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Re-evaluation of titanium dioxide (E 171) as a food additive

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)

Abstract

The present Opinion deals with the re-evaluation of the safety of titanium dioxide (TiO_2, E 171) when used as a food additive. From the available data on absorption, distribution and excretion, the EFSA Panel on Food Additives and Nutrient Sources added to Food concluded that the absorption of orally administered TiO_2 is extremely low and the low bioavailability of TiO_2 appears to be independent of particle size. The Panel concluded that the use of TiO_2 as a food additive does not raise a genotoxic concern. From a carcinogenicity study with TiO_2 in mice and in rats, the Panel chose the lowest no observed adverse effects levels (NOAEL) which was 2,250 mg TiO_2/kg body weight (bw) per day for males from the rat study, the highest dose tested in this species and sex. The Panel noted that possible adverse effects in the reproductive system were identified in some studies conducted with material which was either non-food-grade or inadequately characterised nanomaterial (i.e. not E 171). There were no such indications in the available, albeit limited, database on reproductive endpoints for the food additive (E 171). The Panel was unable to reach a definitive conclusion on this endpoint due to the lack of an extended 90-day study or a multigeneration or extended-one generation reproduction toxicity study with the food additive (E 171). Therefore, the Panel did not establish an acceptable daily intake (ADI). The Panel considered that, on the database currently available and the considerations on the absorption of TiO_2, the margins of safety (MoS) calculated from the NOAEL of 2,250 mg TiO_2/kg bw per day identified in the toxicological data available and exposure data obtained from the reported use/analytical levels of TiO_2 (E 171) would not be of concern. The Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI).

Keywords: titanium dioxide, E 171, anatase, rutile, food colour

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Summary

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion re-evaluating the safety of titanium dioxide (TiO_2, E 171) when used as a food additive.

TiO_2 is a food colour authorised as a food additive in the European Union (EU). It was previously evaluated by the Scientific Committee on Food (SCF) in 1975 and 1977, by the Joint FAO/WHO Expert Committee of Food Additives (JECFA) in 1969. In 1969, JECFA allocated an acceptable daily intake (ADI) 'not limited except for good manufacturing practice'. In 1975, the SCF did not establish an ADI for TiO_2, whereas in 1977, the SCF included TiO_2 in the category 'colours for which an ADI was not established but which could be used in food'. The Panel is aware that the European Chemical Agency (ECHA) is carrying out an evaluation for a proposal for harmonised classification and labelling (CLH) on TiO_2, for which the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) is the Rapporteur on behalf of the French Member State Competent Authority. ANSES prepared a report in which concluded that TiO_2 should be considered as being potentially carcinogenic to humans when inhaled and thus be classified Carc. Cat 1B – H350i. However, it also concluded that there was no carcinogenic concern after oral or dermal administration. A public consultation on this report is currently underway.

In nature, TiO_2 exists in different crystalline forms; anatase and rutile are the two most important natural forms. The food additive TiO_2 (E 171) is a white to slightly coloured powder and it is insoluble in water and in organic solvents (Commission Regulation (EU) No 231/2012).

The Panel noted that, according to the data provided by interested parties and from the literature, TiO_2 (E 171) as a food additive would not be considered as a nanomaterial according to the EU Recommendation on the definition of a nanomaterial (i.e. '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 1–100 nm').

The Panel was aware of the extensive database on TiO_2 nanomaterials, however, most of these data were not considered relevant to the evaluation of TiO_2 as the food additive (E 171) in this opinion. Therefore, the Panel considered these data could not be directly applied to the evaluation of the food additive.

From the available data on absorption, distribution and excretion, the Panel concluded that:

- the absorption of orally administered TiO_2 is extremely low;
- the bioavailability of TiO_2 (measured either as particles or as titanium) is low;
- the bioavailability measured as titanium appeared to be independent of particle size;
- the vast majority of an oral dose of TiO_2 is eliminated unchanged in the faeces;
- a small amount (maximum of 0.1%) of orally ingested TiO_2 was absorbed by the gut-associated lymphoid tissue (GALT) and subsequently distributed to various organs and elimination rates from these organs were variable.

The Panel further concluded that there were significant and highly variable background levels of titanium in animals and humans, which presented challenges in the analysis at the low levels of titanium uptake reported and could complicate interpretation of the reported findings.

The Panel 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*.

The Panel noted that possible adverse effects in the reproductive system were identified in some studies conducted with material which was either non-food-grade or inadequately characterised nanomaterial (i.e. not E 171). There were no such indications in the available, albeit limited, database on reproductive endpoints for the food additive (E 171). The Panel was unable to reach a definitive conclusion on this endpoint due to the lack of an extended 90-day study as in the Guidance for submission of food additives (EFSA ANS Panel, 2012) or a multigeneration or extended-one generation reproduction toxicity study with the food additive (E 171). Therefore, the Panel did not establish an ADI.

From a carcinogenicity study with TiO_2 in mice and in rats, the Panel chose the lowest no observable adverse effect level (NOAEL) reported which was 2,250 mg TiO_2/kg body weight (bw) per day for males from the rat study, the highest dose tested in this species and sex.

For the safety assessment of TiO_2 used as a food additive, based on information reported in the examined literature and information supplied following calls for data taking into account the following considerations:

- the food additive E 171 mainly consists of microsized TiO_2 particles, with a nanosized (< 100 nm) fraction less than 3.2% by mass;
- the absorption of orally administered TiO_2 particles (micro- and nanosized) in the gastrointestinal tract is negligible, estimated at most as 0.02–0.1% of the administered dose;
- no difference is observed in the absorption, distribution and excretion of orally administered micro- and nanosized TiO_2 particles;
- no adverse effect resulting from the eventual accumulation of the absorbed particles is expected based on the results of long-term studies which did not highlight any toxicity up to the highest administered dose;
- the uncertainties in the toxicological database arising from limitations in the available reproductive toxicity studies;

The Panel considered that an ADI should not be established, and that a margin of safety (MoS) approach would be appropriate (EFSA ANS Panel, 2012).

To assess the dietary exposure to TiO_2 (E 171) from its use as a food additive, the exposure was calculated based on: maximum levels of data provided to EFSA (defined as the *maximum level exposure assessment scenario*) and reported use levels (defined as the *refined exposure assessment scenario*) as provided by industry and the Member States.

Based on the available dataset, the Panel calculated two refined exposure estimates based on different assumptions: a *brand-loyal consumer scenario*, in which it is assumed that the population is exposed over a long period of time to the food additive present at the maximum reported use/analytical levels for one food category and to a mean reported use/analytical level for the remaining food categories; and a *non-brand-loyal scenario*, in which it is assumed that the population is exposed over a long period of time to the food additive present at the mean reported use/analytical levels in all relevant food categories.

For the *maximum level exposure assessment scenario*, at the mean, the exposure estimates ranged from 0.4 mg/kg bw per day for infants and the elderly to 10.4 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 1.2 mg/kg bw per day for the elderly to 32.4 mg/kg bw per day for children.

For the *refined estimated exposure scenario*, in the *brand-loyal scenario*, the exposure estimates ranged, at the mean, from 0.4 mg/kg bw per day for infants and the elderly to 8.8 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 1.1 mg/kg bw per day for the elderly to 30.2 mg/kg bw per day for children. In the *non-brand-loyal scenario*, the exposure estimates ranged, at the mean, from 0.2 mg/kg bw per day for infants and the elderly to 5.5 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.5 mg/kg bw per day for the elderly to 14.8 mg/kg bw per day for children.

In the case of TiO_2, the Panel did not identify brand loyalty to a specific food category and therefore the Panel considered that the non-brand-loyal scenario covering the general population was the more appropriate and realistic scenario for risk characterisation because it is assumed that the population would probably be exposed long term to food additives present at the mean reported use/analytical levels in processed food.

The Panel noted that the lowest MoS calculated from the NOAEL of 2,250 mg TiO_2/kg bw per day identified in the available toxicological data and exposure data obtained from the reported use/analytical levels of TiO_2 (E 171) considered in this opinion is above 100. In the Guidance for submission of food additives (EFSA ANS Panel, 2012), the Panel considered that, for non-genotoxic and non-carcinogenic compounds 'a MoS of 100 or more between a NOAEL or BMDL and the anticipated exposure would be sufficient to account for uncertainty factors for extrapolating between individuals and species'. Consequently, the Panel considered that on the database currently available and the considerations on the absorption of TiO_2 the margins of safety calculated from the NOAEL of 2,250 mg TiO_2/kg bw per day identified in the toxicological data available and exposure data obtained from the reported use/analytical levels of TiO_2 (E 171) considered in this opinion would not be of concern.

The Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI).

The Panel recommended that:

- In order to enable the Panel to establish a health-based guidance value (ADI) for the food additive TiO_2 (E 171), additional testing could be performed. An extended 90-day study or a multigeneration or extended-one generation reproduction toxicity study according to the current OECD guidelines could be considered. Such studies should be performed with TiO_2 (E 171) complying with the EU specifications and additionally including a characterisation of the particle size distribution of the test material. However, in deciding on actual testing, considerations of animal welfare need to be balanced against the improvement in the toxicological database within a tiered testing approach.
- The EU specifications for TiO_2 (E 171) should include a characterisation of particle size distribution using appropriate statistical descriptors (e.g. range, median, quartiles) as well as the percentage (in number and by mass) of particles in the nanoscale (with at least one dimension < 100 nm), present in TiO_2 (E 171) used as a food additive. The measuring methodology applied should comply with the EFSA Guidance document (EFSA Scientific Committee, 2011).
- The maximum limits for the impurities of the toxic elements (arsenic, lead, mercury and cadmium) in the EU specification for TiO_2 (E 171) should be revised in order to ensure that TiO_2 (E 171) as a food additive will not be a significant source of exposure to those toxic elements in foods.

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Background as provided by the European Commission

Regulation (EC) No 1333/2008[1] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union (EU). In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the EU before 20 January 2009 has been set up under the Regulation (EU) No 257/2010[2]. This Regulation also foresees that food additives are re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[3] of 2001. The report 'Food Additives in Europe 2000'[4] submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with a highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of adoption of Regulation (EU) 257/2010, the 2003 Terms of References are replaced by those below.

Terms of Reference as provided by the European Commission

The Commission asks EFSA to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

Assessment

1. Introduction

The present Opinion deals with the re-evaluation of the safety of titanium dioxide (TiO_2, E 171) when used as a food additive.

TiO_2 (E 171) is authorised as a food additive in the EU in accordance to Annex with Annex II to Regulation (EC) No 1333/2008[1] in both anatase and rutile forms (Commission Regulation (EU) No 231/2012[5]).

TiO_2 (E 171) has been previously evaluated by the EU SCF in 1975 and 1977, by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969 (JECFA, 1970) and by EFSA in 2004. It has also been reviewed by TemaNord in 2002.

The Panel noted the Scientific Committee on Consumer Safety (SCCS) Opinion on TiO_2 (nanoform) (SCCS, 2013a,b), and the recent commentary on this Opinion (SCCS and Chaudhry, 2015). However, the Panel noted that the aim of these reports was to provide an answer to the question of the

[1] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.
[2] Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19–27.
[3] Report from the Commission on Dietary Food Additive Intake in the European Union, Brussels, 1.10.2001, COM (2001) 542 final.
[4] Food Additives in Europe 2000, Status of safety assessments of food additives presently permitted in the EU, Nordic Council of Ministers, TemaNord 2002:560.
[5] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes Annexes II and III to Regulation (EC) No 1331/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p. 1.

European Commission on whether the use of TiO_2 in its nanoform as an ultraviolet (UV) filter in cosmetic products (e.g. sunscreens), at a concentration up to a maximum of 25.0% (250 g/kg product), was safe for consumers. Therefore, the Panel considered that the conclusions of the report cannot be extrapolated to the safety evaluation of TiO_2 (E 171) as a food additive.

The Panel on Food Additives and Nutrient Sources added to Food (ANS) was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that had become available since then and information available following public calls for data.[6,7] The Panel noted that not all of the original studies on which previous evaluations were based were available for this re-evaluation.

2. Technical data

2.1. Identity of the substance

TiO_2 (E 171), Chemical Abstracts Service (CAS) Registry number 13463-67-7, European Inventory of Existing Commercial Chemical Substances (EINECS) number 236-675-5 and Colour Index (C.I.) number 77891, is an inorganic substance with the molecular formula TiO_2 and a molecular weight of 79.88 g/mol. The titanium atom is coordinated octahedrally with oxygen, but the position of the octahedral structure differs in the different crystalline forms (Diebold, 2003).

In nature, TiO_2 exists in different crystalline forms, anatase and rutile being the two most important natural forms: anatase (tetragonal, CAS Registry number 1317-70-0), rutile (tetragonal, CAS Registry number 1317-80-2) and brookite (orthorhombic, CAS Registry number 12188-41-9). Rutile is the thermodynamically stable form of TiO_2 (Kuznesof, 2006). TiO_2 also exists in an amorphous form (Mathews, 1976). Anatase rapidly transforms to rutile at a temperature $> 700°C$. Rutile melts at temperatures between 1,830 and 1,850°C (Kirk-Othmer, 1997, 2006).

Pure TiO_2 is a white powder that gives a white background colour. TiO_2 particles reflect light (pearlescent) over the majority of the visible spectrum and achieve opacity (i.e. making products impenetrable to light) by causing multiple reflections and refractions.

The food additive TiO_2 (E 171) is a white to slightly coloured powder (Commission Regulation (EU) No 231/2012). It is insoluble in water and organic solvents. It dissolves slowly in hydrofluoric acid and in hot concentrated sulfuric acid [JECFA, 2009; Commission Regulation (EU) No 231/2012].

Several synonyms exist for the different crystalline forms of TiO_2. Some of the more common synonyms for the pigment are: C.I. Pigment White 6, C.I. No 77891, Titania, INS No. 171, titanium white and titanium (IV) oxide [IARC (International Agency for Research on Cancer) (2010)].

2.1.1. Particle size and particle size distribution of TiO_2

Interested parties provided analytical data on the particle size characteristics of TiO_2 (E 171; anatase or rutile) used as a food/feed additive (Doc. provided to EFSA n. 6; Doc. provided to EFSA n. 15; Doc. provided to EFSA n. 9; Doc. provided to EFSA n. 12; Doc. provided to EFSA n. 19). The particle size distributions were determined using different analytical methods (dynamic light scattering (DLS), X-ray disc centrifugation (XSDC), transmission electron microscopy (TEM) and scanning electron microscopy (SEM)) and details of the analytical procedures were provided. The data are shown in Tables 1 and 2.

[6] Call for scientific data on food colours to support re-evaluation of all food colours authorised under the EU legislation. Published: 8 December 2006. Available online: http://www.efsa.europa.eu/en/dataclosed/call/afc061208.htm

[7] Call for food additives usages level and/or concentration data in food and beverages intended for human consumption. Available online: http://www.efsa.europa.eu/en/dataclosed/call/130327.htm

Table 1: Data submitted by industries to EFSA on the particle size characteristics of TiO_2 as food/feed grade

Submitted by			Colorcon (2015; Doc. provided to EFSA n. 9)[a]	TDMA (2015; Doc. provided to EFSA n. 19)[b],[c]				TDMA (2015; Doc. provided to EFSA n. 19)[b],[d]		Interested party 1 (2012; Doc. provided to EFSA n. 15)[b]					
Crystal form			**Anatase**	**Anatase**			**Rutile**	**Anatase**		**Anatase (Sample 1)**			**Rutile (Sample 2)**		
Analytical method applied	DLS HD	Median particle size (d_{50}) (nm)	336	143	160	168	191								
		% particles by number < 100 nm	ND	12	ND	ND									
		% particles by mass < 100 nm		2	ND	ND	ND								
	XSDC HD	Median particle size (d_{50}) (nm)	176	151	166	179	202	168	202	230	250	250	330	340	340
		% particles by number < 100 nm	< 1	8	9	3	ND	ND	ND	14	5	4	< 1	< 1	< 1
		% particles by mass < 100 nm		1	< 1	< 1	ND	ND	< 1	0	0	0	0	0	0
	XSDC AECD	Median particle size (d_{50}) (nm)		121	135	148	169	137	122						
		% particles by number < 100 nm		32	29	20	3	17	26						
	TEM	Median particle size (d_{50}) (nm)	113	115	131	146	165	142	112						
		% particles by number < 100 nm	< 36	33	17	15	11	16	39						
	SEM	Median particle size (d_{50}) (nm)		123	134	147	172								
		% particles by number < 100 nm		23	19	12	10								

DLS HD: dynamic light scattering hydrodynamic diameter; ND: not detected; XSDC HD: X-ray scanning disc centrifugation hydrodynamic diameter; XSDC AECD: X-ray disc scanning centrifugation area equivalent circular diameter; TEM: transmission electron microscopy; SEM: scanning electron microscopy.
(a): Not clear if results are expressed based on particle number or by mass.
(b): Results expressed on a number basis.
(c): Data gathered with TDMA samples.
(d): Data generated with IACM samples, 2013.

Table 2: Data provided by CEFIC in 2011 (Doc. provided to EFSA n. 6)

E 171 TiO_2 products	Particles size of batches of TiO_2 E 171										
	A	**A**	**B**	**C (lot 1)**	**C (lot 1)**	**C (lot 1)**	**C (lot 2)**	**C (lot 2)**	**D**	**E**	**F**
Measured average particle size (nm)	390	680	556	577	394	225	441	190	169	420	550
Weight % < 100 nm	0.15	0.00	0.00	0.00	0.40	0.90	0.40	3.10	3.20	0.05	0.50
Measurement method	Spinning disc centrifuge	Laser diffraction	Laser diffraction	Laser diffraction	Spinning disc centrifuge	TEM	Spinning disc centrifuge	TEM	TEM	Spinning disc centrifuge	Laser diffraction
Method of dispersion	Ultra Turrax	Ultra Turrax	Ultrasonic probe	Ultrasonic probe	Ultrasonic probe	Ultrasonic probe	Ultrasonic probe	Ultrasonic probe	High-shear/ high-speed mixer	Ultrasonic probe	Ultrasonic probe
Dispersion parameter	9,500 rpm/ 60 s	9,500 rpm/60 s	200 W/120 s	350 W/120 s	55 W/600 s	350 W/60 s	55 W/600 s	350 W/60 s	2,500 rpm/ 4 min speed mixer + manual rub out	9,500 rpm/60 s	50 W/180 s
Dispersion aid	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

TEM: transmission electron microscopy.

According to CEFIC (2011a; Doc. provided to EFSA n. 5):

> 'data on the particle size distribution of titanium dioxide will always vary depending on the measurement method. Optimum light scattering (i.e. whitening power) requires a primary particle size of approximately half the wavelength of the light to be scattered (i.e. half of 400–700 nm for visible light). Products with a mean primary particle size in the nano range (< 100 nm) would not be suitable and would not be supplied for this application'.

This statement from CEFIC is in line with Wang et al. (2007b) who reported that TiO_2 became transparent when its particle size was < 100 nm. In addition, CEFIC (Doc. provided to EFSA n. 5) reported that:

> 'There has been no significant change in the particle size of products supplied for the food market, however, as with other particulate materials, there will be a distribution of primary particle sizes around the average value and it is possible that a small fraction of the primary particles would be below 100 nm. It is indicated that in practice any products supplied would be aggregated so the actual particle size would be larger than the primary particle size'. (Doc. provided to EFSA n. 5)

CEFIC (2011b) provided information on the measured average particle size of 11 commercial samples of TiO_2 (E 171) in dispersions, using different methods of dispersion (ultra Turrax, ultrasonic probe, high-shear/high-speed mixer) and different measurement methods (laser diffraction, spinning disc centrifuge, TEM). The results showed an average particle size of 169–680 nm; the smaller particle sizes were reported from application of the TEM measurement technique. The weight percentage of particles with a size < 100 nm ranged between 0.0% and 3.2% (Doc. provided to EFSA n. 6; Table 2).

Limited information from anatase and rutile (E 171) samples was submitted by Interested party 1 (2012; Doc. provided to EFSA n. 15, Table 1).

Colorcon (2015; Doc. provided to EFSA n. 9) provided information on one sample of anatase (E 171) analysed by DLS, XSDC and TEM. The Panel noted that, when using XSDC, the median particle size (d_{50}) value was significantly lower than that obtained with DLS. However, using the former method and even after sonication of the suspension, < 1% of the particles had a size below 100 nm. As regards the data obtained with TEM, it was noted that dispersed TiO_2 showed an aggregated morphology with very few individual particles observed. At higher magnification, the diameters of the discrete particles within the aggregate were predominantly in the range of 80–180 nm. The d_{50} of these discrete (but aggregated) particles was found to be 113 nm and ~ 36% had a diameter of < 100 nm.

TDMA (2015; Doc. provided to EFSA n. 19) provided a report on the analysis of commercial E 171 and pigmentary TiO_2 (Table 1).

The Panel noted the difficulty of comparing the data available from different sources of information, resulting from the use of different analytical methodologies. Therefore, the results from the TDMA 2015 report (Doc. provided to EFSA n. 19) were considered to be most appropriate for assessing the possible presence of the nanoparticle fraction in titanium dioxide (E 171) for the following reasons: six samples of six 'anonymised' commercial products of the food additive E 171 were analysed; all samples had at least some methods of dispersion of the particles in common; four of the samples were analysed with DLS hydrodynamic diameter (HD), XSDC HD, X-ray disc scanning centrifugation area equivalent circular diameter (XSDC AECD), SEM and TEM, and two of them by XSDC HD, XSDC AECD and TEM. The Panel noted that the results on the percentage of nanoparticles by number for each sample were lower when DLS HD (from non-detected to 12%) and XSDC HD (from non-detected to 9%) were used, whereas the maximum percentage of nanoparticles by number were reported when TEM (from 11% to 39%) or XSDC AECD (from 3% to 32%) were used.

Additional information on the particle size characteristics of 'food-grade' TiO_2 gathered from the public literature is given in Table 3.

