3	130	153	200	Industry	[2, 3]
		9	0.2	1779	12500	Analytical data	[6, 11, 19]
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms, excluding chewable forms	16	2	2626	12000	Industry	[2]
		28	52	13495	26950	Analytical data	[2, 11]
1.1	Unflavoured pasteurised and sterilised (including UHT) milk	10	0.1	44.2	434	Analytical data	[6, 11]
1.2	Unflavoured fermented milk products, including natural unflavoured buttermilk (excluding sterilised buttermilk), non-heat-treated after fermentation	7	0.1	47.6	83	Analytical data	[6, 8, 11]
1.7.2	Ripened cheese	2	1.1	1.4	1.7	Analytical data	[6]
4.2.1	Dried fruits and vegetables	1		11.6		Analytical data	[6]
4.2.6	Processed potato products	1		3.0		Analytical data	[6]
5.1	Cocoa and chocolate products as covered by Directive 2000/36/EC	19	0.3	503	4620	Analytical data	[5-7, 15]
9.1	Unprocessed fish and fisheries products	9	1.7	8.1	20	Analytical data	[12]
11.4	Table-top sweeteners	2	0.3	0.3	0.3	Analytical data	[5]

The approach implemented by the GECU has several limitations and uncertainties.

The food products considered in this study come from different markets (Europe, China, United States, Australia, Jordan, Korea). The GECU did not limit its analysis to data from food products manufactured exclusively in Europe, since some food products are from brands distributed internationally. In addition, many products imported from different countries are now available in grocery stores or specialised supermarkets.

Food products were analysed using different analytical techniques (ICP-MS, ICP-HRMS, ICP-AES, UV spectrophotometry, etc.). Measurement validation data were not systematically specified except for the limits of detection (LOD) and quantification (LOQ).

For some data, the LODs and LOQs of the methods reported in the publications had to be calculated using data from the experimental protocols. Significant differences in LODs and LOQs were observed between the various techniques, however, despite these differences, no technique was excluded. In cases where E171 was not detected (value < LOD) or could not be quantified (value < LOQ) in a food matrix, the GECU decided to assign as the E171 concentration level, respectively, the value of the LOD or the LOQ of the technique used in the measurement (upper-bound approach). In cases where E171 was not detected in the food matrix and the LODs and LOQs were not specified, the data were not analysed.

In some cases, the description of the food products did not enable them to be clearly categorised according to the food categories defined in the Food Additives Regulation (FCS categories). Indeed, some "chocolate" products may be found in several food categories. In these cases, the food products were included in the most general food categories in terms of description.
Food products such as "raw milk" or "ice", whose description did not correspond to any food category, were excluded (seven out of 292 data items excluded). The GECU points out that the E171 concentrations were low (less than 0.5 mg/kg) for these excluded data.

Some of the analysed data came from food categories for which the use of E171 is not authorised by the regulations. These detected quantities can be explained by "carry-over", i.e. E171 is added to the final food via other ingredients. Detection of E171 in these food categories may also be explained by analytical background noise. Lastly, difficulties in categorising food products, as set out above, may also explain some of these results.

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