Table 3: Data on the particle size of food-grade TiO_2 from the literature

	Peters et al. (2014)	Theissmann et al. (2014)	Yang et al. (2014)	Weir et al. (2012)	Athinarayanan et al. (2015) – Periasamy et al. (2015)
Samples[(a)]	• Food-grade TiO_2 materials (E 171) – 7 samples • 24 food products • 3 personal care products	• KRONOS K1171 a food-grade pigment with an anatase structure • KRONOS K2360 a pigment with a rutile structure for use in coatings and paints	• Food-grade TiO_2 (E 171) – 5 samples • Synthetic TiO_2 (P 25)	• Food-grade TiO_2 (E 171) – 1 sample • Synthetic TiO_2 (P 25) • Consumer products	• Food-grade TiO_2 (E 171) • Food products (confectionary)
Analytical method(s) used	• SEM • Flow field-flow fractionation (combined with inductively coupled mass spectrometry) • Single-particle inductively coupled mass spectrometry	• SEM	• TEM • PALS	• SEM • DLS	• TEM • DLS
Results (SEM/TEM)	• Size distribution in the range of 30–600 nm • 10% particles < 100 nm	• Equivalent circle diameter: 146 nm • Minimum Feret diameter: 133 nm	• Average diameters: 106–132 nm • 17–35% particles < 100 nm	• Mean particle size: 110 nm (range 30–400 nm) • 36% particles < 100 nm	• Spherical particles with a diameter of 30–250 nm
Results (DLS/PALS)			• Mean hydrodynamic size: 127–504 nm • 0% particles < 100 nm (four samples) and 29% particles < 100 nm (one sample)	• Mean diameter: 150 nm with a primary peak at 225 nm but a shoulder at 37 nm	• Average size of TiO_2 particles: 152 nm[(b)] • Average size of TiO_2 particles: 42 nm[(c)]
Comments	• Limitation: particles below 20 nm are excluded		• Primary particles aggregate in ultrapure water (DLS/PALS)		

SEM: scanning electron microscopy; TEM: transmission electron microscopy; DLS: dynamic light scattering; PALS: phase analysis light scattering.
(a): Results are reported only for the food-grade samples.
(b): Athinarayanan et al. (2015).
(c): Periasamy et al. (2015).

Weir et al. (2012) used TEM to analyse one single batch of food-grade TiO_2 and reported that at least 36% of the particles (it was not specified whether this refers to the weight or the number of particles) had a particle size < 100 nm.

Using SEM analysis of seven TiO_2 E 171 types, Peters et al. (2014) reported that ~ 10% of the particles had a size < 100 nm.

Theissmann et al. (2014) used a microscopic imaging methodology (similar to TEM) and determined that the d_{50} primary particle size of anatase TiO_2 food-grade was in the range of 133–146 nm.

Yang et al. (2014) analysed five different samples of food-grade TiO_2 using TEM and DLS. Four of the samples contained TiO_2 in the anatase form, whereas one sample contained both rutile and anatase. TEM was used to determine the number-based particle size distributions and the average diameters were shown to be in the range of 106–132 nm. The five samples contained 17–35% nanosized particles, based on the size distribution with a confidence level of 95%. However, when suspended in water, the mean hydrodynamic sizes of the five samples were in the range of 127–504 nm, as determined by DLS. The hydrodynamic diameter distributions of four samples showed that all particle sizes were > 100 nm, whereas in one sample, 29% of particles were < 100 nm.

Athinarayanan et al. (2015) and Periasamy et al. (2015) reported the results of the characterisation by TEM of TiO_2 from two different food products (confectionary) and a commercial TiO_2, (E 171). The TEM images of titanium dioxide from the food products or commercial E 171 showed the presence of spherical particles with a diameter range from 30 to 250 nm. Athinarayanan et al. (2015) also reported that the analysis by DLS showed an average particle size of TiO_2 of 152 nm, whereas Periasamy et al. (2015) reported that the average particle size of TiO_2 was 42 nm.

The Panel noted that determination of the fraction of TiO_2 nanoparticles in the food additive (E 171) is method-dependent. In addition, the Panel noted that according to the data provided by industries and from the literature, TiO_2 (E 171) as a food additive would not be considered as a nanomaterial according to the EU Recommendation on the definition of a nanomaterial.[8]

For the sake of comparison, the particle size characteristics of the substances used in the major toxicological studies described in 'Section 3' are given in Table 4.

Overall, the Panel noted that the great majority of the data indicates that in aqueous media, TiO_2 is present in the form of agglomerates and/or aggregates.

The Panel noted that the information on the percentage of nanoparticles by mass was limited (Doc. provided to EFSA n. 6 and 19). According to CEFIC (2011b; Doc. provided to EFSA n. 6), the weight percentage of particles with a size below 100 nm ranged from 0% to 3.2% (maximum value analysed by TEM). The percentage of particles by mass with a size below 100 nm was also provided in the TDMA (2015) report (Doc. provided to EFSA n. 19) when DLS HD and XSDC HD were used (from non-detected to 2%).

For the purpose of estimating the exposure to TiO_2 nanoparticles from the use of TiO_2 (E 171) as a food additive, the Panel considered that the highest reported percentage value of 3.2% of nanoparticles (< 100 nm) by mass, could reasonably be used to address in a conservative way a preliminary estimate.

[8] In Commission Recommendation of 18 October 2011 on the definition of nanomaterial, 2011/696/EU nanomaterials are defined as follows: '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 1–100 nm.

Table 4: Data on the particle size of TiO_2 as used in toxicological studies

Reference	Warheit et al. (2015b)[a]			US National Cancer Institute (NCI, 1979)	Warheit et al. (2015a)		Tassinari et al. (2014)	Mohammadipour et al. (2014)
Type of study	Acute oral toxicity	28-day study rat	Subchronic 90-day study rat (OECD TG 408)	Carcinogenicity study in mice and rat	Developmental toxicity study in pregnant rats (OECD 414)		Short-term oral exposure to rats	Developmental toxicity study in pregnant rats
TiO_2 grade	Ultrafine Alumina and silica coated	Uncoated pigment grade	Alumina-coated pigment grade	Purity 98%, unitane 0–220	Three samples of ultrafine	Three samples of pigment grade	< 25 nm (Sigma-Aldrich, UK)	Nanopowder purchased from Nano Lima, Co. (Iran) Purity: 99% Particle size: 10 nm
Crystal form	79% Rutile 21% Anatase	Rutile	Rutile	Anatase	Anatase and/or rutile		Anatase	Anatase
Analytical method used	TEM[b]				TEM[b]		TEM and SEM	TEM
d_{50} (number %)	73	173	145		19–23	120–165		
Number % particles < 100 nm	73	11	21		100	11–27	13	
Other parameters				Arithmetic mean diameter 180–320 nm			The size distribution was dominated by agglomerates with mean diameter up to 1.6 μm 48% of particles were in the range of 100–300 nm Average diameter of the size distribution: 284 nm SSA = 45–55 m^2/g	Particle diameters were in the nanosize range SSA > 150 m^2/g

OECD TG: Organisation for Economic Co-operation and Development Testing Guidelines; TEM: transmission electron microscopy; SEM: scanning electron microscopy; SSA: specific surface area.
(a): For further details on the synthesis and characterisation, reference is made to Hu et al. (2011a,b) and Yang et al. (2014).
(b): Detailed particle size data are included in the papers using different analytical methods. For comparison purpose, only TEM results are reported in this table.

2.2. Specifications

Specifications have been defined in Commission Regulation (EU) No 231/2012 laying down specifications for food additives and by JECFA (2012).

The purity of TiO_2 (E 171), on a dry basis, is specified as not less than 99% on an alumina (aluminium oxide)- and silica (silicon dioxide)-free basis; the total content of aluminium oxide and/or silicon dioxide is not more than 2%, either alone or combined [Commission Regulation (EU) No 231/2012; JECFA, 2012].

Table 5 shows the specifications for TiO_2 (E 171) according to Commission Regulation (EU) No 231/2012 and JECFA (2012).

Table 5: Specifications for TiO_2 (E 171) according to Commission Regulation (EU) No 231/2012 and JECFA (2012)

	Commission Regulation (EU) No 231/2012	**JECFA (2012)**
Definition	Titanium dioxide consists essentially of pure anatase and/or rutile titanium dioxide, which may be coated with small amounts of alumina and/or silica to improve the technological properties of the product The anatase grades of pigmentary titanium dioxide can only be made by the sulfate process, which creates a large amount of sulfuric acid as a by-product. The rutile grades of titanium dioxide are typically made by the chloride process Certain rutile grades of titanium dioxide are produced using mica (also known as potassium aluminium silicate) as a template to form the basic platelet structure. The surface of the mica is coated with titanium dioxide using a specialised patented process Rutile titanium dioxide, platelet form is manufactured by subjecting titanium dioxide (rutile)-coated mica nacreous pigment to extractive dissolution in acid followed by an extractive dissolution in alkali. All of the mica is removed during this process and the resulting product is a platelet form of rutile titanium dioxide	Produced by either the sulfate or the chloride process. Processing conditions determine the form (anatase or rutile structure) of the final product In the sulfate process, sulfuric acid is used to digest ilmenite ($FeTiO_3$) or ilmenite and titanium slag. After a series of purification steps, the isolated titanium dioxide is finally washed with water, calcined and micronised In the chloride processes, (a) titanium-containing mineral is reacted with chlorine gas under reducing conditions to form anhydrous titanium tetrachloride, which is subsequently purified and converted to titanium dioxide either by direct thermal oxidation or by reaction with steam in the vapour phase; (b) titanium-containing mineral is reacted with concentrated hydrochloric acid to form a solution of titanium tetrachloride, which is further purified and hydrolysed to get titanium dioxide. The compound is filtered, washed and calcined. Commercial titanium dioxide may be coated with small amounts of alumina and/or silica to improve the technological properties of the product
Assay	Content not less than 99% on an alumina and silica-free basis	Not less than 99.0% on the dried basis and on an aluminium oxide and silicon dioxide-free basis
Description	White to slightly coloured powder	White to slightly coloured amorphous powder
Identification		
Solubility	Insoluble in water and organic solvents. Dissolves slowly in hydrofluoric acid and in hot concentrated sulfuric acid	Insoluble in water, hydrochloric acid, dilute sulfuric acid and organic solvents. Dissolves slowly in hydrofluoric acid and hot concentrated sulfuric acid
Colour reaction		Add 5 mL sulfuric acid to 0.5 g of the sample, heat gently until fumes of sulfuric acid appear, then cool. Cautiously dilute to about 100 mL with water and filter. To 5 mL of this clear filtrate, add a few drops of hydrogen peroxide; an orange-red colour appears immediately

	Commission Regulation (EU) No 231/2012	JECFA (2012)
Purity		
Loss on drying	Not more than 0.5% (105°C, 3 h)	Not more than 0.5% (105°C, 3h)
Loss on ignition	Not more than 1.0% on a volatile matter-free basis (800°C)	Not more than 1.0% (800°C) on the dried basis
Aluminium oxide and/or silicon dioxide	Total not more than 2.0%	Not more than 2%, either singly or combined
Acid-soluble substances/ matter soluble in 0.5 N HCl	Not more than 0.5% on an alumina and silica-free basis and, in addition, for products containing alumina and/or silica, not more than 1.5% on the basis of the product as sold	Not more than 0.5%; Not more than 1.5% for products containing alumina or silica Suspend 5 g of the sample in 100 mL of 0.5 N hydrochloric acid and place on a steam bath for 30 min with occasional stirring. Filter through a Gooch crucible fitted with a glass fibre filter paper. Wash with three 10 mL portions of 0.5 N hydrochloric acid, evaporate the combined filtrate and washings to dryness, and ignite at a dull red heat to constant weight
Water-soluble matter	Not more than 0.5%	Not more than 0.5%
Antimony	Not more than 2 mg/kg after an extraction with 0.5 N HCl	Not more than 2 mg/kg (impurities soluble in 0.5 N hydrochloric acid)
Arsenic	Not more than 1 mg/kg after an extraction with 0.5 N HCl	Not more than 1 mg/kg (impurities soluble in 0.5 N hydrochloric acid)
Cadmium	Not more than 1 mg/kg after an extraction with 0.5 N HCl	Not more than 1 mg/kg (impurities soluble in 0.5 N hydrochloric acid)
Lead	Not more than 10 mg/kg after an extraction with 0.5 N HCl	Not more than 10 mg/kg (impurities soluble in 0.5 N hydrochloric acid)
Mercury	Not more than 1 mg/kg after an extraction with 0.5 N HCl	Not more than 1 mg/kg (impurities soluble in 0.5 N hydrochloric acid)

The International Agency for Research in Cancer (IARC, 2010) stated that natural rutile and anatase contain impurities of up to ~ 2% including iron, chromium, vanadium, aluminium, niobium, tantalum, hafnium and zirconium. It further stated that, as most commercial titanium dioxide is manufactured from natural material by dissolution of the parent mineral and reprecipitation as fine particles with the structure of anatase or rutile, most but not all of these chemical impurities are generally removed (IARC, 2010). However, the Panel recommends that limits for these elements should be included in the EU specifications for TiO_2 (E 171). JECFA specifications for TiO_2 were set in 2012 (JECFA, 2012). The JECFA specifications in 2004 referred only to the sulfate process for the production of TiO_2, whereas both the sulfate and chloride processes are mentioned in the 2006, 2009, 2010 and 2012 specifications (JECFA, 2006a, 2009, 2010, 2012).

The Panel noted that, according to the EU specifications for TiO_2 (E 171), impurities of the toxic elements arsenic, lead, mercury and cadmium are accepted up to concentrations of 1, 10, 1 and 1 mg/kg, respectively. Contamination at those levels could have a significant impact on the exposure to these metals, for which the intake is already close to the health-based guidance values established by EFSA (EFSA CONTAM Panel, 2009a,b, 2010, 2012).

The Panel noted that there are no set limits for the particle size of TiO_2 in the EU specifications (Commission Regulation (EU) No 231/2012), and therefore characterisation of the particle size in the food additive E 171 should be included among the specifications. The full characterisation should include the particles size distribution, together with determination and quantification of any nanoparticulate material.

The Panel noted that the manufacturing process for powdered or particulate food additives resulted in material with a range of sizes. Although the median size of the particles is generally significantly greater than 100 nm, a small fraction will always be, and has been, with at least one dimension below 100 nm. The material used for toxicological testing would have contained this nanofraction. The test

requirements stipulated in the current EFSA guidance documents and the European Commission guidelines for the intended use in the food/feed area apply in principle to unintended nanoforms, as well as to engineered nanomaterials. Therefore, the Panel considers that, in principle, for a specific food additive containing a fraction of particles with at least one dimension below 100 nm, adequately conducted toxicity tests should be able to detect hazards associated with this food additive, including its nanoparticulate fraction. The Panel considers that for the re-evaluation of food additives, this procedure would be sufficient for evaluating constituent nanoform fraction in accordance with the recommendation of the EFSA Nano Network in 2014 (EFSA, 2015).

2.3. Manufacturing process

The principal raw materials for manufacturing TiO_2 include ilmenite (iron titanium oxide, $FeTiO_3$), naturally occurring rutile (TiO_2) or titanium slag. TiO_2 (E 171) is manufactured to obtain either the anatase or the rutile crystal structures (Commission Regulation (EU) No 231/2012).

Titanium pigment is extracted from the raw material via either the sulfate process or the chloride process.

- In extraction via the sulfate process, there are three main stages. The ore (usually ilmenite) is dissolved in sulfuric acid to form a mixture of sulfates. Most of the TiO_2 from the ore is solubilised as a titanium oxysulfate. Iron is removed from the solution in view of the required white colour of the final product. The titanyl oxysulfate is then hydrolysed in solution to give insoluble, hydrated TiO_2. The isolated TiO_2 is washed with water, calcined and micronised. However, due to environmental issues (i.e. the production of a large amount of sulfuric acid as a by-product) and also cost issues associated with the sulfate process, currently, the chloride process predominates (Kirk-Othmer, 1997, 2006).
- In extraction via the chloride process, there are two main stages. In a first step, the dry ore is reacted with chlorine to produce titanium tetrachloride. In a second step, titanium tetrachloride is oxidised by burning it in oxygen with another combustible gas (often carbon monoxide). By adding seed crystals, the TiO_2 is formed as a fine solid in a gas stream and is filtered out of the gases. The reaction products are cooled by mixing with chlorine gas. The product is further washed, calcined, milled and coated (Kirk-Othmer, 1997, 2006).

Both anatase and rutile TiO_2 can be produced by the sulfate process depending on the specific processing conditions. To produce anatase specifically, titanium oxysulfate is hydrolysed and neutralised under alkaline conditions. Rutile is typically produced by the chloride process (Kirk-Othmer, 2006).

The rutile form can be formed into platelets on a mica (potassium aluminium silicate) template, which is removed by extractive dissolution in acid and then alkali. The specific properties of the TiO_2 are determined by the thickness of the TiO_2 layer and the process used to coat the mica substrate (EFSA, 2005).

2.4. Methods of analysis in food

Leone (1973) used a spectrophotometric method described by Kolthoff and Sandell (1952) to determine TiO_2 in cheese.

Hamano et al. (1990) described a colorimetric procedure for the determination of small amounts of TiO_2 (10–100 mg TiO_2/kg) in processed cheese, chocolate and chewing gum.

Lomer et al. (2000) used inductively coupled plasma optical emission spectrometry to determine TiO_2 in 25 foodstuffs, including confectionery, cheese, chewing gum, sauces and dressings, mustard and beverage whiteners. The limits of detection were 2–7.5 μg/kg, depending on spectral integration times, and the signal was linear up to 5 mg/kg.

Scotter (2011) describes a number of methods for the determination of TiO_2 in food and feed, but stresses that there are very few literature references to the determination of TiO_2 in foods.

2.5. Reaction and fate in food

TiO_2 (E 171) is highly stable to heat, light, oxygen and pH, making it unaffected by almost any food processing (Scotter, 2011). In any food application, its role is as an insoluble whitening agent (Emerton, 2008).

2.6. Case of need and proposed uses

Maximum levels of TiO_2 (E 171) have been defined in Annex II to Regulation (EC) No 1333/2008[1] on food additives, as amended. In this document, these levels are named maximum permitted levels (MPLs).

Currently, TiO_2 (E 171) is an authorised food additive in the EU at quantum satis[9] (QS) in all 51 foods. TiO_2 (E 171) as such is permitted to be used in seaweed-based fish analogues, in fish paste and crustacean paste, in precooked crustaceans and in smoked fish. TiO_2 (E 171) is also included in Group II of food colours authorised at QS.

Table 6 summarises foods that are permitted to contain TiO_2 (E 171) and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

Table 6: Maximum permitted levels of TiO_2 (E 171) in foods according to Annex II to Regulation (EC) No 1333/2008

Food category number	Food category name	E-number/ group	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat-treated products	Group II		QS
01.5	Dehydrated milk as defined by Directive 2001/114/EC	Group II	Except unflavoured products	QS
01.6.3	Other creams	Group II	Only flavoured creams	QS
01.7.1	Unripened cheese, excluding products falling in category 16	Group II	Only flavoured unripened cheese	QS
01.7.3	Edible cheese rind	Group II		QS
01.7.4	Whey cheese	Group II		QS
01.7.5	Processed cheese	Group II	Only flavoured processed cheese	QS
01.7.6	Cheese products, excluding products falling in category 16	Group II	Only flavoured unripened products	QS
01.8	Dairy analogues, including beverage whiteners	Group II		QS
03	Edible ices	Group II		QS
04.2.4.1	Fruit and vegetable preparations, excluding compote	Group II	Only *mostarda di frutta*	QS
04.2.4.1	Fruit and vegetable preparations, excluding compote	E 171	Only seaweed-based fish roe analogues	QS
04.2.5.3	Other similar fruit or vegetable spreads	Group II	Except *crème de pruneaux*	QS
05.2	Other confectionery including breath-refreshening microsweets	Group II		QS
05.3	Chewing gum	Group II		QS
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Group II		QS
06.3	Breakfast cereals	Group II	Only breakfast cereals other than extruded, puffed and/or fruit-flavoured breakfast cereals	QS
06.5	Noodles	Group II		QS
06.6	Batters	Group II		QS
06.7	Precooked or processed cereals	Group II		QS
07.2	Fine bakery wares	Group II		QS
08.2.3	Casings and coatings and decorations for meat	Group II	Except edible external coating of pastourmas	QS

[9] Article 3 (2) of Regulation (EC) No 1333/2008 'quantum satis' shall mean that no maximum numerical level is specified and substances shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled'.

Food category number	Food category name	E-number/ group	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
09.2	Processed fish and fishery products, including molluscs and crustaceans	Group II	Only surimi and similar products and salmon substitutes	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	E 171	Only fish paste and crustacean paste	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	E 171	Only precooked crustacean	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	E 171	Only smoked fish	QS
09.3	Fish roe	Group II	Except sturgeons' eggs (caviar)	QS
12.2.2	Seasonings and condiments	Group II	Only seasonings, for example curry powder, tandoori	QS
12.4	Mustard	Group II		QS
12.5	Soups and broths	Group II		QS
12.6	Sauces	Group II	Excluding tomato-based sauces	QS
12.7	Salads and savoury-based sandwich spreads	Group II		QS
12.9	Protein products, excluding products covered in category 1.8	Group II		QS
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC, excluding products from food category 13.1.5	Group II		QS
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	Group II		QS
13.4	Foods suitable for people intolerant to gluten as defined by Regulation (EC) No 41/2009	Group II		QS
14.1.4	Flavoured drinks	Group II	Excluding chocolate milk and malt products	QS
14.2.3	Cider and perry	Group II	Excluding *cidre bouché*	QS
14.2.4	Fruit wine and made wine	Group II	Excluding *wino owocowe markowe*	QS
14.2.5	Mead	Group II		QS
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Group II	Except spirit drinks as defined in Article 5(1) and sales denominations listed in Annex II, paragraphs 1–14 of Regulation (EC) No 110/2008 and spirits (preceded by the name of the fruit) obtained by maceration and distillation, Geist (with the name of the fruit or the raw material used), London Gin, Sambuca, Maraschino, Marrasquino or Maraskino and Mistrà	QS
14.2.7.1	Aromatised wine-based products as defined by Regulation (EEC) No 1601/91	Group II	Except Americano, bitter vino	QS
14.2.7.2	Aromatised wine-based drinks	Group II	Except bitter soda, sangria, claria, zurra	QS
14.2.7.3	Aromatised wine-product cocktails	Group II		QS

Food category number	Food category name	E-number/ group	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	Group II		QS
15.1	Potato-, cereal-, flour- or starch-based snacks	Group II		QS
15.2	Processed nuts	Group II		QS
16	Desserts, excluding products covered in categories 1, 3 and 4	Group II		QS
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms, excluding chewable forms	Group II		QS
17.2	Food supplements supplied in a liquid form	Group II		QS
17.3	Food supplements supplied in a syrup-type or chewable form	Group II		QS

MPL: maximum permitted level; QS: quantum satis.

2.7. Reported use levels or data on analytical levels of TiO_2 (E 171) in food

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a level lower than the MPL. Therefore, information on actual use levels is required for performing a more realistic exposure assessment, especially for those food additives for which no MPL is set and which are authorised according to QS.

In the framework of Regulation (EC) No 1333/2008 on food additives and Commission Regulation (EU) No 257/2010 regarding the re-evaluation of approved food additives, EFSA issued a public call for concentration data (usage and/or analytical data) on TiO_2 (E 171).[7]

In response to this public call, updated information on the actual use levels of TiO_2 (E 171) in foods was made available to EFSA by industry and the Member States (MSs).

2.7.1. Summarised data on reported use levels in foods provided by industry

Industry provided EFSA with data on use levels (n = 61) of TiO_2 (E 171) in foods for 14 of the 51 food categories in which TiO_2 is authorised.

Updated information on the actual use levels of TiO_2 in foods was made available to EFSA by FoodDrinkEurope (FDE) (Doc. provided to EFSA n. 10), the International Chewing Gum Association (ICGA) (Doc. provided to EFSA n. 13), the Association of the European Self-Medication Industry (AESGP) (Doc. provided to EFSA n. 1) and Capsugel (Doc. provided to EFSA n. 3).

Appendix A provides data on the use levels of TiO_2 (E 171) in foods, as reported by industry.

2.7.2. Summarised data on concentration levels in foods from the Member States

In total, 28 analytical results were reported to EFSA by one country (Austria) for foods intended for particular nutritional uses (FCS Category 13) and food supplements (FCS Category 17). Foods were sampled between 2007 and 2012. Complete information on the methods of analysis (e.g. validation) was not made available to EFSA, but all samples were derived from accredited laboratories.

Foods classified in the FCS 13 (n = 2) were described as foods for sports people without further detail, and could not be used in the current assessment.

Appendix B shows the analytical results for TiO_2 (E 171) in foods as reported by MSs (full set of reported data and positive samples only).

2.8. Summarised data extracted from the Mintel GNDP database

Mintel's Global New Products Database (GNPD) is an online database, which monitors product introductions in consumer packaged goods markets worldwide. It contains information of over two million food and beverage products of which more than 800,000 are or have been available on the European food market. Mintel started covering the EU's food markets in 1996, having 20 out of its 28 member countries presented in the GNPD.[10]

For the purpose of this Scientific Opinion, GNPD[11] was used for checking the labelling of products containing TiO_2 (E 171) within the EU's food products as GNPD shows the compulsory ingredient information presented in the labelling of products.

According to Mintel, TiO_2 (E 171) is labelled on more than 6,500 products. The use of TiO_2 increased constantly until 2014. In the last 5 years, TiO_2 has been labelled on more than 3,500 foods or drinks, mainly in chewing gums, cakes and pastries, and confectionary (pastilles, gums, jellies and chews).

Appendix C presents the percentage of food products labelled with TiO_2 (E 171) between 2011 and 2015, out of the total number of food products per food subcategories according to Mintel food classification.

2.9. Information on existing authorisations and evaluations

TiO_2 was evaluated by JECFA in 1969 (JECFA, 1970), the SCF in 1975 and 1977, and by EFSA in 2004. It was also reviewed by TemaNord in 2002. The British Industrial Biological Research Association (BIBRA) issued a toxicity profile on TiO_2 in 1990.

In 1969, JECFA did not establish a limit on the intake of TiO_2 (anatase and rutile forms were not distinguished), considering that the available information indicated '. . .that it is free from toxic effects on account of its insolubility and inertness'. An acceptable daily intake (ADI) 'not limited except for good manufacturing practice,' was allocated (JECFA, 1970).

In 1975, the SCF did not establish an ADI for TiO_2 because they 'felt able to accept the use of this colouring matter for the surface and mass colouring of sugar confectionary only, without the need for further investigations'. In a later SCF evaluation (1977), it was indicated that new information on other potential uses and specifications had been presented to the Committee, and subsequently, they included TiO_2 in the category 'colours for which an ADI was not established but which could be used in food'.

In 2004, the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and materials in Contact with Food (AFC Panel) evaluated the safety in use of platelet forms of rutile TiO_2 as an alternative to the permitted anatase form. The AFC Panel concluded that the bioavailability of these forms was essentially the same, that the toxicological database would, therefore, be applicable to either form and that the platelet forms of rutile TiO_2 could be used to replace anatase TiO_2 in any of its current applications (EFSA, 2005).

In 2000, the Scientific Committee on Cosmetics and Non-Food Products (SCCNFP)[12] evaluated TiO_2 as a cosmetic product. The SCCNFP concluded that TiO_2 is photocatalytic in UV light, but that it did not give rise to concern for human use (SCCNFP, 2000). The SCCS issued an Opinion on TiO_2 (nano form) in 2013, and a commentary on this Opinion was released in 2015. The aim of these reports were to provide an answer to the question of the European Commission on whether the use of TiO_2 in its nanoform as a UV filter in cosmetic products (e.g. sunscreens), at a concentration up to maximum 25.0%, was safe for the consumers.

In 2002, TemaNord reviewed TiO_2 and concluded that 'the available data do not currently meet requirements. However, the inertness of the substance and the lack of absorption and tissue storage does not warrant further testing or a re-evaluation of the safety in use of this compound'.

In the USA, a platelet form of rutile TiO_2 is currently permitted for use in aqueous film coating systems for food and drug use under Code of Federal Regulations Title 21CFR73.575. This regulation states that TiO_2 may be used as a food colour provided that it does not exceed 1% of the weight of the food (Food and Drug Administration (FDA), 2002). In 2006, the FDA amended the colour additive regulation to allow the use of TiO_2-coated mica-based pearlescent pigments (identified as the colour additive 'formed by depositing titanium salts onto mica, followed by heating to produce TiO_2 in mica')

[10] Missing Bulgaria, Cyprus, Estonia, Latvia, Lithuania, Luxembourg, Malta and Slovenia.
[11] http://www.gnpd.com/sinatra/home/ accessed on 19/5/2016.
[12] Presently called 'Scientific Committee on Consumer Products (SCCP)'.

as a colour additive for foods (FDA, 2006). TiO_2-coated mica-based pearlescent pigments are authorised for use up to 1.25% by weight in the following food categories: 'cereals, confections and frostings, gelatin desserts, hard and soft candies (including lozenges), nutritional supplement tablets and gelatin capsules and chewing gum' (FDA, 2006).

In Japan, TiO_2 is used without limitations other than for certain food categories in which it is not permitted (JECFA, 2006b). In India, TiO_2 is only authorised for use in chewing gum and bubble gum at not more than 1%, and in powdered concentrate mixes for fruit drinks at not more than 100 mg/kg (Kuznesof, 2006).

In 2010, IARC re-evaluated TiO_2 and revised the classification as 'possibly carcinogenic to humans (Group 2B)' based on an excess incidence of lung tumours in inhalation studies. It was stated that 'No increases were observed among mice and hamsters exposed intratracheally. Other studies that used different routes of administration did not observe excesses in tumour incidence' (IARC, 2010).

In 2015, the Organisation for Economic Co-operation and Development (OECD) published different Series on the Safety of Manufactured Nanomaterials, among which there is a dossier on titanium dioxide (TiO_2) manufactured nanomaterials. Detailed information on results and tests performed can be found in the technical dossiers of the particular TiO_2 nanomaterials (OECD, 2015).

In the very recent Scientific Report by the Food Standard Agency of New Zealand (FSANZ) (2016) on 'The potential health risks associated with nanotechnologies in existing food additives', it is reported that all forms of TiO_2 (nano- and microsized) in the diet are poorly absorbed from the gastrointestinal tract. There are few studies investigating the toxicity of TiO_2 by dietary exposure (grade or particle size not specified) reporting no evidence of carcinogenicity or systemic toxicity. Nevertheless, there is some evidence that oral exposure to nano-TiO_2 (non-food-grade) by gavage can result in small increases in tissue titanium potentially associated with a range of tissue effects. Overall, this review concluded that there is limited information available to support a contemporary risk assessment of nano-TiO_2 in food. There are no epidemiology studies available regarding possible associations with adverse health outcomes. However, the long history of use has not given rise to reports of adverse effects.

The Panel is aware that the European Chemical Agency (ECHA) is carrying out an evaluation for a proposal for harmonised classification and labelling (CLH) on TiO_2, for which the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) is the Rapporteur on behalf of the French Member State Competent Authority. ANSES prepared a report in which concluded that TiO_2 should be considered as being potentially carcinogenic to humans when inhaled and thus be classified Carc. Cat 1B – H350i. However, it also concluded that there was no carcinogenic concern after oral or dermal administration. A public consultation on this report is currently underway.[13]

2.10. Exposure

2.10.1. Food consumption data used for exposure assessment

2.10.1.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with national data on food consumption at a detailed level. Competent authorities in European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment'; EFSA, 2011a). New consumption surveys recently added to the Comprehensive Database were also taken into account in this assessment.[14,15]

The food consumption data gathered by EFSA were collected using different methodologies and thus direct country-to-country comparisons should be interpreted with caution. Depending on the food category and the level of detail used for exposure calculations, uncertainties could be introduced owing to possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless, the EFSA Comprehensive Database represents the best available source of food consumption data across Europe at present.

[13] http://echa.europa.eu/harmonised-classification-and-labelling-consultation/-/substance-rev/13832/term; http://echa.europa.eu/web/guest/harmonised-classification-and-labelling-consultation
[14] Available online: http://www.efsa.europa.eu/en/press/news/150428.htm
[15] Available online: http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

Food consumption data from the following population groups, infants, toddlers, children, adolescents, adults and the elderly, were used for the exposure assessment. For the current assessment, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Table 7).

Table 7: Population groups considered for the exposure estimates of TiO_2 (E 171)

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From more than 12 weeks up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, UK
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Finland, Germany, Italy, the Netherlands, Spain, UK
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden, UK
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Italy, Latvia, Spain, Sweden, UK
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Spain, Sweden, UK
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Romania, Sweden, UK

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the EFSA guidance on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the food categorisation system (FCS) as presented in Annex II of Regulation (EC) No 1333/2008, part D, to perform exposure estimates. In practice, FoodEx food codes were matched to the FCS food categories.

2.10.1.2. Food categories considered for the exposure assessment of TiO_2

The food categories in which the use of TiO_2 (E 171) is authorised were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system food codes), at a detailed level (up to FoodEx Level 4) (EFSA, 2011b).

Some food categories are not referenced in the EFSA Comprehensive Database and therefore could not be taken into account in the current estimate. This might result in an underestimation of the exposure. The food categories that were not taken into account are described below (in ascending order of the FCS codes):

- 01.7.3. edible cheese rind,
- 01.7.6. cheese products (excluding products falling in category 16), only flavoured unripened products,
- 04.2.4.1. fruit and vegetable preparations, excluding compote, only *mostarda di frutta*,
- 04.2.4.1. fruit and vegetable preparations, excluding compote, only seaweed-based fish analogue,
- 05.4. decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4, only decorations, coatings and sauces, except fillings and only fillings,
- 06.6. batters,
- 06.7. precooked or processed cereals,
- 08.2.3. casings and coatings and decorations for meat,
- 14.2.4. fruit wine and made wine,
- 14.2.5. mead.

It has to be mentioned that these food categories could be country-specific products (*mostarda di frutta*) or could be included in other food categories taken into account with the EFSA Comprehensive

Database (edible cheese rind with the ripened cheeses) or should represent minor food consumption amounts (seaweed-based fish analogue, batters, mead, etc.).

In addition, food categories for which no or inadequate reported use/analytical levels were available were not considered in the exposure assessment. This concerns 25 food categories, which are presented in Appendix C.

The Panel noted that if TiO_2 is nevertheless used in those food categories for which reported use/analytical levels were not available, the calculated refined exposure assessment might result in an underestimation of the exposure to TiO_2. The current exposure assessment takes into consideration a percentage of the foods in which TiO_2 is authorised and that is dependent on the individuals. The Panel calculated that between 60% and 80% of food (by weight), authorised to contain TiO_2 according to Annex II, was reported to potentially contain TiO_2 as a food additive

Overall, during the current exposure estimate, 10 out of 51 food categories were not taken into account because they are not referenced in the EFSA Comprehensive Database and 25 food categories were not included in the exposure assessment due to a lack of data. Thus, in the current exposure estimate, 35 out of 51 food categories are not taken into account.

2.10.2. Exposure to TiO_2 (E 171) from its use as a food additive

The Panel estimated chronic exposure to TiO_2 (E 171) for the following population groups: infants, toddlers, children, adolescents, adults and the elderly. Dietary exposure to TiO_2 (E 171) was calculated by multiplying TiO_2 (E 171) concentrations for each food category (Appendix D) by their respective consumption amount per kilogram of body weight (bw) for each individual in the Comprehensive Database. The exposure per food category was subsequently added to derive an individual total exposure per day. These exposure estimates were averaged over the number of survey days, resulting in an individual average exposure per day for the survey period. Dietary surveys with only 1 day per subject were excluded as they are considered as not adequate to assess repeated exposure.

This was carried out for all individuals per survey and per population group, resulting in distributions of individual exposure per survey and population group (Table 7). Based on these distributions, the mean and 95th percentiles of exposure were calculated per survey and per population group. High percentile exposure was calculated only for those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile of exposure (EFSA, 2011a). Therefore, in the current assessment, high levels of exposure for infants from Italy and for toddlers from Belgium, Italy and Spain were not included.

Assessment of exposure to TiO_2 (E 171) was carried out by the ANS Panel based on the maximum levels of data provided to EFSA (defined as the maximum level exposure assessment scenario), and reported use levels (defined as the refined exposure assessment scenario), as provided by industry and the MSs.

2.10.2.1. Maximum level exposure assessment scenario

The regulatory maximum level exposure assessment scenario is based on the MPLs as set in Annex II to Regulation (EC) No 1333/2008 and listed in Table 6. As TiO_2 (E 171) is authorised according to QS in all food categories, a 'maximum level exposure assessment' scenario was estimated based on the maximum reported use levels provided by industry or high level of analytical data provided by the MSs, as described in the EFSA Conceptual framework (EFSA ANS Panel, 2014), whichever was highest or available. This exposure scenario can consider only food categories for which data were available to the Panel.

The Panel considers the exposure estimates derived following this scenario as the most conservative as it is assumed that the consumer will be continuously (over a lifetime) exposed to TiO_2 (E 171) present in food at maximum reported use levels/high level of analytical data.

2.10.2.2. Refined exposure assessment scenario

The refined exposure assessment scenario is based on reported use levels by industry and analytical results submitted to EFSA by the MSs. This exposure scenario can only consider food categories in which the above data were available to the Panel.

Appendix D summarises the concentration levels of TiO_2 (E 171) used in the refined exposure assessment scenario. Based on the available dataset, the Panel calculated two estimates based on different model populations:

1) The brand-loyal consumer scenario: It was assumed that a consumer is exposed long term to the food additive present at the maximum reported use/analytical levels for one food category. This exposure estimate is calculated as follows:
   - combining food consumption with the maximum of the maximum reported use levels or the maximum of the analytical results, whichever was highest or available, for the main contributing food category at the individual level;
   - using the mean of the typical reported use levels or the mean of analytical results, whichever was highest or available, for the remaining food categories.
2) The non-brand-loyal consumer scenario: It was assumed that the population is exposed long term to the food additive present at the mean reported use/analytical levels in food. This exposure estimate is calculated using the mean of the typical reported use levels or the mean of analytical results for all food categories.

In the two refined exposure assessment scenarios, the concentration levels considered by the Panel were extracted from the whole dataset (i.e. reported use levels and analytical results). To consider left-censored analytical data (i.e. analytical results below the limit of detection (LOD) or below the limit of quantification (LOQ)), the substitution method as recommended in the 'Principles and Methods for the Risk Assessment of Chemicals in Food' (WHO, 2009) and the EFSA scientific report 'Management of left-censored data in dietary exposure assessment of chemical substances' (EFSA, 2010) was used. In the current Opinion, analytical data below LOD or LOQ were assigned half of LOD or LOQ, respectively (medium bound). Subsequently, per food category, the mean or median, whichever is highest, medium bound concentration was calculated.

If both reported use levels and analytical results were available for the same food category, the most reliable value was used.

2.10.2.3. Dietary exposure to TiO_2 (E 171)

Table 8 summarises the estimated exposure to TiO_2 (E 171) from its use as a food additive for all six population groups (Table 7). Detailed results by population group and survey are presented in Appendix E.

Table 8: Summary of dietary exposure to TiO_2 (E 171) from its use as a food additive using the maximum level exposure assessment scenario and refined exposure scenarios, in six population groups (min–max across the dietary surveys in mg/kg bw per day)

	Infants (12 weeks – 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (> 65 years)
Maximum level exposure assessment scenario						
Mean	0.4–1.9	1.2–9.2	1.8–10.4	0.8–6.7	0.6–6.8	0.4–4.5
95th percentile	1.4–9.6	4.0–19.3	4.9–32.4	3.1–23.5	2.2–15.0	1.2–10.7
Refined estimated exposure scenario						
Brand-loyal scenario						
Mean	0.4–1.8	1.1–7.6	1.5–8.8	0.7–5.9	0.5–5.7	0.4–3.9
95th percentile	1.2–9.2	3.6–14.7	4.1–30.2	2.5–21.2	1.9–13.6	1.1–9.2
Non-brand-loyal scenario						
Mean	0.2–0.8	0.6–4.6	0.9–5.5	0.4–4.1	0.3–4.0	0.2–2.8
95th percentile	0.7–3.9	2.0–6.8	2.4–14.8	1.3–10.8	1.1–9.7	0.5–7.0

For the maximum level exposure assessment scenario, at the mean, the exposure estimates ranged from 0.4 mg/kg bw per day for infants and the elderly to 10.4 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 1.2 mg/kg bw per day for the elderly to 32.4 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the brand-loyal scenario, the exposure estimates ranged at the mean from 0.4 mg/kg bw per day for infants and the elderly to 8.8 mg/kg bw per day

for children. At the 95th percentile, exposure estimates ranged from 1.1 mg/kg bw per day for the elderly to 30.2 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the non-brand-loyal scenario, the exposure estimates ranged at the mean from 0.2 mg/kg bw per day for infants and the elderly to 5.5 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.5 mg/kg bw per day for the elderly to 14.8 mg/kg bw per day for children.

For the purpose of providing an indicative estimate of nanoparticles of titanium dioxide from the use of E 171 as a food additive, the Panel considered that the highest reported weight percentage value of 3.2% of nanoparticles by mass could reasonably be used in a conservative way to address this issue. Table 9 summarises the estimated exposure to nanoparticles from the use of TiO_2 as a food additive for all six population groups.

Table 9: Summary of exposure to nanoparticles (present at a level of 3.2% by weight in TiO_2 (E 171)) from the use of TiO_2 as a food additive using the maximum level exposure assessment scenario and refined exposure scenarios in six population groups (min–max across the dietary surveys in mg/kg bw per day)

	Infants (12 weeks – 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (> 65 years)
Maximum level exposure assessment scenario						
Mean	0.01–0.06	0.04–0.30	0.06–0.33	0.03–0.21	0.02–0.22	0.01–0.14
95th percentile	0.04–0.31	0.13–0.62	0.16–1.04	0.10–0.75	0.07–0.48	0.04–0.34
Refined estimated exposure scenario						
Brand-loyal scenario						
Mean	0.01–0.06	0.03–0.24	0.05–0.28	0.02–0.19	0.02–0.18	0.01–0.12
95th percentile	0.04–0.29	0.11–0.47	0.13–0.97	0.08–0.68	0.06–0.44	0.03–0.29
Non-brand-loyal scenario						
Mean	0.01–0.03	0.02–0.15	0.03–0.18	0.01–0.13	0.01–0.13	0.01–0.09
95th percentile	0.02–0.13	0.06–0.22	0.08–0.47	0.04–0.35	0.04–0.31	0.02–0.23

For the maximum level exposure assessment scenario, at the mean, the exposure estimates to nanoparticles ranged from 0.01 mg/kg bw per day for infants and the elderly to 0.33 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.04 mg/kg bw per day the infant and elderly to 1.04 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the brand-loyal scenario, the exposure estimates ranged at the mean from 0.01 mg/kg bw per day for infants and the elderly to 0.28 mg/kg bw per day for children. At 95th percentile, exposure estimates ranged from 0.03 mg/kg bw per day for the elderly to 0.97 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the non-brand-loyal scenario, the exposure estimates ranged at the mean from 0.01 mg/kg bw per day for infants, adolescents, adults and the elderly to 0.18 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.02 mg/kg bw per day for infants and the elderly to 0.47 mg/kg bw per day for children.

2.10.3. Main food categories contributing to exposure to TiO_2 (E 171) using the maximum level exposure assessment scenario

Table 10 summarises the main food categories contributing to exposure to TiO_2 (E 171) using the maximum level exposure scenario for all six population groups.

Table 10: Main food categories contributing to exposure to TiO_2 (E 171) using maximum levels (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

Food category number	Foods	Range of % contribution to the total exposure (number of surveys)[a]					
		Infants	Toddlers	Children	Adolescents	Adults	The elderly
03	Edible ices	20.9 (1)	5.7–14.9 (7)	6.3–29.9 (18)	5.3–31.1 (12)	5.1–18.2 (7)	9–13.8 (2)
05.2	Other confectionery, including breath-refreshening microsweets	14.3 (1)	7.3–37.4 (8)	5.5–61.2 (18)	7.2–71.9 (15)	5.2–38.8 (10)	5.2–25.4 (7)
05.3	Chewing gum			6.7–9.6 (3)	7.2–13.3 (3)	12.9 (1)	10.6 (1)
07.2	Fine bakery wares	5.6–81.3 (4)	7.5–43.4 (8)	6.1–34.0 (16)	5.5–28.1 (15)	5.2–20.5 (13)	5.9–20.3 (13)
12.5	Soups and broths	40.0 (1)	5.2–10.5 (3)	8.7–8.8 (2)	7.7 (1)	7.2–11.6 (3)	6.7–17.7 (6)
12.6	Sauces	18.0–66.6 (5)	12.8–58.9 (9)	11.6–53.4 (16)	6.4–58.1 (17)	12–58.3 (17)	11–57.8 (14)
12.7	Salads and savoury-based sandwich spreads			7.8–44.9 (4)	10.2–41.6 (3)	5.3–54.4 (6)	6–48.1 (3)
14.1.4	Flavoured drinks	13.6 (1)	5.8–12.4 (5)	5.6–11.8 (12)	5.8–22.8 (12)	5.1–16.4 (9)	5.7–13.2 (3)
15.2	Processed nuts	5.6–24.6 (3)	7–24.4 (4)	5.4–11.8 (8)	5.1–14.4 (11)	5.5–54.1 (16)	5.9–50.9 (12)
16	Desserts, excluding products covered in categories 1, 3 and 4	7.9–15.6 (2)	5.3–12.5 (3)	5.3–5.6 (2)			
17	Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants and young children	7.5–81.0 (3)	7.9–50.5 (4)	5.4–9.8 (4)	6.5 (1)	6.0–21.0 (8)	11.9–42.4 (6)

(a): The total number of surveys may be greater than the total number of countries as listed in Table 7 because some countries submitted more than one survey for a specific population.

2.10.4. Main food categories contributing to exposure to TiO_2 (E 171) using the refined exposure assessment scenarios

Table 11 summarises the main food categories contributing to exposure to TiO_2 (E 171) using the brand-loyal refined exposure scenario for all six population groups.

Table 11: Main food categories contributing to exposure to TiO_2 (E 171) using the brand-loyal refined exposure scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

Food category number	Foods	Range of % contribution to the total exposure (number of surveys)[a]					
		Infants	Toddlers	Children	Adolescents	Adults	The elderly
03	Edible ices	21.4 (1)	7.4–14.8 (6)	5.3–32.9 (18)	5.2–34.5 (7)	5.6–18.9 (5)	7.7–14.4 (2)
05.2	Other confectionery, including breath-refreshening microsweets	12.6 (1)	6.7–40.2 (8)	5.1–70.3 (18)	5.8–81.5 (15)	6.0–42.4 (8)	5.2–26 (7)
05.3	Chewing gum			6–8.5 (2)	6.8–11.4 (2)	13.4 (1)	11.4 (1)
07.2	Fine bakery wares	8.9–81.8 (3)	5.9–44.0 (7)	5.3–32.8 (14)	5.1–26.6 (11)	5.0–18.4 (8)	5.8–18.3 (11)
12.5	Soups and broths	42.6 (1)	5.9–12.8 (3)	5.6–10.6 (3)	5.1–8.9 (2)	5.0–13.5 (4)	7.6–19.8 (6)
12.6	Sauces	15.2–69.6 (5)	10.5–63.4 (9)	9.7–58.3 (16)	6.5–65.0 (16)	10.0–63.2 (17)	8.7–62.3 (14)
12.7	Salads and savoury-based sandwich spreads			9.0–50.4 (4)	11.7–46.7 (3)	6.0–59.4 (6)	6.3–54.7 (3)
14.1.4	Flavoured drinks	13.2 (1)	5.2–11.4 (3)	5.1–9.5 (11)	6.2–21.2 (10)	6.4–14.5 (6)	5.0–11.9 (3)
15.2	Processed nuts	5.7–26.9 (3)	7.8–30.3 (4)	5.7–12.3 (9)	5.2–16.5 (12)	5.5–58.5 (15)	5.2–53.5 (13)

Food category number	Foods	Range of % contribution to the total exposure (number of surveys)[a]					
		Infants	Toddlers	Children	Adolescents	Adults	The elderly
16	Desserts, excluding products covered in categories 1, 3 and 4	7.2–15.8 (2)	7.3–13.7 (2)	5.2–5.4 (2)			
17	Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants and young children	7.7–83.9 (3)	8.5–52.9 (4)	6.8–10.8 (3)	7.8 (1)	6.8–22.2 (8)	12–45.6 (6)

(a): The total number of surveys may be greater than the total number of countries as listed in Table 7 because some countries submitted more than one survey for a specific population.

Table 12 summarises the main food categories contributing to exposure to TiO_2 (E 171) using the non brand-loyal refined exposure scenario for all six population groups.

Table 12: Main food categories contributing to exposure to TiO_2 (E 171) following the non-brand-loyal exposure scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

Food category number	Foods	Range of % contribution to the total exposure (number of surveys)[a]					
		Infants	Toddlers	Children	Adolescents	Adults	The elderly
03	Edible ices	21.8 (1)	5.7–17.3 (8)	6.2–30 (18)	5.2–31.2 (14)	5.5–17.3 (7)	5.8–13.2 (3)
05.2	Other confectionery including breath-refreshening microsweets	7.1 (1)	5.1–24.2 (7)	6.8–46.7 (14)	5.7–61.1 (13)	6.2–24.1 (5)	10.1–16 (3)
05.3	Chewing gum				5.0–7.5 (2)	7.0 (1)	5.4 (1)
07.2	Fine bakery wares	6.2–82.6 (4)	7.6–50.6 (8)	5.4–38.2 (16)	6.5–28.4 (14)	5.6–19.6 (14)	6.0–19.4 (13)
09.2	Processed fish and fishery products, including molluscs and crustaceans			6.2 (1)	5.4 (1)		
12.5	Soups and broths	9.5–59.3 (2)	6.6–21 (6)	5.4–18.5 (6)	5.9–12.1 (6)	7.4–22.3 (6)	8.0–33.5 (7)
12.6	Sauces	12.8–52.7 (5)	9.1–46.7 (9)	8.4–44.8 (16)	9.5–47.6 (16)	8.2–47.4 (17)	7.5–48.6 (14)
12.7	Salads and savoury-based sandwich spreads			15.3–56.3 (4)	8.0–54.3 (4)	6.2–66.3 (7)	10.5–61.1 (3)
14.1.4	Flavoured drinks	16.3 (1)	5.4–16.9 (7)	5.7–15.3 (13)	5.6–27.0 (15)	5.4–18.3 (10)	8.3–15 (3)
15.2	Processed nuts	6.1–28.6 (3)	10.2–28.7 (4)	6.5–14.2 (9)	5.8–17.2 (12)	5.1–58.4 (16)	5.1–55.9 (13)
16	Desserts excluding products covered in categories 1, 3 and 4	12.2–22.8 (2)	5.5–19.0 (5)	5.4–9.3 (3)	6.1 (1)		
17	Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children	8.9–85.9 (3)	9.4–53.7 (4)	7.0–16.8 (4)	8.7–9.1 (2)	7.5–26.5 (8)	13–48.7 (6)

(a): The total number of surveys may be greater than the total number of countries as listed in Table 7 because some countries submitted more than one survey for a specific population.

2.10.5. Uncertainty analysis

Uncertainties in the exposure assessment of TiO_2 (E 171) have been discussed above. In accordance with the guidance provided in the EFSA Opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 13.

Table 13: Qualitative evaluation of influence of uncertainties on the dietary exposure estimate

Sources of uncertainty	Direction
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Correspondence of reported use levels and analytical data to the food items in the EFSA Comprehensive Food Consumption Database: uncertainties about which types of food the levels refer to	+/–
Food categories selected for the exposure assessment: exclusion of food categories due to missing FoodEx linkage (n = 10/51 food categories)	–
Food categories included in the exposure assessment: data not available for certain food categories which were excluded from the exposure estimates (n = 35/51 food categories)	–
Concentration data: • levels considered applicable for all items within the entire food category, • not representative of foods on the EU market (coming from one Member State)	+ +/–
Maximum level exposure assessment scenario: – exposure calculations based on the maximum (reported use from industries or analytical data from Member States)	+
Refined exposure assessment scenarios: – exposure calculations based on the maximum or mean levels (reported use from industries or analytical data from Member States)	+/–
Uncertainty in possible national differences in use levels of food categories	+/–
Exposure to nanoparticles: uncertainties on the percentage of nanoparticles	+

+: uncertainty with potential to cause over-estimation of exposure; –: uncertainty with potential to cause underestimation of exposure; EU: European Union.

Overall, the Panel noted that not all the food categories in which use of TiO_2 (E 171) is authorised were taken into account in the current exposure estimate. The Panel, therefore, considered that the uncertainties identified would, in general, result in an underestimation of the exposure to TiO_2 (E 171) if all food categories according the regulation had the reported uses. The Panel noted that the usage data submitted by industries for food categories and considered in its estimates were for some of them confirmed, when comparing with the qualitative information as described in the Mintel database.

The Panel also noted that the uncertainties identified in its estimates of exposure to nanoparticles that could be present in TiO_2 used as a food additive, would result in an overestimation because in these estimates it was assumed that nanoparticles were present in all considered food categories at the maximum reported percentage value (3.2% by mass).

3. Biological and toxicological data

In their review, Walkey and Chan (2012) indicated that when small particles, such as nanomaterials enter a physiological environment, they rapidly adsorb proteins from the biological fluids forming a protein 'corona'. This protein corona alters the size, aggregation state and interfacial composition of a nanomaterial, giving it a biological identity that is distinct from its synthetic identity. The biological identity determines the physiological response, including signalling, kinetics, transport, accumulation, and toxicity. The structure and composition of the protein corona depends on the synthetic identity of the nanomaterial (size, shape and composition), the nature of the physiological environment (blood, interstitial fluid, cell cytoplasm, etc.) and the duration of exposure. The Panel considered that these elements should be taken into account when interpreting the biological and toxicological data on nano- and microsized materials after oral intake. However, the Panel wants to emphasise that E 171 is not an (engineered) nanomaterial. The Panel was aware of the extensive database on TiO_2 nanomaterials,

however, most of these data were not considered relevant to the evaluation of TiO_2 as the food additive (E 171) in this opinion. Therefore, the Panel considered these data could not be directly applied to the evaluation of the food additive.

A large number of animal experimental studies (80 publications in PubMed) has been published from the Medical College of Soochow University (Suzhou, China) describing effects of nanosized TiO_2 on various organ systems. The Panel noted that the publications of Gui et al. (2013); Zhao et al. (2014); Hu et al., 2011b) were retracted from the journals by the Editor due to deficiencies and inadequate reporting of the data (Hu et al., 2011a; Gui et al., 2015; Zhao et al., 2015). These deficiencies were the use of the same 5% standard deviation or standard error for all measured values and thus the real variation and statistical significance of the results cannot be evaluated. The Panel noted that the same data handling was also found in other publications on TiO_2 nanoparticles from this group (e.g. Hu et al., 2010; Cui et al., 2011; Gui et al., 2011; Gao et al., 2012, 2013; Sheng et al., 2013, 2014; Sang et al., 2014). The Panel evaluated these publications but did not consider them as appropriate for risk assessment in the present evaluation.

References of the toxicological studies with coated nanoparticles considered by the Panel are given in Appendix F.

3.1. Absorption, distribution and excretion

Numerous studies on the absorption, distribution and excretion of inhaled TiO_2 particles from animal experiments and human exposure are available in the literature. However, the Panel considered that this route of exposure was not directly relevant to the safety evaluation of TiO_2 as a food additive and therefore further details on exposure via inhalation were not considered in this Opinion. The general consensus is that small amounts of TiO_2, when under a nanoform, can enter the systemic circulation from the lungs (Jin and Berlin, 2008).

3.1.1. Absorption

Reports in the literature on studies with animals indicate that a primary port of entry into the body for orally absorbed micro- and nanoparticulates from the undamaged intestine was the gut-associated lymphoid tissue (GALT), represented by Peyer's patches and the follicle-associated epithelium overlying Peyer's patches. Follicle-associated epithelium contains a population of phagocyte cells (M cells) that are responsible for absorbing particulates. Uptake also takes place, but to a limited extent, across normal epithelial cells (enterocytes) and by paracellular means. Quantitative models have shown that particle binding to the apical membrane of M cells was followed by rapid internalisation (Florence, 1997; Hussain et al., 2001; des Rieux et al., 2006; Emond, 2011).

In general, smaller particles, < 1 μm (1,000 nm), lead to higher absorption rates. Particles > 1 μm were effectively trapped in the Peyer's patches. At this size, the particles were not translocated into the systemic circulation. Oral absorption was influenced by different particle characteristics (e.g. diameter, surface chemistry, surface ligands, shape and elasticity, physical and chemical stability) (Hussain et al., 2001). Particles > 3 μm (3,000 nm) were phagocytosed and stayed sequestered in the gastrointestinal tract cells (Emond, 2011).

3.1.1.1. *In vitro*

In a study by McCracken et al. (2013), TiO_2 nanoparticles (particle size 21 nm; surface area 35–65 m^2/g; purity > 99.5%) were dispersed in simulated digestion media and placed in contact with a Caco-2 cell monolayer (C2BBe1) isolated from a human colon cancer. The nanoparticles were added to the cells at a dose of 10 $\mu g/cm^2$. Aggregates of negatively charged particles appeared in the culture media, but the charge became positive in the presence of pepsin (pH 2). The same particles became strongly negative in a simulated intestinal digestive solution, whereas a corona made of bile salts/proteins was identified on the particles. TEM indicated the internalisation of TiO_2 particles to occur. The authors indicated that, based on assays on necrosis, apoptosis, membrane damage and mitochondrial activity, no toxicity was exhibited by TiO_2 particles suspended in the media at loading levels of 10 $\mu g/cm^2$. The authors further indicated that although no toxicity was exhibited, internalisation of the particles by the epithelial cells may result in the circulation and migration of the particles to other parts of the body.

In a study by Chaudhry et al. (2013; Doc. provided to EFSA n. 7) (published as MacNicoll et al., 2015), the potential of microsized TiO_2 and of TiO_2 nanoparticles to cross the gastrointestinal–epithelial barrier was tested. A coculture of human enterocytes (Caco-2 cells) and M cells was used as test

model system. Translocation of TiO_2 nano- and microparticles, dispersed in ovalbumin solution was studied in a transwell system. For comparison, the smallest particles were also tested without ovalbumin in the medium in agglomerated form (dispersed by sonication in water). The integrity of the cell monolayer, the viability of the cells and the translocation of TiO_2 were determined. The TiO_2 particles were characterised using TEM and limited DLS analysis.

Three sizes of TiO_2 particles obtained from one producer and a 25-nm TiO_2 nanomaterial from a second producer were tested.

Table 14: Characteristics of tested TiO_2 materials (Chaudhry et al., 2013; Doc. provided to EFSA n. 7)

Material	Description	Measured particle size	Use in tests
TiO_2-anatase; purity 99.7%	Nominal particle size: < 25 nm	~ 15 nm (~ 250–400 nm when in agglomerated form)	*In vitro*
TiO_2-rutile; purity 99.5%	Nominal particle size: < 100 nm	~ 40–50 nm (submicron-sized when in agglomerated form)	*In vitro* and *in vivo*
TiO_2-rutile; purity 99.5%	Nominal particle size: < 5,000 nm (< 5 μm)	Up to 5 μm	*In vitro* and *in vivo*
TiO_2 mixture: anatase (80%)/rutile (20%); purity 99.5%	Nominal particle size: 23.9 nm	~ 25 nm (~ 125 nm when in agglomerated form)	*In vitro*

The characteristics of the test materials are given in Table 14.

The authors concluded that TiO_2 nanoparticles are very agglomerative in nature; it was not straightforward to obtain, or keep, the nanoparticles within narrow size ranges. The study provided evidence of a lack of any significant TiO_2 translocation above the limit of detection across the gut epithelium model whether it was in the micro- or nanosized forms. The TiO_2 particles seemed to settle between or in the cells, because analytical measurements showed titanium in the cell fractions, but not in the basolateral fraction.

3.1.1.2. *In vivo*

Studies in the mouse

Gu et al. (2015) orally administered 64 mg microsized TiO_2/kg bw per day (> 100 nm in size) to CD-1 mice, and examined the effects on plasma glucose levels. They showed that titanium levels were not changed in blood, liver and pancreas. No histopathological changes in liver or pancreas were observed. The authors concluded that their results indicated that microsized TiO_2 cannot be absorbed after oral administration and consequently, cannot affect plasma glucose levels in mice.

Studies in the rat

In a study by Fournier (1950) (cited by JECFA, 1970), rats (species, sex and number of animals not stated) given a diet of either 0.2, 1 or 2% TiO_2 (not further specified) (equivalent to 236, 1,180 and 2,360 mg TiO_2/kg bw per day, respectively)[16] for 7 days did not appear to absorb TiO_2 from the gastrointestinal tract. In the same study, it was reported that no titanium was found in the blood, liver, kidney and urine of rats given 660 mg TiO_2/kg bw per day for 15 days (sensitivity of analysis 10 μg).

Jani et al. (1994) investigated the uptake of rutile TiO_2 particles (particle size 500 nm) from the rat gastrointestinal tract. Six adult female Sprague–Dawley rats (average weight: 150 g; age: 12–14 weeks) were administered 12.5 mg TiO_2/kg bw per day (0.1 mL of a 2.5% w/v suspension) by oral gavage for 10 days. Organs and tissues, such as Peyer's patches, small intestine, colon, mesentery network and nodes, peritoneal tissue, liver, spleen, heart and kidney, were removed for histological examination, SEM and spectrometric analysis for titanium using inductively coupled plasma emission spectroscopy. Histopathological examination showed the presence of particles, proved to contain TiO_2 by chemical analysis, in all major tissues of the GALT, and demonstrated that TiO_2 particles (500 nm) were translocated to systemic organs such as the liver and the spleen. TiO_2 particles were also found in the lung and peritoneal tissues, but were not detected in the heart or kidney. The authors calculated, based on inductively coupled plasma measurements of titanium levels, that 6.5% of the total dose of TiO_2 particles (size range of 500 nm) administered orally over 10 days was taken up. The authors concluded that the uptake of rutile TiO_2 particles occurs primarily via Peyer's patches and that

[16] Calculated by the Panel according to EFSA Scientific Committee (2012).

the particles subsequently translocate to the mesentery network where they accumulate in the mesenteric lymph nodes. Some particles then entered the general circulation and were taken up by the liver and the spleen.

Onishchenko et al. (2012) studied the penetration of TiO_2 nanoparticles (rutile; physical characteristics not given) into enterocytes, after administration of water dispersions of the test material (rutile dispersion; 50 mg/cm^3) into an isolated loop of Wistar rat small intestine. Penetration was shown *in vivo* using TEM. After 3-h exposure using electron diffraction, rutile nanoparticles were identified in the apical regions of the cells under plasma membranes and in deeper parts of the cytoplasm as solitary objects or small aggregations. The data indicated that the rutile TiO_2 nanoparticles, administered into the gastrointestinal tract, penetrated the small intestinal epithelial barrier.

In a study by Chaudhry et al. (2013; Doc. provided to EFSA n. 7) cited above (published as MacNicoll et al., 2015), the absorption of TiO_2 was further studied in rats bred, fed and maintained in titanium-controlled environment (strain not given; five groups/six rats per type of material) receiving a single oral dose of TiO_2 (4.6 mg TiO_2/kg bw) in the form of nanosized particles (two anatase and one rutile) and microsized particles (rutile). The characteristics of the test materials are given in Table 14. Following oral administration of TiO_2, samples of blood, urine and faeces were collected at appropriate time intervals. When the particles were submitted to pH values mimicking gastrointestinal tract biological conditions, no appreciable dissolution (titanium release) was observed. No significant difference in the amounts of titanium in the urine from the control (microsized) and treated (nanosized) groups was found during the 96 h post-treatment period. The bulk of the titanium (not quantified) was found in the faeces. Titanium concentrations in blood, urine or tissues were not significantly increased. It was concluded by the authors that absorption/translocation to blood, urine and faeces, and distribution to various organs (liver, kidney, spleen, heart, brain, gastrointestinal tract) was very limited.

Cho et al. (2013) studied the fate of spherical nanoparticles (80% anatase, 20% rutile) after oral administration to Sprague–Dawley rats. The measured particles size (using SEM) was 26.4 ± 6.1 nm and the hydrodynamic particle size was 37.8 ± 0.4 nm. Samples were administered for 13 weeks (7 days/week) at doses of 0, 260, 521 and 1,042 mg TiO_2/kg bw per day. The durability of the particles under gastrointestinal-mimicking conditions was demonstrated. Samples of blood, tissues (liver, kidneys, spleen and brain), urine and faeces were obtained at necropsy. The absorption of TiO_2 nanoparticles was shown to be extremely low. Tissue distribution data showed that TiO_2 nanoparticles were not significantly increased in sampled organs, even in the group receiving the highest dose (1,042 mg/kg bw per day). Titanium concentrations were not significantly increased in the urine. Very high concentrations of titanium were detected in the faeces.

In a study by Geraets et al. (2014) on the tissue distribution, elimination and oral absorption of different TiO_2 nanoparticles in Wistar rats, five different TiO_2 samples were tested (NM-100, NM-101, NM-102, NM-103 and NM-104) after oral or intravenous administration. The characteristics of the test materials used are given in Table 15.

Table 15: Characteristics of materials obtained from the JRC Nanomaterials (NM) Repository (JRC, 2011)

NM code	Type of material	Mean particle size (nm)	Primary particle (nm)	Specific area (mm^2/g)
NM-100	TiO_2	267	42–90	10
NM-101	TiO_2	38	6	320
NM-102	TiO_2, anatase	132	20	90
NM-103	TiO_2 thermal hydrophobic	186	20	60
NM-104	TiO_2 thermal hydrophilic	67	20	60

Animals were dosed either orally (gavage) or intravenously (injection, tail vein) once (three males per group, four TiO_2 nanomaterials and controls) or during five consecutive days (three males per group, four TiO_2 nanomaterials and controls); in addition, for the NM-101 test material, three females per group and controls were dosed.

For the oral route study, the single dose groups received a dose of 2.3 mg TiO_2/animal (calculated by the authors to be equivalent to 6.8–8.6 mg TiO_2/kg bw depending on the actual weight of the (male) animals). The repeated dose groups received five consecutive daily doses (day 1–5) of 2.3 mg TiO_2/mL per animal, resulting in a cumulative dose range of 34.1–42.4 mg TiO_2/kg bw for males and 54.5–59.9 mg TiO_2/kg bw for females.

The rats were killed and tissue sampling was carried out 24 h after the last exposure (day 2 or 6). Liver, spleen and mesenteric lymph nodes were selected as target tissues for titanium analysis.

For the intravenous study, the single-dose groups received a dose of 8.4–9.8 or 12.4–14.1 mg TiO_2/kg bw, for male and female animals, respectively. The repeated intravenously treated animals received five consecutive daily doses (day 1–5) for a cumulative dose range (taking into account the actual weight of the animals) of 42.3–49.4 and 61.2–71.9 mg TiO_2/kg bw for male and female animals, respectively.

Blood and tissue samples were collected from day 2 to day 90 after administration.

From the data on absorption, it was concluded that titanium levels in liver and spleen could only be measured above the limit of detection (30 ng/g tissue) in some rats. Titanium could be detected in the mesenteric lymph nodes, although the levels were very low. When compared with data from non-exposed animals it was shown that some minor, but very limited, absorption occurred in the gastrointestinal tract. No increase in titanium levels was observed in the other tissues.

3.1.2. Distribution

3.1.2.1. Studies in the mouse

Wang et al. (2007b) compared the biodistribution of different sized TiO_2 particles ((25, 80 and 155 nm (fine) in CD-1 (ICR) mice (40 males/40 females). The animals were randomly divided into four groups: one control and three experimental groups receiving a single oral (gavage) dose of the different particles sizes at a level of 5 g TiO_2 suspension/kg bw. Two weeks after treatment, titanium concentrations were analysed in different tissue samples from female mice only.

Titanium accumulated mainly in the liver: 3970 $\pm$ 1670 ng titanium/g in the 80 nm group, 106 $\pm$ 8 ng titanium/g in the 25 nm group, and 107 $\pm$ 25 ng titanium/g in the 155 nm (fine) group. In the kidneys, the titanium concentrations were as follows: ~ 440 ng titanium/g in the 80 nm group (statistically significantly different from control; $p < 0.05$), ~ 375 ng titanium/g in the 25 nm group, ~ 170 ng titanium/g in the 155 nm (fine) group and ~ 150 ng titanium/g in the control group. Titanium concentrations for animals receiving the 155 nm particle suspension were highest in the spleen ($p < 0.05$ compared with control), followed by the lung and brain ($p < 0.05$ compared with control). In the red blood cell, titanium concentrations were ~ 130 ng titanium/g for the 25 nm group, ~120 ng titanium/g for the 80 nm and 155 nm (fine) groups and ~ 80 ng titanium/g for the control group.

As regards biodistribution, the experiment showed that TiO_2 is mainly retained in the liver, spleen, kidneys and lungs, indicating that TiO_2 particles can be transported to other tissues and organs after uptake via the gastrointestinal tract. Furthermore, a basal level of TiO_2 of 150 ng/g TiO_2 in kidney and 80 ng/g in the red blood cell was demonstrated in control animals.

3.1.2.2. Studies in the rat

Lloyd et al. (1955) tested TiO_2 (particle size not given) as an index material for determining the digestibility of a rat diet. Albino male rats (n = 30, 60 days old) were fed a diet containing 0.25% TiO_2 (equivalent to ~ 295 mg TiO_2/kg bw per day) for 6 days.[16] Another group of rats (n = 30) were fed a diet containing 0.25% chromium(III) oxide, but there was no control group. The faeces of 10 of the 30 rats receiving the diet for 6 days were collected individually and daily for 13 days after the initial consumption. Twenty other rats were divided equally into four groups and after the 6-day feeding period; total faeces per group were collected for 7 days (total food consumption was also noted for this 7-day period). For the 30 rats on the TiO_2 diet, an average of 92% of the administered TiO_2 was recovered. The authors noted that some TiO_2 (8%) was unaccounted for which they treated as absorbed and hypothesised that delayed excretion could be due to accumulation of titanium in some part of the gastrointestinal tract.

West and Wyzan (1963) (as reported in IPCS, 1982) fed male and female rats (no further details given) a diet containing TiO_2 (100 g TiO_2/kg diet; particle size not given) for ~ 32 days. A statistically significant amount of titanium was found only in the muscles (0.06 mg/kg wet weight in males and 0.11 mg/kg wet weight in females); no retention was observed in the liver, spleen, kidney, bone, plasma or erythrocytes.

A bioavailability study (Colorcon, 2003 as reported by EFSA in 2004) performed in Sprague–Dawley (Cr:CD® BR) rats using four test substances of TiO_2 (no information on particle size given): rutile TiO_2 (thick platelet), rutile TiO_2 (thin platelet), rutile TiO_2 (amorphous) and anatase TiO_2 (amorphous). Groups (three animals/sex per time-point, aged 6–10 weeks) were fed *ad libitum* either a control diet

or a diet containing one of the four types of TiO_2, which were given at a concentration of 200 mg TiO_2/kg diet (equivalent to ~ 30 mg TiO_2/kg bw per day).[17] These TiO_2-containing diets were fed to the rats for seven consecutive days and were then replaced by the control diet for a maximum of 72 h before sacrifice. The control diet administered during the treatment phase contained a mean concentration of 9 mg TiO_2/kg wet weight and the control diet administered after the treatments contained a mean concentration of 7 mg TiO_2/kg wet weight. Groups of animals were killed at 1, 24 and 72 h after withdrawal from the treatment diet, and the titanium contents of the liver, kidneys, muscle, whole blood, urine and faeces were determined. The main route of titanium excretion was via the faeces. Faecal excretion in each collection interval (0–24, 24–48, 48–72 h) was similar for all TiO_2-treated groups. The mean total amounts of titanium excreted in the faeces during 0–72 h after withdrawal of the TiO_2-treated diet were in the range of 1.4–2.2 mg/animal for male rats and 1.1–1.3 mg/animal for female rats, accounting for means of 39–63% of the daily dose. Urinary excretion of titanium was equivalent to < 2% daily dose/L of urine for all groups and was generally below the limit of quantification (< 0.04 mg/L). Whole-blood concentrations of titanium from all groups were < 0.04 mg/L and concentrations of titanium in liver, kidney and muscle were generally below the LOD(< 0.1 to < 0.2 mg/kg wet weight) or in the range of 0.1–0.3 mg/kg wet weight for most animals treated with either the control diet or a diet containing TiO_2. The bioavailability study showed that there was no difference in the systemic absorption of the four forms of TiO_2 following dietary administration at a nominal concentration of 200 mg TiO_2/kg (based on a LOQ < 0.04 mg/L for urinary excretion).

In the study by Onishchenko et al. (2012) cited above, the effect of the administration of water dispersions of TiO_2 nanoparticles with an anatase structure (not further specified) and of micron-sized TiO_2 particles (food additive E 171; crystal structure not indicated) at low (1 mg/kg) and high (100 mg/kg) doses for 28 days was studied in Wistar rats. Titanium in basal amounts, characteristic of a great number of biological objects, was present in the liver tissue of rats fed a standard semisynthetic diet. Administration of the water dispersions induced no appreciable increase in these basal values. A similar result was observed in animals treated with rutile nanoparticles at the low dose (1 mg/kg). However, the titanium concentration in the liver increased significantly (almost doubling) in rats receiving intragastric water dispersions of rutile nanoparticles at the high dose (100 mg/kg), which, according to the authors, could indicate its penetration through the intestinal barrier. The Panel noted that the authors did not reveal the size characteristics of the nanoparticulate test material.

In a study by Chaudhry et al. (2013; Doc. provided to EFSA n. 7) (published as MacNicoll et al., 2015) described in Section 3.1.1, rats were administered by gavage a single dose of different TiO_2 nano- and larger particles dispersed in water (see Table 14). Animals were killed at different time intervals during the 96 h post-treatment and tissues (liver, brain, heart, kidney and spleen) were sampled. Based on titanium determination (LOD = 1 ng/g), no translocation of TiO_2 was observed in any of the treatments applied and tissues selected.

Tassinari et al. (2014) studied the effect of short-term oral exposure to TiO_2 nanoparticles in Sprague–Dawley rats with a focus on the reproductive and endocrine systems and spleen. In the study, anatase nanoparticles with two different morphologies were used, i.e. spherules with primary sizes ranging from 20 to 60 nm and irregular-shaped particles ranging from 40 to 60 nm. Moreover, large agglomerates and chains of spherules were also observed to be present. The test materials were administered, by gavage, for five consecutive days at doses of 0, 1 and 2 mg TiO_2/kg bw. An increase in the titanium concentration was found in the spleen and ovaries of treated animals compared with controls, even though the titanium tissue levels remained low (control, 0.036 ng/g fresh weight; 1 mg/kg bw dose, 0.040 ng/g fresh weight; 2 mg/kg bw dose, 0.046 ng/g fresh weight) and were similar to the levels reported in controls and were within the values reported by Wang et al. (2007a). In the spleen of treated animals, TiO_2 aggregates of 200–400 nm (in high-dose females) were identified and quantified (2–3 $\times$ 10^4 particles/mm^2 vs < 1 $\times$ 10^4 particles/mm^2 in controls).

Geraets et al. (2014) concluded that after both single and repeated intravenous exposure, titanium (not further specified in terms of purity, nanosized distribution) is rapidly distributed from the systemic circulation to all tissues evaluated (i.e. liver, spleen, kidney, lung, heart, brain, thymus and reproductive organs). Liver was identified as main target tissue, followed by spleen and lung. Total recovery (expressed as % nominal dose), measured 24 h after single or repeated exposure, ranged from 64% to 95%. Based on calculations using different scenarios (i.e. using LOD or half the LOD for

[17] A grade of sufficient purity to meet or exceed requirements of the United States National Formulary (NF) (merged with the United States Pharmacopeia, USP-NF).

the non-detects; correcting tissue levels for background levels; using only the positive liver titanium levels), the authors estimated that ~ 0.02% of the administered dose of TiO_2 was distributed in the tissues. The Panel agreed with this conclusion of the authors.

3.1.3. Excretion

From the Geraets et al. (2014) study (see above), it was concluded that, following intravenous administration, a decrease in titanium in the investigated organs was observed over the 90-day period, although > 50% of the administered dose was still present at the end, indicating a long half-life (28–248 days for the liver). Titanium levels in liver, the tissue exhibiting the highest levels, showed a decrease during that period for all nanoparticles tested, together with a concomitant increase in spleen, in which the final titanium level was higher than in liver. Only minor differences in kinetic profiles were observed, both after single and repeated exposure.

The authors of the study further indicated that the titanium levels measured in the faeces of intravenously treated (single and repeated dose) animals revealed no clear differences between TiO_2-exposed animals and vehicle-treated controls. Furthermore, no increase in titanium levels in urine was observed.

At day 90 post-exposure, titanium levels in spleen were higher than in liver (expressed as μg/g tissue). This would be consistent with a redistribution of the TiO_2 nanoparticles between liver and spleen and slow elimination. The Panel noted that there were only a few sampling times during the post-exposure period.

The Panel also noted that although tissue half-life was estimated, it was not possible to determine the excretion pathway.

3.1.4. Human studies

West and Wyzan (1963) (as reported in International Programme on Chemical Safety (IPCS), 1982), gave five male volunteers 5 g of National Formulary grade TiO_2 suspended in milk on three consecutive days.[18] Urine samples were collected for 5 days after the start of ingestion. No detectable change in urinary titanium levels was detected, which suggests the absence of any significant absorption of the titanium ion, although accumulation in the body cannot be excluded.

Böckmann et al. (2000) measured blood titanium levels in males (24–66 years old) after oral administration of TiO_2 (23 or 46 mg) either as anatase (median particle size, 160 nm) in gelatin capsules or as a powder (median particle size, 380 nm). Pretreatment background blood levels had titanium levels ranging from 6 to 18 μg/L. After TiO_2 administration, blood samples were taken over 24 h (i.e. 0, 15 and 30 min and 1, 2, 4, 8, 12 and 24 h) and the titanium level in the blood was measured. The authors reported concentration–time data, from which the Panel calculated the area under the blood concentration/time course (AUC) as a measure of absorbed amount. The AUC of five subjects (median: 17,573.25 μg/L × min) having taken the gelatin capsules (160 nm particles) was higher than for the two subjects (AUC: 9,384 and 10,519.5 μg/L × min) having taken the powder (380 nm particles). According to these authors, this indicates that there might be an influence of particle diameter on the extent of absorption of TiO_2, however, the Panel noted the median particle size of both particles studied were greater than 100 nm. The authors reported that the blood concentration/time correlation showed the type of curve characteristic of a persorption mechanism of absorption.

In a study by Jones et al. (2015), human volunteers (four males and five females; aged 30–56 years) received a 5 mg/kg bw single oral dose of TiO_2 (particle sizes: 15 nm (anatase; ~ 100% by number < 50 nm), 100 nm (rutile; 95% by number between 48 and 154 nm) and < 5000 nm (rutile; 100% by number > 100 nm) dispersed in water. Doses were administered at least 4 weeks apart. All urine samples were collected in timed collections over a 4-day period starting 24 h before dosing and ending 72 h post-dose, and analysed for titanium content after hydrolysis. Blood samples were collected before dosing and at 2, 4, 24 and 48 h after dosing and analysed for titanium content, full blood count and liver function tests. The study demonstrated that very little TiO_2 at all nanosizes tested was absorbed gastrointestinally after an oral dose at a maximum estimate of 0.1% of the administered dose. There was no demonstrable difference in absorption for any of the three particle sizes tested. Because of the very low absorption and the variable endogenous titanium levels, no classic absorption and elimination curve was observed in any of the studies. A dose of 5 mg/kg bw was well tolerated (both clinically and biochemically) by all volunteers for all particle sizes.

[18] 'NF grade' is the purity standard as defined in the US National Formulary.

In another recent published study (Pele et al., 2015), seven human volunteers were given a single oral dose of 100 mg TiO_2 (particle size (d_{50}), 260 nm). Venous blood was sampled up to 10 h post-administration, TiO_2 particles were identified by dark field microscopy (reflectance) and ^{47}Ti was measured by inductively coupled plasma mass spectrometry. An unquantified fraction of TiO_2 particles was detected in blood with a peak of absorption observed at 6 h, which paralleled the titanium concentration in blood (~ 10 μg/mL, which decreased to 5 μg/mL at 10 h). Whether the particles are transported within or outside immune cells requires confirmation.

Based on the above dataset, the Panel considered that:

- TiO_2 was chemically stable under physicochemical conditions that mimic the gastrointestinal situation. No release of titanium ions was shown to occur.
- The vast majority of orally administered TiO_2 was excreted in the faeces.
- TiO_2 particles did not cross the gastrointestinal–epithelial barrier models by diffusion *in vitro*, but there was minimal translocation into the cells, which varied with the model system used.
- Nano/microsized TiO_2 particles were absorbed to a limited extent from the gastrointestinal tract (bioavailability estimated at 0.02–0.1%), essentially through the GALT. However, there were uncertainties regarding the real physical state (primary size, aggregation/agglomeration, protein corona) of the absorbed particles and estimates were based on measurements of titanium ion. Furthermore, evaluating the data overall, the Panel considered that there were no differences in the extent of absorption related to particle size. The Panel noted that the very low bioavailability and variable background basal levels of TiO_2 in tissues not only made it difficult to interpret the results, but also prevented accurate determination of kinetic parameters such as elimination half-life.
- Absorbed nano/microsized TiO_2 particles were distributed in different organs, by order of decreasing concentration: mesenteric lymph nodes, liver, spleen, kidney, lungs, heart and reproductive organs (testes and ovaries).
- After intravenous administration of nano- and microsized TiO_2 particles, studying four different particles, titanium was poorly excreted via urine and higher titanium concentrations were observed in tissues than in blood. After repeated intravenous dosing over 6 days, titanium concentrations in tissues were higher than after single intravenous administration. A long-term redistribution of titanium from the liver to the spleen has been shown to occur, which emphasises the role of the mononuclear phagocyte system in particle processing. The decline of titanium concentrations in the tissues was slow; the authors calculated half-lives of between 28 and 650 days, depending on the TiO_2 particles and tissue. The Panel noted that titanium absorbed after oral TiO_2 administration would have the same kinetic pattern as TiO_2 administered by repeated i.v. The Panel also noted that after oral administration, direct evidence for higher concentrations in the tissues was lacking, which may be due to the low bioavailability, high variability of intake and high background (basal) tissue levels of titanium. The slow elimination of titanium after intravenous administrations indicates the potential for a low but steady increase in titanium tissue levels with time for absorbed titanium after oral administration.

3.2. Toxicological data

3.2.1. Acute oral toxicity

The acute oral median lethal dose (LD_{50}) value for TiO_2 was > 10 g TiO_2/kg bw per day for mice and > 25 g/kg bw per day for rats (Hallagan et al., 1995; SCCNFP, 2000).

Three different TiO_2 particle sizes (25, 80 and 155 nm) were administered by gavage with a single dose of 5,000 mg TiO_2/kg bw in CD-1 (ICR) mice in accordance with OECD 420, by oral gavage (Wang et al., 2007b). After 2 weeks, TiO_2 particles showed no obvious acute toxicity. Female animals exposed to nanosized TiO_2 showed hepatotoxicity characterised by changes in aspartate amino transferase/alanine amino transferase ratio and lactate dehydrogenase activity, and hydropic degeneration around the central vein and focal necrosis of hepatocytes. In addition, nephrotoxicity (increased blood urea nitrogen levels) was also observed in these groups. These changes were not seen in mice treated with TiO_2 particles of 155 nm.

3.2.2. Short-term and subchronic toxicity

3.2.2.1. Studies in the mouse

The US National Cancer Institute (NCI, 1979) performed a subchronic toxicity (90-day) dose range-finding study in B6C3F1 mice to estimate the maximum tolerated doses of TiO_2 (anatase; particle size not given) to be used in a carcinogenesis study in the mouse. Doses of 6,250, 12,500, 25,000, 50,000 or 100,000 mg TiO_2/kg diet were administered (equivalent to 1,344, 2,688, 5,375, 10,750 or 21,500 mg TiO_2/kg bw per day for female mice and 1,056, 2,113, 4,225, 8,450 or 16,900 mg TiO_2/kg bw per day for male mice, respectively).[16] TiO_2 had a purity of minimum 98%. Ten males and 10 females were administered the test substance at each dose, and 10 males and 10 females received basal diets for 13 consecutive weeks. There were no deaths, and dosed animals had mean bw gains that were comparable with those of the controls. No gross or microscopic pathology was found that could be related to the administration of anatase in the mice.

The Panel noted that the study was only briefly described in the NCI (1979) report and that no haematological parameters and no biochemical parameters in urine and blood were measured.

3.2.2.2. Studies in the rat

West and Wyzan (1963) (as reported in JECFA, 1970) fed a group of 10 male and 10 female rats (strain not given) 100 mg National Formulary Grade TiO_2/kg diet for 30–34 days.[17] A second, untreated group was used as a control. All animals remained healthy and normal. Weight gain and food intake were comparable for the two groups. At autopsy, no relevant gross pathology was observed. No evidence of an increase in titanium content was found in any of the seven different tissues analysed (no further details) except muscle, where the increase was 0.1 mg/kg compared with tissues from the control animals.

In a study that was in line with OECD Test Guideline 407 for 'Repeated Dose 28-Day Oral Toxicity Study in Rodents', three groups of five young male Sprague–Dawley Crl:CD(SD) rats were given daily gavage doses of either pure water (control) or 24,000 mg/kg bw of one of two similar non-coated pigment-grade forms of rutile with a d_{50} of 173 nm; one form was described as 'research grade' and the other was 'commercial grade'. One rat from each of the test groups died prematurely due to misdosing (perforation of the oesophagus). There were no treatment-related effects on food intake, body weight, clinical signs, haematology, serum clinical chemistry, organ weights, gross pathology or histopathology. Particles found in intestinal lymphoid tissue were not regarded as an adverse effect. There were no differences in response to the two forms of the test material. The no observable adverse effect level (NOAEL) for the study was 24,000 mg/kg bw per day for both forms of TiO_2 tested. Although this study was not performed with TiO_2 (E 171), its results are useful as supporting evidence in the assessment of the use of TiO_2 as a colouring agent for food and feeds (Warheit et al., 2015b).

The NCI (1979) performed a subchronic toxicity (90-day) dose range-finding study in Fischer 344 rats to estimate the maximum tolerated doses of TiO_2 (anatase; particle size not given) to be used in a carcinogenesis study in the rat. Doses of 6,250, 12,500, 25,000, 50,000 or 100,000 mg TiO_2/kg diet were administered (equivalent to 569, 1,138, 2,275, 4,550 or 9,100 mg TiO_2/kg bw per day for female rats and 506, 1,013, 2,025, 4,050 or 8,100 mg TiO_2/kg bw per day for male rats, respectively).[16] TiO_2 had a purity of minimum 98%. Ten males and 10 females were administered the test substance at each dose, and 10 males and 10 females received basal diets for 13 consecutive weeks. There were no deaths, and dosed animals had mean body weight gains that were comparable with those of the controls. No gross or microscopic pathology was found that could be related to the administration of the test substance in the rats.

The Panel noted that the study was described only briefly in the NCI (1979) report and that no haematological parameters and no biochemical parameters in urine and blood were measured.

The Panel noted that there was rather limited information available on the short-term and subchronic toxicity on the food additive TiO_2 (E 171).

In a well-performed 28-day gavage study in rats with non-coated pigment-grade TiO_2 (rutile form; d_{50} 173 nm) at a dose of 24,000 mg TiO_2/kg bw, no treatment-related effects were observed (Warheit et al., 2015b). Particles found in intestinal lymphoid tissue were not regarded as an adverse effect. The NOAEL for the study was 24,000 mg/kg bw per day. Although the study was not performed with food-grade TiO_2, the Panel considered the results useful as supporting evidence in the assessment of the use of TiO_2 as food additive (E 171) colouring agent for food and feeds.

In a 90-day feeding study, doses up to 16,900 mg TiO_2/kg bw per day for male mice and up to 8,100 mg TiO_2/kg bw per day for male rats did not result in differences in body weight or in relevant gross or microscopic pathology compared with the control (NCI, 1979). However, no haematological parameters and no biochemical parameters in urine and blood were measured.

3.2.3. Genotoxicity

3.2.3.1. *In vitro*

In an early study, TiO_2 was reported to be negative in a rec-assay with *Bacillus subtilis* for genotoxicity using a M45 recombination-deficient strain (Kada et al., 1980). The Panel noted that such a test system has not been validated and considered this information not relevant for risk assessment.

In a screening study of 63 carcinogenic and non-carcinogenic chemicals, TiO_2 (CAS Registry number 13463-67-7, particle size not specified) was tested for mutagenicity in a bacterial reverse mutation assay using the plate-incorporation procedure in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA1538, and *Escherichia coli* WP2 *uvrA*, in the absence and in the presence of exogenous metabolic activation S9 liver preparations from uninduced and Aroclor 1254-induced F344 rats, B6C3F1 mice and Syrian hamsters. Dose levels were selected at half-log intervals and never exceeded 10 mg/plate. Clear negative results were observed for TiO_2 (Dunkel et al., 1985). The Panel noted that in this, and in the other genotoxicity assays performed within the validation exercise coordinated by the National Toxicological Programme (NTP) (Dunkel et al., 1985; Tennant et al., 1987; Ivett et al., 1989; Myhr and Caspary, 1991; Shelby et al., 1993; Shelby and Witt, 1995), the sample of TiO_2 tested was received from the NTP repository of the chemicals tested in carcinogenicity bioassays. According to the NCI-CG-TR 97, the sample was an anatase TiO_2 white pigment designated Unitane® 0.220.

Tennant et al. (1987) assayed TiO_2 (particle size not given, see above) in the Ames *Salmonella*/microsome mutagenicity assay, in the assays for chromosomal aberrations and sister chromatid exchanges (SCEs) in a Chinese hamster ovary (CHO) cell line, and in the mouse lymphoma L5178Y cell mutagenicity assay. Standard protocols developed by the NTP of the NCI were used for the selected assays. Negative results for TiO_2 were reported in any of the four short-term tests (STTs) selected. The highest negative dose levels assayed were as follows: 10,000 μg/plate in the Ames test, 25 μg/mL in the assays for chromosomal aberrations and SCEs, and 1.6 μg/mL in the mouse lymphoma L5178Y cell mutagenesis assay.

Ivett et al. (1989) studied the genotoxicity of TiO_2 (particle size not given, see above) in a SCE assay and in a chromosomal aberration assay in CHO cells, both in the absence and presence of rat liver S9. Cells were exposed for 25 and 2 h in the SCE assay and for 8 and 2 h in the chromosomal aberration assay in the absence and presence of rat liver S9, respectively. In both assays, a top-dose level of 25 μg/mL (equivalent to 313 μM) was selected based on the solubility of the test material. Reported results indicated that TiO_2 did not induce SCE or chromosomal aberrations in mammalian cells *in vitro*.

Myhr and Caspary (1991), in a following screening study on 31 coded compounds, tested TiO_2 (particle size not given, see above) for its mutagenicity in an *in vitro* mammalian cell gene mutation assay in L5178Y mouse lymphoma cells at the thymidine kinase (TK) locus in both the absence and presence of S9. The test compound was administered for 48 h at dose levels ranging from 1.56 to 50 μg/mL. Negative results were reported in any treatment conditions.

In the study by Miller et al. (1995), TiO_2 (particle size not given) was assessed for its genotoxic potential in an *in vitro* micronucleus assay in CHO cells in both the absence and presence of rat S9. Dose levels ranged from 0.025 to 10 μg/mL in the absence of S9 and from 0.25 to 10 μg/mL in its presence, and treatment times were 48 and 3 h, respectively. Top-dose levels were selected according to cytotoxic effects, which were based on a reduction of cell density by at least 25% of concurrent control values. However, precipitation of TiO_2 was observed at concentrations of $\geq$ 0.5 and $\geq$ 1.0 μg/mL in the absence and in the presence of S9, respectively. Micronuclei were scored in at least 1,000 mononucleated cells from each culture. Results obtained indicated that TiO_2 was not able to induce micronuclei in CHO cells.

Linnainmaa et al. (1997) assessed the induction of micronuclei in a rat liver epithelial cell line by two ultrafine (UF1 and UF2) TiO_2 preparations. The test material consisted of uncoated anatase (UF1, average particle size 20 nm), rutile coated with aluminium hydroxide and stearic acid (UF2, average particle size 20 nm), and pigmentary TiO_2 (average particle size 170 nm). Treatments were conducted for 21 h alone or in combination with UV irradiation (365 nm). Dose levels evaluated

ranged from 5 to 20 μg/cm^2. Cytochalasin B (1 μg/mL) was added to the culture for the last 20 h. The reported results indicate that TiO_2 (pigmentary or ultrafine) alone or in combination with UV light did not induce chromosomal damage measured as induction of micronuclei. However, the Panel noted that the spontaneous frequencies of micronuclei in the untreated controls were markedly high (53–71 micronuclei/1,000 binucleated cells) indicating elevated genomic instability of the cell line employed, and on this basis, the Panel considered the results reported in this study of limited relevance for risk assessment.

Nakagawa et al. (1997) investigated the photogenotoxicity of TiO_2 particles in a single-cell gel electrophoresis Comet assay with mouse lymphoma L5178Y cells, a microbial mutation assay with *S.* Typhimurium, a mammalian cell mutation assay with L5178Y cells and a chromosomal aberration assay with Chinese hamster CHL/IU cells. The following TiO_2 particles were tested in the single-cell gel electrophoresis assay: anatase-p-25, (average size 21 nm) and anatase-WA, (average size 255 nm); rutile-WR, (average size 255 nm) and rutile-TP-3, (average size 420 nm). In the TiO_2 Comet assay WA, WR and TP-3 were tested at concentrations from 250 to 2,000 μg/mL and p-25 was tested at five concentrations from 2.1 to 800 μg/mL. In the chromosomal aberration assay in CHL cells, only p-25 was tested at concentrations from 25 to 800 μg/mL, in the absence of UV radiation and at concentration from 0.78 to 28.5 μg/mL in the presence of UV radiation. In bacteria (*S.* Typhimurium strains TA100, TA98 and TA102), only p-25 TiO_2 particles were tested from 6,750 to 54,000 μg/plate with and without UV radiation. Results obtained showed that p-25 and TP-3 induced primary DNA damage only when UV irradiated (minimum effective concentrations of 12.5 and 200 μg/mL, respectively); WA particles (50–3,200 μg/mL) were also positive without irradiation, but only at the highest tested dose, whereas WR particles were negative in the same dose range. Negative results were observed with p-25 nanoparticles in bacteria (500–4,000 μg/plate) and in the L5178Y mouse lymphoma gene mutation assays (250–2,000 μg/mL). Positive results were obtained with p-25 in an *in vitro* chromosomal aberration assay in Chinese hamster cells (minimum effective concentration 12.5 μg/mL), only in the presence of UV irradiation.

Lu et al. (1998) studied the effect of TiO_2 (particle size not indicated) for the induction of SCE and micronuclei in CHO-K1 cells. TiO_2 was administered for 24 h, at dose levels of 1, 2 and 5 μM for SCE and at 5, 10, 15, 20 μM for 18 and 24 h in the conventional and cytokinesis-block micronuclei analysis, respectively. Selection of top-dose levels was based on a reduction in colony-forming ability. Results obtained indicated that TiO_2 induced dose-related and statistically significant increases in SCE compared with concurrent untreated control cultures. Dose-related and statistically significant increases were also observed for induction of micronuclei both in the conventional micronuclei analysis and in the cytokinesis-block micronuclei analysis. However, higher levels of micronuclei (2.5- to 3-fold increases) were observed with and without the cytokinesis-block micronuclei.

Rahman et al. (2002) reported the effects of ultrafine TiO_2, particle size $\leq$ 20 nm, and fine TiO_2, particle size $>$ 200 nm, on chromosomal damage in Syrian hamster embryo cells (SHE) monitored by the formation of micronuclei. Cells were treated on coverslips at concentrations of 0.5, 1, 5 and 10 μg/cm^2 for 12, 24, 48, 66 and 72 h. DNA was stained with bisbenzimide at 1 μg/mL and micronuclei scored at $\times$630 magnification under a fluorescence microscope. For further micronuclei analyses, kinetochores were stained with CREST serum to allow discrimination of clastogenic effects from aneuploidy. Results obtained revealed significant increases in micronuclei induction by ultrafine TiO_2 at a dose of 1 μg/cm^2 at sampling times for 24, 48, 66 and 72 h, whereas fine TiO_2 did not induce significant increases in micronuclei. Furthermore, kinetochore analyses revealed no significant increases in the kinetochore-positive micronuclei compared with micronuclei in the untreated control, indicating that induced micronuclei arise mainly from clastogenic and not aneugenic events.

Wang et al. (2007a) evaluated the cytotoxic and genotoxic activity of ultrafine TiO_2 particles (particle size not specified) in human lymphoblastoid WIL2-NS cells. Cells were incubated for 6, 24 and 48 h with 0, 26, 65 and 130 μg/mL ultrafine TiO_2; cytotoxicity was evaluated by the methyl tetrazolium cytotoxicity (MTT) assay, apoptosis assay by the flow cytometry, and genotoxicity by the cytokinesis block micronucleus assay, by the Comet assay and by the hypoxanthine–guanine phosphoribosyltransferase gene mutation assay. Significant decreases in viability and proliferation, and increase in apoptosis were seen at the highest doses. In genotoxicity assays, increased incidence of micronuclei ($\sim$ 2.5-fold at 130 μg/mL), olive tail moment ($\sim$ 5-fold increases at 65 μg/mL) and hypoxanthine–guanine phosphoribosyltransferase mutations ($\sim$ 2.5-fold increases at 130 μg/mL) were observed in cells following exposure to ultrafine TiO_2.

Türkez and Geyikoğlu (2007) evaluated the potential genotoxic effects of TiO_2 (particle size not indicated) in human whole-blood cultures. Blood samples were obtained from four young non-smoking

and healthy donors, and pooled for treatment. SCE and micronuclei were scored as genetic endpoints. Dose levels of 1, 2, 3, 5, 7.5 and 10 μM were administered to blood cultures for 72 h. For SCE, 5-bromo-2′-deoxiuridine at 20 μM was added from the beginning of culture. For micronuclei analyses, cytochalasin B (6 μg/mL) was added 44 h from the beginning of culture. Results obtained showed dose-related and statistically significant increases in both SCE and micronuclei, indicating the potential genotoxicity of TiO_2. These results were confirmed in a second study (Turkez, 2011), in which the role of oxidative stress was suggested based on the observed reduction in TiO_2 genotoxicity in presence of ascorbic acid.

Warheit et al. (2007) tested TiO_2 particles (79% rutile, 2% anatase; median particle sizes of 140 nm) for mutagenicity in a bacterial reverse mutation test in *S.* Typhimurium strains TA98, TA100, TA1535 and TA1537, and in *E. coli* strain WP2uvrA in the absence and presence of metabolic activation (Aroclor-induced rat liver S9). Negative results were reported up to 5,000 μg/plate. The same test item was also negative in a chromosome aberrations test in CHO cells in the absence and presence of metabolic activation (Aroclor-induced rat liver S9). The test item was analysed without S9 up to 2,500 and 100 μg/mL in the 4- and 20-h treatment, respectively, whereas with S9, the top dosage was 250 μg/mL.

Karlsson et al. (2009) compared the toxicity of nano- and micrometre particles of some metal oxides, and nano- and micrometre particles of TiO_2 (average particle size 63 nm and 1 μm, respectively) by assessing DNA damage and DNA oxidative lesions in the human alveolar type II-like cell line A549. To study DNA damage in forms of DNA strand breaks and alkali labile sites, the alkaline version of the Comet assay was used. For analyses of oxidative DNA lesions, mainly oxidised purines, the enzyme formamidopyrimidine DNA glycosylase was applied to the Comet assay. When A549 cells were treated with nano- and micrometre particles of TiO_2 for 4 h at 40 and 20 μg/cm^2, statistically significant increases in DNA damage compared with untreated controls were observed for both nano- and micrometre particles. However, micrometre particles caused markedly higher levels of DNA damage compared with nanoparticles. By contrast, for oxidative DNA damage, no significant increases in oxidised purines were observed for both nano- and micrometre particles.

Xu et al. (2009) assessed the genotoxicity of TiO_2 particles of different size distributions (anatase form, size 5 nm, 40 nm and 325 mesh, applied in the dose range 0.1–30 μg/mL) using gpt delta transgenic mouse primary embryo fibroblasts. Mutation frequencies were investigated at redBA and gam loci, sensitive to kilobase deletion mutations. TiO_2 nanoparticles (both 5 and 40 nm) significantly increased mutation yield at 0.1 μg/mL and above, with no clear relation with the dose applied. The effect was abrogated by the concurrent treatment with the endocytosis inhibitor Nystatin.

Bhattacharya et al. (2009) evaluated the genotoxicity and oxidative stress induced by TiO_2 nanoparticles (anatase; size < 100 nm) in human lung fibroblasts (IMR-90) and human bronchial epithelial cells (BEAS-2B). TiO_2 nanoparticles (2–50 μg/cm^2) did not induce detectable DNA damage, as evaluated by Comet assay, although they increased both oxidative damage (8-hydroxy 2′-deoxyguanosine (8-OH-dG)) and the intracellular generation of reactive oxygen species (ROS).

In the study by Falck et al. (2009), the *in vitro* genotoxicity of nanosized TiO_2 rutile and anatase was assessed in comparison with fine TiO_2 rutile in human bronchial epithelial BEAS-2B cells using the single-cell gel electrophoresis (Comet) assay and the cytokinesis-block micronucleus test. BEAS-2B cells were exposed to eight doses (1–100 μg/cm^2) of titanium oxide nanosized rutile (99.9% < 5 nm), nanosized anatase (99.7%; < 25 nm) or fine rutile (99.9%; < 5 μm) for 24, 48 and 72 h. Fine rutile reduced cell viability at lower doses than nanosized anatase, which was more cytotoxic than nanosized rutile. In the Comet assay, nanosized anatase and fine rutile induced DNA damage at several doses for all treatment times. The lowest doses inducing DNA damage were 1 μg/cm^2 for fine rutile and 10 μg/cm^2 for nanosized anatase. Nanosized rutile showed a significant induction in DNA damage only at 80 and 100 μg/cm^2. Only nanosized anatase could elevate the frequency of micronucleated BEAS 2B cells, producing a small but significant increase at 10 and 60 μg/cm^2 (with no dose dependency).

Di Virgilio et al. (2010) analysed the cytotoxicity and genotoxicity of titanium oxide nanoparticles (20 ± 7 nm) on CHO-K1 cells using the Neutral Red and MTT assays, and by the SCE and micronuclei assays. Results showed a dose-related cytotoxic and genotoxic effects, with micronuclei frequencies significantly increased at 0.5 and 1 μg/mL, and SCE significantly increased at 1–5 μg/mL TiO_2. Cytotoxicity, evidenced also by the absence of metaphases, was observed at higher concentrations.

Landsiedel et al. (2010) investigated the genotoxicity of coated rutile TiO_2 nanoparticles (size 10×50 nm) in standard OECD *in vitro* and *in vivo* test systems. No genotoxicity was observed *in vitro* in the *Salmonella* gene mutation test (at 20–5000 μg/plate) and in the V79 micronucleus test

(at 75–300 μg/mL), or *in vivo* in Comet assays on alveolar lavage cells from rats exposed by inhalation 6h/day for 5 days to 10 mg/m^3 TiO_2 nanoparticles.

Using a Hep-2 cell line, Osman et al. (2010), evaluated the cytotoxicity and genotoxicity of TiO_2 nanoparticles using the MTT and Neutral Red assays, and the Comet and the cytokinesis-block micronucleus assays, respectively. Concentration- and time-dependent cytotoxicity and increases in DNA and cytogenetic damage were observed (no further details available).

Shukla et al. (2011) evaluated the cytotoxic and genotoxic activity of TiO_2 nanoparticles (anatase; average diameter 50 nm) in the human epidermal cell line (A431). A mild cytotoxic response of TiO_2 nanoparticles was observed using the MTT and Neutral Red uptake assays after 48 h of exposure. A statistically significant ($p < 0.05$) induction in DNA damage was observed using the formamidopyrimidine DNA glycosylase-modified Comet assay in cells exposed to 0.8 μg/mL TiO_2 nanoparticles (2.20 ± 0.26 vs control 1.24 ± 0.04) and higher concentrations for 6 h. A significant ($p < 0.05$) induction in micronucleus formation was also observed at the above concentration (14.7 ± 1.2 vs control 9.3 ± 1.0). TiO_2 nanoparticles elicited a significant cytotoxicity, evaluated using the MTT and Neutral Red assays, and reduced glutathione level with a concomitant increase in lipid hydroperoxides and ROS.

Wang et al. (2011) examined oxidative stress as well as cyto- and genotoxicity induced by TiO_2 nanoparticles (100% anatase; < 25 nm) in CHO-K1 cells following 60 days of continuous exposure at 0, 10, 20 or 40 μg/mL. The results of the study showed that oxidative stress increased in a concentration-dependent manner in short-term (2 days) cultures, whereas long-term cultures had lower levels of oxidative stress. The primary ROS appeared to be superoxide, because ROS indicators were lowered on addition of superoxide dismutase. No cyto- or genotoxic effects were apparent using the MTT, Trypan Blue exclusion and colony-forming assays for viability, and the Comet and *hprt* gene mutation assays for genotoxicity. According to the authors, CHO cells appear to adapt to chronic exposure to nano-TiO_2 and to detoxify excess ROS, possibly through upregulation of superoxide dismutase in addition to reduction of particles uptake.

Hackenberg et al. (2011) evaluated the *in vitro* geno- and cytotoxicity of TiO_2 anatase nanoparticles (diameter 15–30 nm) in peripheral blood lymphocytes from 10 male donors. TEM was performed to describe particle morphology and size, the degree of particle aggregation, and their intracellular distribution. Cells were exposed to nanoparticles in increasing concentrations of 20, 50, 100 and 200 μg/mL for 24 h. Cytotoxic effects were analysed by the Trypan Blue exclusion test and the single-cell microgel electrophoresis (Comet) assay was applied to detect DNA strand breaks, alkali labile sites and repair intermediates. Particles displayed a strong tendency to form aggregates, despite dispersive treatments. The Trypan Blue exclusion test did not show any decrease in lymphocyte viability, and there was no evidence of genotoxicity in the Comet assay for any of the tested concentrations, despite particles being detected in the cytoplasm as well as in the nucleus of treated cells.

Jugan et al. (2012) characterised the genotoxic potential of TiO_2 nanoparticles of different sizes and crystalline phases in the human lung cell line A549. Test material consisted of spherical anatase (nano) particles with average diameters of 12, 25 and 140 nm (A12, A25 and A140), and spherical rutile nanoparticles with average diameters of 20 and 68 nm (R20 and R68). Cells were exposed for various lengths of time (4, 24 and 48 h), and cytotoxicity, oxidative stress and genotoxicity were evaluated using a set of complementary techniques (MTT and clonogenic assays for cytotoxicity, Comet and micronuclei assays and γ-H2AX immunostaining for genotoxicity, and 8-OH-dG analysis, titration of intracellular ROS, glutathione content, antioxidant enzyme activities for oxidative stress). Mild cytotoxicity was observed after 48 h treatment with nanoparticles (A12, A25, R20 at 1–100 μg/mL), whereas no or borderline toxicity was elicited by R68 and A140. Increased intracellular ROS levels and genotoxicity were observed in the Comet assays with all particles after 4 h treatment (100 μg/mL), which decreased at later times. At the same dose, increased 8-OH-dG levels were observed in cells treated with A12, A25, A68 and R20, but not with A140. Negative results were obtained with all particles in micronucleus and γ-H2AX assays (50, 100 and 200 μg/mL). In conclusion, this work showed that TiO_2 particles with different sizes and crystalline phases could elicit oxidative stress and induce the formation of transient DNA lesions detectable by Comet assay – but not with the γ-H2AX immunostaining specific for DNA double-strand breaks – which did not result in clastogenic or aneugenic events visualised as micronuclei.

The lung adenocarcinoma epithelial cell line A549 was also used by Toyooka et al. (2012) in an *in vitro* study on the genotoxicity of TiO_2 anatase microparticles (diameter 5000 nm) and nanoparticles (diameter 5 nm). Genotoxicity elicited by treatments (1–100 μg/mL) was evaluated based on the

phosphorylation of the histone H2AX (γ-H2AX). Both TiO_2 particles generated γ-H2AX foci, which was more remarkable with the smaller particles. The flow cytometric analysis showed that γ-H2AX generation was independent of cell-cycle phase, and cells that incorporated larger amounts of TiO_2 particles had more γ-H2AX foci. Low levels of intracellular ROS were detected, even if large amounts of TiO_2 particles were taken up. By contrast, the generation of γ-H2AX was attenuated by coating the surface of TiO_2 particles with bovine serum albumin. According to the study authors, these results suggested that smaller TiO_2 particles were easy to incorporate into cells and generated cell-cycle phase-independent γ-H2AX, which was dependent on the condition of the TiO_2 surface, but not on the formation of ROS.

TiO_2 nanoparticles induced cytotoxicity and DNA damage in human amnion epithelial (WISH) cells, as investigated by Saquib et al. (2012). Crystalline, polyhedral rutile TiO_2 nanoparticles (diameter 30 nm) were characterised using X-ray diffraction, UV–visible spectroscopy, Fourier transform infrared spectroscopy and TEM analyses. The Neutral Red uptake and MTT assays revealed a concentration-dependent cytotoxic effect of TiO_2 nanoparticles over a concentration range of 0.625–10 μg/mL. Cells exposed to TiO_2 nanoparticles (10 μg/mL) exhibited a significant reduction (46.3% and 34.6%; $p < 0.05$) in catalase activity and glutathione level, respectively. Treated cells showed a 1.87-fold increase in intracellular ROS generation and a 7.3% ($p < 0.01$) increase in G_2/M cell-cycle arrest compared with the untreated control. Cells treated with TiO_2 nanoparticles also demonstrated the formation of DNA double-strand breaks with a 14.6-fold ($p < 0.05$) increase in the Olive tail moment value at 20 μg/mL concentration (highest dose tested), under neutral Comet assay conditions.

Woodruff et al. (2012) assessed the genotoxicity of 10 nm uncoated sphere TiO_2 nanoparticles with an anatase crystalline structure using the *Salmonella* reverse mutation assay (Ames test) and the single-cell gel electrophoresis (Comet) assay in TK6 cells. For the Ames test, *Salmonella* strains TA102, TA100, TA1537, TA98 and TA1535 were preincubated with eight different concentrations of TiO_2 nanoparticles for 4 h at 37°C, ranging from 0 to 4,915.2 μg per plate. No mutation induction was found. TEM and energy-dispersive X-ray spectroscopy analyses showed that the TiO_2 nanoparticles were not able to enter the bacterial cell. For the Comet assay, TK6 cells were treated with 0–200 μg/mL TiO_2 nanoparticles for 24 h at 37°C. Although the TK6 cells did take up TiO_2 nanoparticles, no significant induction of DNA breakage or oxidative DNA damage was observed in treated cells using the standard alkaline Comet assay and the endonuclease III and human 8-hydroxyguanine DNA-glycosylase (hOGG1)-modified Comet assay, respectively.

Guichard et al. (2012) studied the *in vitro* cytotoxicity and genotoxicity of commercially available nanosized and microsized anatase TiO_2 and rutile TiO_2 in SHE cells. Samples had the following characteristics: anatase, 14 ± 4 nm; anatase, 160 ± 48 nm; rutile, 62 ± 24 nm; and rutile, 530 ± 216 nm. The particle concentrations in the different tests varied between 0.5 and 200 μg/cm^2. In acellular assays, TiO_2 particles were able to generate ROS. At the same mass dose, all nanoparticles produced higher levels of ROS than their microsized counterparts. Measurement of particle size in the SHE culture medium showed that primary nanoparticles and microparticles are present in the form of micrometric agglomerates of highly polydispersed size. Uptake of primary particles and agglomerates by SHE exposed for 24 h was observed for all samples. TiO_2 samples were found to be cytotoxic, anatase TiO_2 and rutile TiO_2 nanoparticles being found to induce higher cytotoxicity than their microparticle counterparts after 72 h of exposure. Over this treatment time, anatase TiO_2 nanoparticles also produced more intracellular ROS compared with the microparticles. However, similar levels of DNA damage were observed in the Comet assay after 24 h of exposure to anatase nanoparticles and microparticles. Rutile microparticles were found to induce more DNA damage than the nanoparticles. None of the samples tested showed significant induction of micronuclei formation after 24 h of exposure. In agreement with previous size-comparison studies, the authors suggested that *in vitro* cytotoxicity and genotoxicity induced by metal oxide nanoparticles are not always higher than those induced by their bulk counterparts.

Magdolenova et al. (2012) investigated the effect of dispersion on the cytotoxicity and genotoxicity of TiO_2 nanoparticles (rutile/anatase; particle size, 15–60 nm). Two protocols giving TiO_2 nanoparticle dispersions with different stability and agglomeration states were assessed: TK6 human lymphoblast cells, EUE human embryonic epithelial cells and Cos-1 monkey kidney fibroblasts were used to assess cytotoxicity (by Trypan Blue exclusion, proliferation activity and plating efficiency assays) and genotoxicity (Comet assay). DNA strand breaks were detected by the alkaline Comet assay. DNA oxidation lesions (especially 8-oxo-7,8-dihydroguanine) were measured using a modified Comet assay including incubation with the specific repair enzyme formamidopyrimidine DNA glycosylase. TiO_2 nanoparticle dispersion with large agglomerates (3-min sonication and no serum in stock solution)

induced DNA damage in all three cell lines, whereas TiO_2 nanoparticles dispersed with agglomerates < 200 nm (fetal serum in stock solution and sonication for 15 min) had no effect on the genotoxicity. An increased level of DNA oxidation lesions detected in Cos-1 and TK6 cells indicated that the leading mechanism by which TiO_2 nanoparticles trigger genotoxicity was most likely oxidative stress. The results showed that the dispersion method used could influence the results of toxicity studies. Therefore, according to the authors, at least two different dispersion procedures should be incorporated into assessment of cyto- and genotoxic effects of nanoparticles.

Demir et al. (2013a) evaluated the genotoxic activity of TiO_2 nanoparticles (anatase; spherical shape with average diameter 2.3 nm) in human peripheral blood lymphocytes and cultured human embryonic kidney (HEK293) cells by means of a modified alkaline Comet assay with/without formamidopyrimidine DNA glycosylase and endonuclease III in order to detect also oxidised DNA bases. Both human peripheral blood lymphocytes and cultured embryonic kidney cells were incubated with TiO_2 nanoparticles at concentrations of 1, 10, or 100 μg/mL. In both cell types, a significant induction in DNA damage (similar with/without endonuclease III and formamidopyrimidine DNA glycosylase) was only observed at the highest concentration of 100 μg/mL. The ionic form of TiO_2 was completely inactive.

The same author (Demir et al., 2013b) reported the results of a study with TiO_2 nanoparticles (anatase; mean diameter 2.3 nm) and microparticles in the wing somatic mutation and recombination assay in *Drosophila melanogaster*. Larvae were fed TiO_2 particles at concentrations ranging from 0.1 to 10 mM. The results obtained did not show any significant increases in the frequency of wing spots, indicating that exposure to TiO_2 nanoparticles by feeding was unable to elicit genotoxicity detectable by the wing spot assay of *D. melanogaster*.

The influence of medium composition on the physicochemical characteristics and genotoxicity of TiO_2 nanoparticles (86% anatase, 14% rutile; size 27.5 nm) was assessed in a study by Prasad et al. (2013). In this work, the influence of TiO_2 nanoparticle agglomeration, cellular interaction and cell-cycle stage on the induction of genotoxicity was evaluated in human lung epithelial cells using three different nanoparticle-treatment media: keratinocyte growth medium (KGM) plus 0.1% bovine serum albumin (KB); a synthetic broncheoalveolar lavage fluid containing phosphate-buffered saline, 0.6% bovine serum albumin and 0.001% surfactant (DM); or KGM with 10% fetal bovine serum (KF). The Comet assay showed that TiO_2 nanoparticles (10–100 μg/mL) induced similar amounts of DNA damage in all three media, independent of the amount of agglomeration, cellular interaction or cell-cycle changes. By contrast, TiO_2 nanoparticles induced micronuclei only in KF, which is the medium that facilitated the lowest amount of agglomeration, the greatest amount of nanoparticle cellular interaction, and the highest population of cells accumulating in the S phase.

Setyawati et al. (2013) investigated the potential cytotoxicity and genotoxicity of TiO_2 nanoparticles (73–85% anatase; diameter 22 nm) in the human skin fibroblast cell line (BJ). The nanoparticles were first characterised by size, morphology and surface charge, and cytotoxicity was evaluated by monitoring the proliferation of treated BJ cells. Genotoxicity was evaluated based on the induction of phosphorylation of histone H2AX, a cellular marker of DNA double-strand break recognition and repair. TiO_2 nanoparticles induced dose-dependent cytotoxicity (dose range 10–1,000 μg/mL) and genotoxicity (at both 10 and 500 μg/mL, the two doses assayed) in this test system.

Shukla et al. (2013) evaluated the genotoxicity of TiO_2 nanoparticles (anatase; size range 30–70 nm) in the human liver cell line HepG2. Treatment with TiO_2 nanoparticles induced significant ($p < 0.05$) DNA damage in Comet assay at 10 μg/mL and above, with a possible increase in oxidative (formamidopyrimidine DNA glycosylase-dependent) damage even at the lowest dose of 1 μg/mL. Increased micronucleus frequency was observed at 20 μg/mL. The genotoxicity observed was attributed by the study authors to the generation of ROS, with concomitant reduced glutathione levels and increase in lipid peroxidation. Increased expression of p53, BAX, Cyto-c, Apaf-1, caspase 9 and caspase 3, and a decreased level of Bcl-2 were also observed by immunoblotting, indicating that TiO_2-induced apoptosis occurs via the caspase-dependent pathway.

Srivastava et al. (2013) evaluated apoptosis, oxidative stress and genotoxicity induced by TiO_2 particles (< 25 nm) in the human lung cancer cell line A549. Tetrazolium bromide salt and lactate dehydrogenase release assays were used to measure cytotoxicity. Genotoxicity was evaluated by the cytokinesis block micronucleus assay and apoptosis was assessed by the formation of apoptotic bodies and altered expression of p53, p21, Bax, Bcl-2 and cleaved caspase 3. Cells exposed to TiO_2 particles (10 and 50 μg/mL) for 6–24 h showed dose-related induction of cytotoxicity, oxidative stress (as shown by increase intracellular ROS and lipid peroxidation, and decrease catalase and glutathione activity), apoptotic bodies (up to twofold) and micronuclei (up to threefold).

Tavares et al. (2014) evaluated the genotoxicity of a set of TiO_2 nanoparticles in human lymphocytes using the cytokinesis-blocked micronucleus assay. Four TiO_2 nanoparticles were assessed: NM-102 (anatase; size 28 nm), NM-103 (rutile; size 22 nm), NM-104 (rutile; size 19 nm) and NM-105 (85% anatase, 15% rutile; size 20 nm. The morphology and size of the nanoparticles were characterised using TEM, whereas the hydrodynamic particle size distributions were determined by DLS. Particles were dispersed using a standardised procedure and applied up to the limit allowed by the dispersibility in the vehicle (0.5% ethanol and bovine serum albumin in water), corresponding to a final concentration of 250 μg/mL. Additional lower doses of 125, 45, 15 and 5 μg/mL were tested. Statistical comparison of the results showed weak (two- to threefold), but significantly increased frequencies of micronuclei for NM-102 at a dose of 125 μg/mL, for NM-103 at 5 and 45 μg/mL, and for NM-104 at 15 and 45 μg/mL; no significant effect was observed for NM-105. None of the tested TiO_2 NMs induced a dose-dependent effect. Cell viability and cell-cycle progression, assessed by RI and cytokinesis-block proliferation indices were not affected by treatments. The study authors highlight as differential genotoxicity was observed for closely related NMs, indicating the need for investigating the toxic potential of each NM individually, instead of assuming a common mechanism and equal genotoxic effects for a set of similar NMs.

3.2.3.2. *In vivo*

Shelby et al. (1993), in a survey study, tested 49 chemicals in a mouse bone marrow micronucleus test via three daily exposures by intraperitoneal injection. TiO_2 (particle size not specified) was tested for its clastogenicity in an *in vivo* mouse bone marrow micronucleus test. B6C3F1 mice were administered, for three consecutive days, doses of 250, 500 and 1,000 mg TiO_2/kg bw on the first trial, and 500, 1,000 and 1,500 mg TiO_2/kg bw on the second trial. Mice were killed 24 h after the third injection. Micronuclei were analysed in bone marrow and peripheral blood erythrocytes in the first trial and in bone marrow erythrocytes in the second trial. The initial test was positive by trend analysis in the bone marrow cells at 1,000 mg TiO_2/kg bw, showing significantly elevated levels of micronuclei at this dose level. The repeat study was trend negative, as were results from scoring blood samples in the first trial. However, due to the elevated levels of micronucleated immature erythrocytes at 1,000 mg TiO_2/kg both in the peripheral blood samples and in the repeat bone marrow test, the overall results were considered positive. Trend analyses performed following decoding of slides and excluding the upper dose level from the repeat bone marrow study showed significant effects ($p = 0.002$) at 1,000 mg TiO_2/kg bw. However, although the available data showed significant increases and a linear trend, the effect is not marked and the highest mean value obtained for induction of micronuclei falls within historical range values for untreated controls, and therefore this result should be considered equivocal or of uncertain biological relevance.

In a further study, aiming to compare induction of chromosomal aberrations and micronuclei in the bone marrow of mice using 65 chemicals, Shelby and Witt (1995) also tested TiO_2 (particle size not specified). For the micronucleus test, B6C3F1 mice were administered TiO_2 for three consecutive days at doses of 250, 500 and 1,000 mg TiO_2/kg bw on the first trial, and 500, 1,000 and 1,500 mg TiO_2/kg bw on the second trial. Mice were killed 24 h after the third injection. In the bone marrow chromosomal aberration test, B6C3F1 mice were administered with TiO_2 once by intraperitoneal injection at doses of 625, 1,250 and 2,500 mg TiO_2/kg bw. Mice were killed at sampling times of 17 and 36 h. Animals received colchicine by intraperitoneal injection to accumulate cells in metaphase 2 h before sampling. For the 17 h sampling time, animals were subcutaneously implanted with 5-bromo-2′-deoxyuridine tablets (18 h before the scheduled sampling) to allow selection of first metaphase for scoring. In the first trial for the induction of micronuclei, a significant trend was obtained with the effect significantly elevated at the highest dose. In the second trial for the induction of micronuclei, effects of a similar magnitude were observed, a single-dose level group (1,000 mg TiO_2/kg) was significantly elevated, and the trend test was significant when the high-dose level group was excluded from analysis. Results on chromosomal aberrations were clearly negative at both sampling times.

The Panel noted that the data on micronuclei in bone marrow erythrocytes in the Shelby and Witt (1995) study are identical to the data presented in the earlier Shelby et al. (1993) study.

Trouiller et al. (2009) investigated the genotoxicity, oxidative DNA damage and inflammation of nano-TiO_2 in an *in vivo* study in male and female mice (C57Bl/6Jp^{un}/p^{un}). The test material was a mixture of 75% anatase and 25% rutile TiO_2 with a primary particle size of 21 nm and a mean, agglomerated, particle size of 160 nm. Groups of five male mice were dosed for 5 days with drinking water supplemented with 60, 120, 300 and 300 μg TiO_2/mL, corresponding to 0, 50, 100, 250 and 500 mg TiO_2/kg bw per day. Pregnant dams were dosed in drinking water with 500 mg TiO_2/kg bw

per day for 10 days at gestation days from 8.5 to 18.5 post coitum. In males, a marginal increase of tail moment in peripheral blood cells (~ 0.010 vs 0.013 μm as average, from the graphical representation of data), and a twofold increase in micronuclei in peripheral blood normochromatic erythrocytes, were observed in mice treated with the highest dose tested (500 mg/kg bw per day). At this dose, a slight but significant increase of oxidative DNA damage (8-hydroxy-2′-deoxyguanosine levels) was observed in the liver (~ 4.2 vs 6.4 8-OH-dG/10^6 dG), and the increased expression of proinflammatory cytokine in peripheral blood. A dose-related increase in γ-H2AX positive cells (i.e. with more than four foci) was observed at all tested doses in bone marrow. *In utero* exposure of fetuses via the mothers (five animals per group) was associated with a slight increase in large deletions in offspring (6.42 ± 1.47 vs 8.13 ± 1.70 eyespots in the offspring of control and treated mice, respectively). The authors concluded that TiO_2 nanoparticles were genotoxic and clastogenic *in vivo* in mice, possibly as a consequence of a secondary mechanism associated with inflammation and/or oxidative stress.

The Panel noted, however, that in the above study, the methods implemented had some shortcomings and that therefore their reliability was limited because:

- For the micronucleus assay, the study protocol applied is not appropriate to detect micronuclei in mature (normochromatic) erythrocytes. Micronuclei in mature erythrocytes can be used as endpoint only when the treatment period exceeds the lifespan of erythrocytes, e.g. 4 weeks or more in the mouse (OECD TG474, 2014). In this work, a far shorter treatment period was applied (5 days), with no positive control to demonstrate the efficacy of treatment. Thus, the results reported were not considered a reliable indication of a treatment-related effect.
- The alkaline Comet assay performed in peripheral blood did not include the evaluation of cytotoxicity, which is mandatory in this assay (OECD TG489, 2014). Moreover, due to the exiguity of the difference between treated and control groups, the biological significance of the effect reported should be evaluated based on the distribution of historical control values, which were not available in this study.
- The assessment of genotoxicity in developing embryos was based on method developed in-house, which has not been validated.

Overall, the Panel concluded that this study cannot be used for risk assessment.

Sycheva et al. (2011) treated CBAB6F1 male mice by oral gavage with TiO_2 particles (anatase; microsized, 160 nm; nanosized, 33 nm) at doses of 40, 200 and 1000 mg/kg bw per day, for 7 days. Genotoxic effects were analysed by Comet assay in the cells of brain, liver and bone marrow of mice treated with 40 and 200 mg/kg bw, by the micronucleus assay in bone marrow, and in cells of forestomach, colon and testis with a poly-organ karyological assay (analysis of micronuclei, nuclear protrusions, atypical nuclei, multinucleated cells, mitotic and apoptotic index) in mice treated with 40, 200 and 1,000 mg/kg bw. In Comet assays, an increase of DNA damage was reported in bone marrow cells at both tested doses (40 and 200 mg/kg bw) with both micro- and nanosized TiO_2, and in liver at 200 mg/kg bw with nanoparticles only. An increase in micronuclei was observed in the bone marrow of mice administered 1,000 mg/kg microsized TiO_2, (the highest dose tested), but not with nanoparticles. This increase of less than twofold was considered statistically significant. In the karyological assay, micro- and nanosized TiO_2 increased the mitotic index in forestomach and colon epithelia, the frequency of spermatids with two and more nuclei, and apoptosis in forestomach (only nanosized TiO_2) and testis. According to the authors, this study demonstrated that micro- and nanosized TiO_2 were genotoxic *in vivo* in mice, possibly through an indirect genotoxic mechanism associated with inflammation and/or oxidative stress because no genotoxic effect was observed at the site of direct contact with the particles (forestomach, colon).

However, the Panel noted that some of the methods implemented had some shortcomings and that, therefore, their reliability was limited:

- The micronucleus assay was performed with a limited protocol, based on the analysis of 1,000 immature erythrocytes per animal instead of the 4,000 recommended (OECD 474, 2014); moreover, the statistical analysis of the experimental results, performed by the chi-square test, is incorrect because it does not consider the animal as a statistical unit, as recommended. Finally, the biological significance of the small and not dose-related relative increase in micronucleated cells in treated animals compared with controls should be evaluated based on the distribution of historical control values, which were not available in this study.

- The 'poly-organ karyological assay' is not a validated assay for risk assessment. Moreover, the parameters evaluated, i.e. mitotic index, apoptosis and nuclear abnormalities of spermatids, are not adequate to evaluate genotoxicity.

Overall, the Panel concluded that this study cannot be used for risk assessment.

Sadiq et al. (2012) conducted *in vivo* micronucleus and Pig-A (phosphatidylinositol glycan, class A gene) mutation assays to evaluate the genotoxicity of TiO_2 nanoparticles (anatase; 10 nm) in mice. Groups of five, 6–7-week-old male B6C3F1 mice were treated intravenously for three consecutive days with 0.5, 5.0 and 50 mg TiO_2/kg bw for the two assays. Mouse blood was sampled 1 day before the treatment and on day 4, and weeks 1, 2, 4 and 6 after the beginning of the treatment. Pig-A mutant frequencies were determined at day 1 and weeks 1, 2, 4 and 6, whereas per cent micronucleated reticulocyte frequencies were measured on Day 4 by flow cytometry in 2×10^{e4} CD71-positive reticulocytes/animal. Additional animals were treated intravenously with three daily doses of 50 mg TiO_2/kg bw for the measurement of titanium levels in bone marrow 4, 24 and 48 h after the last treatment. The measurement indicated that the accumulation of nanoparticles reached a peak in the tissue 4 h after the administration, and the levels were maintained for a few days. No increase in either Pig-A mutant frequency or the frequency of per cent micronucleated reticulocytes was detected, although the per cent micronucleated reticulocytes were reduced in the treated animals on day 4 in a dose-dependent manner indicating cytotoxicity of TiO_2 nanoparticles in the bone marrow. A marked positive response was elicited in both the Pig-A and micronucleus assays by the positive control substance ethylnitrosourea. These results suggest that although TiO_2 nanoparticles can reach the mouse bone marrow inducing measurable cytotoxicity, no genotoxic effect detectable by the micronucleus or Pig-A gene mutation assays is elicited.

Xu et al. (2013) reported negative results in a bone marrow micronucleus test on ICR mice administered intravenously with TiO_2 nanoparticles (0, 140, 300, 645 and 1,387 mg/kg bw) 14 days before sacrifice (Xu et al., 2013). However, the Panel noted that the sampling time applied in this study (14 days after treatment) is not appropriate for the test method applied, and considered this study not relevant for risk assessment.

In another recent *in vivo* study (Louro et al., 2014), transgenic C57B1/6 mice harbouring a plasmid containing the bacterial *lacZ* reporter gene were exposed to TiO_2 nanoparticles (anatase; average diameter 22 nm) with two daily intravenous injections at 10 and 15 mg/kg bw. Top dose was the maximum achievable based on concentration of stable nanoparticle dispersion and the administered volume. Micronuclei in reticulocytes were scored in blood smears prepared 42 h after last treatment; gene mutations in *lacZ* and DNA strand breaks (by Comet assay) were assessed in liver and spleen 28 days after treatment. No genotoxic effect was detected, although TEM and light microscopy highlighted the accumulation of nanoparticles and a mild inflammatory response in liver at the time of sacrifice. A marked positive response was elicited in both the Pig-A and micronucleus assays by the positive control substance ethylnitrosourea.

Chen et al. (2014) administered TiO_2 nanoparticles (anatase; 75 ± 15 nm) intragastrically to Sprague–Dawley rats at 0, 10, 50 and 200 mg/kg bw every day for 30 days. DNA damage in bone marrow was evaluated by the micronucleus assay and immunofluorescence detection of histone H2AX phosphorylation. In the same study, the genotoxicity of TiO_2 nanoparticles was assessed with *in vitro* Comet and gene mutation (*hprt* locus) assays in V79 cells treated at 0, 5, 10, 20, 50 and 100 μg/mL. A significant and dose-related increase in γ-H2AX foci in bone marrow cells was observed at the end of treatment, with no concurrent increase in micronuclei in polychromatic erythrocytes (PCE), or deviation in the polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio. *In vitro*, TiO_2 nanoparticles induced a slight increase in tail moment after 24 h treatment with the highest dose, and a significant and dose-related increase of *hprt* gene mutations.

Dobrzynska et al. (2014) injected male Wistar rats intravenously with 5 mg/kg bw TiO_2 nanoparticles (anatase/rutile powder, average size 21 nm). Animals were killed either 24 h, 1 or 4 weeks later, and genotoxicity was evaluated in bone marrow cells by Comet and micronucleus assays. No genotoxicity was detected in bone marrow leukocytes by Comet assays at any sampling time. A significant (threefold) increase in micronucleated polychromatic erythrocytes stained with the conventional May–Grunwald and Giemsa stains was observed at the first sampling time (i.e. 24 h after treatment), but not at later times. However, the Panel noted that the authors also reported no increase in the number of micronuclei in bone marrow reticulocytes stained with Acridine Orange. Because both PCEs and reticulocytes represent the same cell type, i.e. immature erythrocytes detected with different staining procedures, this raises doubts about the biological significance of the positive result reported. Overall, the Panel concluded that this study cannot be used for risk assessment.

El-Ghor et al. (2014) exposed male Swiss Webster mice to nanosized TiO_2 (rutile and anatase; size 45 nm) by intraperitoneal injection once a day for 5 days at 500, 1,000 and 2,000 mg/kg bw. Animals were killed 24 h after last treatment and the genotoxic effect of treatment evaluated by the micronuclei assay in bone marrow PCEs, by Comet assays in bone marrow, brain and liver, and by the single-strand conformation polymorphisms analysis in p53 exons 5–8 (as a surrogate of gene mutation). Moreover, the oxidative stress induced by TiO_2 administration was evaluated by measuring hepatic malondialdehyde level and glutathione, superoxide dismutase, catalase and glutathione peroxidase levels. The results showed a highly significant ($p < 0.001$) and dose-dependent increase in micronuclei in PCEs and Comet parameters (tail length, % DNA and tail moment) in bone marrow, brain and liver cells, and an increased frequency of mutations in p53 exons in brain and liver of treated animals. TiO_2 treatment also resulted in significantly increased ($p < 0.001$) liver malondialdehyde and significantly decreased ($p < 0.001$) hepatic glutathione, superoxide dismutase, catalase and glutathione peroxidase. Coadministration with chlorophyllin (40 mg/kg bw per day) effectively suppressed both oxidative stress and genotoxicity biomarkers, indicating a mechanistic link between ROS generation and TiO_2-induced genotoxicity. The Panel noted that for the micronucleus test, a distinct genotoxic activity of nanosized TiO_2, even greater than the concurrent positive control cyclophosphamide at 25 mg/kg, is described in this paper. No comparable effect has been observed in any other *in vivo* micronucleus test, including those performed by intravenous administration. For the Comet assay, highly significant and dose-dependent increases in tail length, % DNA and tail moment were obtained in the absence of adequate measurements of cytotoxicity, and organ collection was performed 24 h from the last administration and not at 2–6 h as recommended by the relevant OECD Guideline No. 489, which strongly limit the reliability of the test. Furthermore, the screening of mutations in exons 5–8 of the p53 gene is not considered an actual genotoxicity test and has not received adequate validation. Overall, the Panel concluded that the reliability of this study is limited. The Panel also noted that the intraperitoneal route of administration applied in this study is not recommended by OECD guidelines, as non-physiological, and that study results obtained with this route have no relevance for oral risk assessment.

Donner et al. (2016) evaluated three pigment grades (size range 153–213 nm) and three nanoscale (size range 43–47 nm) TiO_2 particle samples (both anatase and/or rutile) in an *in vivo* micronucleus test performed in compliance with OECD Guideline No. 474 (2014) and Good laboratory Practice (GLP). The materials were administered to groups of five male and female rats once by gavage at the doses of 0, 500, 1,000 or 2,000 mg/kg bw. Concurrent control groups received water (vehicle) or cyclophosphamide (positive control). The effect of treatment on micronucleus induction in bone marrow was evaluated by analysing 20,000 peripheral blood reticulocytes by flow cytometry at ~ 48 and 72 h after treatment. No increases in the frequency of micronucleated reticulocytes, and no reduction in the ratio of reticulocytes to total erythrocytes (indicative of cytotoxicity to bone marrow) was detected in rats administered TiO_2. According to the authors, no increase in titanium content was detected by inductively coupled plasma mass spectrometry in blood and liver of rats treated with the highest dose of both nano- and pigment-grade TiO_2 (one sample of each). The Panel noted that the very low intestinal absorption of TiO_2 is consistent with the lack of systemic genotoxicity reported in this study.

Mohamed (2015) investigated the toxic and genotoxic effects of TiO_2 nanoparticles (77% rutile, 22% anatase; average size 46 nm) on the gastric mucosa of orally treated male mice. Five animals per experimental group were orally administered 0, 5, 50 or 500 mg/kg bw TiO_2 nanoparticles in distilled water for five consecutive days and killed 24 h, 1 or 2 weeks after the last treatment. No positive control group was included in the study.

The author reported that the titanium content in gastric cells (measured by inductively coupled plasma mass spectrometry) showed a dose-dependent increase and remained stable over 2 weeks. Treatments caused a remarkable local cytotoxic effect at all dose levels. The histopathological examination revealed, already at the low dosage of 5 mg/kg bw, submucosal oedema after 24 h that developed to ulcerations and mucosal necrosis after 1 and 2 weeks, respectively. The severity of the effects reported in the two other treatment groups (50 or 500 mg/kg bw) was even higher. Several indicators of oxidative stress, as well as of apoptosis (analysed by the colorimetric diphenylamine assay and by laddered DNA fragmentation assay) and DNA damage (measured by comet assay) of gastric cells were found to be increased in a dose- and time-dependent manner.

The Panel noted that the toxic findings reported in this study are clearly in conflict with the results reported by the US NCI carcinogenicity study (NCI, 1979), in which male mice receiving up to 6,500 mg TiO_2/kg bw per day (anatase; particle size not specified; purity 98%) for 103 consecutive weeks did not show at histopathological examination any alteration in a wide range of organs, including stomach.

Moreover, the Panel noted that the reported relatively high and constant concentration of TiO_2 in gastric cells is not consistent with the high turnover of gastric epithelium. Concerning the genotoxicity findings, the Panel noted that the reported DNA fragmentation was observed in conditions associated with evident cytotoxicity, and as such cannot be taken as an evidence of genotoxicity. The secondary origin of DNA damage is also supported by its relative increase with longer intervals after last treatment, which parallels the exacerbation of local toxicity. As to the other genotoxicity results, the Panel noted that the modest increase in single-strand conformation polymorphism of the p53 exons 3 and 8 cannot be taken as an evidence of mutagenicity without confirmatory sequencing data.

Overall, due to these remarkable uncertainties, the Panel concluded that this work should not be considered for risk assessment.

In addition to the above, a few *in vivo* studies were performed using inhalational or intratracheal routes of administration. The Panel noted that such studies, especially when assessing genotoxicity at site of direct contact with nanoparticles, have limited relevance for the safety assessment of oral exposure to TiO_2.

Driscoll et al. (1997) evaluated the role of pulmonary inflammation in driving mutagenesis in rat lungs after *in vivo* instillation of different particles. These included a fine anatase TiO_2 sample (180 nm median diameter, 8.8 m^2/g). Mutagenicity was studied by *hprt*-analysis of lung epithelial cells isolated from the lungs of female SPF F334 Fischer rats, 15 months after intratracheal instillation of particles at 10 or 100 mg/kg. Enhanced *hprt*-mutagenesis was observed with 100 mg/kg, the dose that also elicited persistent lung inflammation, but not with the 10 mg/kg dose. The inflammatory cells obtained by broncheoalveolar lavage from the particle-treated animals were found to induce *hprt*-mutagenesis in a rat lung epithelia cell line *in vitro*.

Rehn et al. (2003) also investigated oxidative DNA damage induction by two samples of TiO_2 in rat lungs after intratracheal instillation at dosages of 0, 0.15, 0.3, 0.6 and 1.2 mg/kg bw per day. The samples used were an untreated TiO_2 and a trimethoxyoctylsilane-treated TiO_2 sample, both ~ 20 nm. Oxidative damage induction was determined after 90 days by immunohistochemical analysis of lung sections using an 8-oxoguanine antibody. Enhanced oxidative DNA damage was not observed with the untreated or silanised TiO_2 nanoparticles. Analysis of markers of pulmonary inflammation and toxicity at 3, 21 and 90 days indicated only mild inflammatory effects.

Lindberg et al. (2012) examined whether inhalation of freshly generated nanosized TiO_2 (74% anatase, 26% brookite; 5 days, 4 h/day) at 0.8, 7.2 and 28.5 mg/m^3 (the highest concentration allowing stable aerosol production) could induce genotoxic effects in C57BL/6J mice locally in the lungs or systematically in peripheral PCEs. DNA damage was assessed by the Comet assay in lung epithelial alveolar type II and Clara cells sampled immediately following the exposure. Micronuclei were analysed by Acridine Orange staining in blood PCEs collected 48 h after the last exposure. A dose-dependent deposition of titanium in lung tissue was seen. Although the highest exposure level produced a clear increase in neutrophils in BAL fluid, indicating an inflammatory effect, no significant effect on the level of DNA damage in lung epithelial cells or micronuclei in PCEs was observed, suggesting no genotoxic effects by the 5-day inhalation exposure to nanosized TiO_2 anatase.

In the work by Saber et al. (2012), DNA-damaging activity and inflammogenicity (pulmonary cell composition and mRNAs) were determined in mice 24 h after intratracheal instillation of a single dose (54 μg) of three TiO_2-based particles (two coated rutile, size 288 and 20 nm; one uncoated anatase; size 12 nm). The coated TiO_2 induced DNA damage, as detected by Comet assay, in lung lining fluid cells. The uncoated TiO_2 was not DNA damaging by the same assay 24 h after exposure despite being highly inflammogenic, suggesting that inflammation is not a prerequisite for the induction of DNA damage in lung cells by TiO_2-based products.

Naya et al. (2012) evaluated the *in vivo* genotoxicity of anatase TiO_2 nanoparticles using the Comet assay after a single or repeated intratracheal instillation in Sprague–Dawley rats. The nanoparticles were instilled at a dosage of 1 or 5 mg/kg bw (single instillation group) and 0.2 or 1 mg/kg bw once a week for 5 weeks (repeated instillation group). Macrophages and neutrophils were detected at sacrifice in the alveolus of the lung in the 1 and 5 mg/kg TiO_2 groups. In the Comet assay, there was no increase in % tail DNA in any of the TiO_2 groups.

Summary of genotoxicity data

In summary, numerous genotoxicity studies with TiO_2 particles of different specifications are available in the literature. The overall results obtained with particles of different size can be summarised as follows:

Microsized TiO_2 particles – in vitro *and* in vivo

A set of *in vitro* and *in vivo* studies, coordinated by the NTP, was performed with a TiO_2 anatase (Unitane® 0-220) with undefined particle size distribution. This material was not genotoxic in gene mutation tests in bacteria and in mammalian cells, in cytogenetic assays *in vitro* (chromosomal aberrations and SCE) (Dunkel et al., 1985; Tennant et al., 1987; Ivett et al., 1989; Myhr and Caspary, 1991) and *in vivo* (micronuclei and chromosomal aberrations in mouse bone marrow by intraperitoneal) (Shelby et al., 1993; Shelby and Witt, 1995). The Panel noted that the same material was non-carcinogenic in the NCI mouse and rat bioassays.

Microsized TiO_2, with a defined size > 100 nm or designed as 'fine rutile or anatase' also produced mixed results in genotoxicity tests *in vitro*: negative in Comet assays in CHL cells (both anatase and rutile, 255 nm, Nakagawa et al., 1997), chromosomal aberrations in CHO cells (anatase; 140 nm, Warheit et al., 2007), micronuclei in SHE cells and in human bronchial epithelial (BEAS-2B) cells (fine particles, Rahman et al., 2002; Falck et al., 2009), micronuclei and H2AX phosphorylation in human lung adenocarcinoma A549 cells (anatase, 140 nm, Jugan et al., 2012).

Conversely, positive results were reported by other authors in Comet assays with A549 cells (anatase, 140 nm, Jugan et al., 2012; fine TiO_2, 1 μm size, Karlsson,2009), BEAS-2B cells (fine rutile; Falck et al., 2009), SHE cells (anatase 160 nm and rutile 530 nm, Guichard et al., 2012), and for H2AX phosphorylation in A549 cells (anatase, 5 μm, Toyooka et al., 2012).

Nanosized TiO_2 particles – in vitro

Both positive and negative results have been reported in the numerous *in vitro* investigations on the genotoxicity of TiO_2 nanoparticles in a variety of experimental systems. As for microsized TiO_2, the crystalline phase and nanoparticle size do not seem to be important determinants of TiO_2 genotoxicity in experimental systems.

Anatase nanoparticles (with various diameters) were tested with negative results in Comet assays in rodent (Nakagawa et al., 1997; Wang et al., 2011) and human cells (Bhattacharya et al., 2009; Hackenberg et al., 2011; Jugan et al., 2012; Vales et al., 2015), gene mutation in rodent cells (Nakagawa et al., 1997; Wang et al., 2011), and micronuclei in rodent (Guichard et al., 2012) and human cells (Jugan et al., 2012; Vales et al., 2015).

However, positive results have been reported from a number of other studies covering also similar genetic endpoints, i.e. Comet assays in various cell types (Falck et al., 2009; Shukla et al., 2011, 2013; Guichard et al., 2012; Jugan et al., 2012; Magdolenova et al., 2012; Demir et al., 2013a; Prasad et al., 2013), micronucleus induction (Falck et al., 2009; Shukla et al., 2011, 2013; Prasad et al., 2013; Tavares et al., 2014) and H2AX phosphorylation (Toyooka et al., 2012; Setyawati et al., 2013).

A similar picture can be drawn for rutile nanoparticles, for which, however, fewer studies are available: negative in micronuclei tests in rodent (Landsiedel et al., 2010; Guichard et al., 2012) and human cells (Falck et al., 2009; Jugan et al., 2012), and in the γH2AX assay in A549 cells (Jugan et al., 2012); positive in Comet assays in rodent (Falck et al., 2009; Guichard et al., 2012) and human cells (Jugan et al., 2012), and in a micronuclei test with human lymphocytes (Tavares et al., 2014).

Additional positive results have been reported from studies with nanosized TiO_2 particles in an undefined crystalline phase. These consist of *in vitro* Comet, micronuclei, SCE and *hprt* assays with various cell lines (Rahman et al., 2002; Wang et al., 2007a; Karlsson et al., 2009; Di Virgilio et al., 2010; Osman et al., 2010; Magdolenova et al., 2012; Prasad et al., 2013; Srivastava et al., 2013).

Overall, the Panel noted that variable results have been obtained in genotoxicity tests *in vitro* with both nano- and microsized TiO_2. The observed discrepancies cannot be explained based on the crystalline phase or size of tested material, or on the specificity of the endpoint of the test system, but are more likely to be related to the variable experimental conditions applied, which greatly affect the aggregation status, availability and ensuing biological activity of particles (see Magdolenova et al., 2012).

Nanosized TiO_2 particles – in vivo

Fewer *in vivo* studies are available, with mixed results. Some evidence of genotoxicity in liver and bone marrow was reported following oral administration of both nano- and microsized TiO_2 particles (Trouiller et al., 2009; Sycheva et al., 2011). The Panel, however, noted a series of shortcomings in these studies, which cast doubts on the reliability of these results.

In another oral *in vivo* study, the intragastric administration of TiO_2 nanoparticles for 30 days to rats resulted in an increase in H2AX phosphorylated loci in bone marrow (an indication of double-strand break DNA repair), with no concurrent increase of chromosome breaks (micronuclei) (Chen et al., 2014).

Negative results were also obtained in a micronucleus assay on rat blood cells after administration by gavage of acute doses of both nano- and microsized TiO_2 particles (Donner et al., 2016).

Other *in vivo* studies have used other routes of exposure. Negative results in gene and chromosomal mutation tests were obtained in rats injected intravenously (Sadiq et al., 2012; Louro et al., 2014). A mild increase in micronuclei in bone marrow, with no concurrent DNA damage detectable by Comet assay, was reported in another recent intravenous study (Dobrzynska et al., 2014), but the Panel noted some inconsistencies in these results which are regarded of questionable biological significance.

Recently, the repeated intraperitoneal administration of TiO_2 nanoparticles has been reported to induce oxidative stress and genotoxicity in mice. The Panel noted that these results are not corroborated by any other *in vivo* study, by intraperitoneal or other routes, and concluded that these results should be considered with caution.

Unspecified particles size

Another commercial TiO_2, with unspecified particle size distribution, provided variable results in cytogenetic assays *in vitro*: positive in the micronucleus assay in human lymphocytes (Türkez and Geyikoğlu, 2007), either negative (Miller et al., 1995) or positive (Lu et al., 1998) in the micronuclei test in CHO cells, and positive in the SCE assay in CHO cells (Lu et al., 1998).

Conclusion on genotoxicity

The Panel concluded that the available mixed results provide some evidence of *in vitro* genotoxicity for TiO_2 micro- and nanoparticles. The Panel noted that most positive results have been reported under experimental conditions associated with the induction of oxidative stress (as shown by increased 8-OH-dG, lipid peroxidation and ROS generation), and that the genotoxic effects observed mainly concern indicator assays (comet and H2AX histone phosphorylation), which in some studies were shown not to be associated with permanent chromosome damage such as chromosome breaks visualised as micronuclei (Falck et al., 2009; Jugan et al., 2012).) In this respect, the Panel noted that the reliability of Comet assay for evaluating nanoparticle-induced genotoxicity has been questioned because of the possible secondary induction of DNA damage by nanoparticles during sample processing (Karlsson et al., 2015). Indeed, comparing the results obtained in intact cells and isolated nuclei, Ferraro et al. (2016) recently demonstrated that most DNA damage elicited by TiO_2 nanoparticles in human epithelial cells was produced during the assay performance (*ex post* damage) rather than during treatment (*ex ante* damage), through the direct interaction of cytoplasm-internalised nanoparticles with DNA in nucleoids.

In vivo, overall negative results have been obtained in genotoxicity studies with microsized TiO_2 pigment. Limited evidence of genotoxicity, if any, is provided by studies with orally administered TiO_2 nanoparticles. Limited or no indication of the genotoxicity of TiO_2 nanoparticles is provided by studies using an intravenous route of administration, which allows maximum exposure of target tissues.

Overall, the Panel concluded that the use of TiO_2 (E 171) as a food additive does not raise a concern with respect to genotoxicity.

3.2.4. Chronic toxicity and carcinogenicity

JECFA (1970) evaluation on TiO_2 reported a study by Lehmann and Herget (1927) in which two guinea pigs, two rabbits, two cats and one dog were fed technical-grade TiO_2 (assay of $\geq$ 99%) for 390 days. From the diets, the dog received 9 g/day (equivalent to 900 mg TiO_2/kg bw per day),[16] the rabbits received a total amount of 1170 g (equivalent to 1.5 g/kg bw per day),[16] the cats received 3 g/day (equivalent to 1.5 g TiO_2/kg bw per day)[16] and the guinea pigs received 0.6 g/day (equivalent to 800 mg/kg bw per day).[16] Two additional cats received 3 g TiO_2 daily for 175 and 300 days, respectively. No adverse effects were seen and histopathological examination revealed no abnormality. Less than 5 mg of titanium was detected in the bile, heart, spleen and skeletal muscle (no further information was available).

The US NCI (NCI, 1979) conducted a carcinogenicity study in groups of both Fischer 344 rats and B6C3F1 mice (50 animals/sex). These studies are summarised below.

3.2.4.1. Mice

Groups of B6C3F1 mice (50 animals/sex) were administered, in the diet, TiO_2 (anatase; particle size not specified, purity 98%) at doses of 0, 25,000 and 50,000 mg/kg diet (equivalent to 0, 3,250,

6,500 mg TiO_2/kg bw per day and 0, 4,175, 8,350 mg TiO_2/kg bw per day for male and female mice, respectively).[16] The study was conducted for 103 consecutive weeks and animals then observed for an additional week. All surviving animals were killed at week 104. A full histopathological evaluation was done and the following tissues were examined microscopically: brain (frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons), pituitary, spinal cord (if neurological signs were present), eyes (if grossly abnormal), oesophagus, trachea, salivary glands, mandibular lymph node, thyroid, parathyroid, heart, thymus, lungs and main stem bronchi, liver, gallbladder, pancreas, spleen, kidney, adrenal, stomach, small intestine, colon, urinary bladder, prostate or uterus, testes or ovaries, sternebrae, femur, or vertebrae including marrow, mammary gland, tissue masses, and any gross lesion. At the end of the study, the test compound had not affected the survival rates of male mice; 80% of the high-dose males survived until the end of the 104-week study, compared with 64% survival in the controls. In female mice, there was a statistically significant dose-related trend for decreased survival ($p = 0.001$, Tarone test). It was reported that in female mice fed 50,000 mg TiO_2/kg diet (equivalent to 8,350 mg TiO_2/kg bw per day),[16] 66% survival was reported until the end of the 104-week study, in comparison with 90% survival in the controls. There was a slight increase in the incidence of hepatocellular carcinomas in high-dose male mice compared with controls, but this was not increased compared with historical control data. Tumour incidences in the dosed groups were not significantly higher than in controls. The study authors concluded that TiO_2 administered orally was not carcinogenic in B6C3F1 mice.

From this study, the Panel identified a NOAEL of 50,000 mg/kg diet, equivalent to 6,500 and 8,350 mg TiO_2/kg bw per day, for male and female mice, respectively, the highest doses tested.

3.2.4.2. Rats

Groups of Fischer 344 rats (50 animals/sex) were administered in the diet TiO_2 (anatase; particle size not specified, purity 98%) at doses of 0, 25,000 and 50,000 mg/kg diet (equivalent to 0, 1,125, 2,250 mg/kg bw per day and 0, 1,450, 2,900 mg/kg bw per day for male and female rats, respectively).[16] The study was conducted for 103 consecutive weeks and the animals were then observed for an additional week. All surviving animals were killed at week 104. A full histopathological evaluation was done and the following tissues were examined microscopically: brain (frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons), pituitary, spinal cord (if neurological signs were present), eyes (if grossly abnormal), oesophagus, trachea, salivary glands, mandibular lymph node, thyroid, parathyroid, heart, thymus, lungs and main stem bronchi, liver, pancreas, spleen, kidney, adrenal, stomach, small intestine, colon, urinary bladder, prostate or uterus, testes or ovaries, sternebrae, femur, or vertebrae including marrow, mammary gland, tissue masses, and any gross lesion. At the end of the study, the test compound had not affected survival rates of male and female rats. Tumour incidences in the dosed groups were not significantly higher than in controls. The study authors concluded that TiO_2 administered orally was not carcinogenic in Fischer 344 rats.

From this study, the Panel identified a NOAEL of 50,000 mg/kg diet, equivalent to 2,250 and 2,900 mg TiO_2/kg bw per day, for male and female rats, respectively, the highest doses tested.

The US National Sanitation Foundation (NSF) International (2005) evaluated non-cancer oral toxicity data for TiO_2, and calculated an oral reference dose of 3 mg/kg per day based on the NCI study (1979) reported above, in which no adverse effects were observed in Fischer 344 rats or B6C3F1 mice fed TiO_2 for 2 years at concentrations up to 50,000 mg/kg. US NSF International applied a composite uncertainty factor of 1,000 (10 each for inter- and intraspecies extrapolation and for database deficiencies) to a NOAEL of 2,680 mg/kg bw per day in rats.

The IARC Monograph (IARC, 2010) concluded that: 'there was inadequate evidence from epidemiological studies to assess whether titanium dioxide causes cancer in humans', but that 'there is sufficient evidence in experimental animals for the carcinogenicity of titanium dioxide' and overall concluded that 'titanium dioxide is possibly carcinogenic to humans (Group 2B)'. However, this conclusion was based on an excess incidence of lung tumours in male and female rats in inhalation studies (Lee et al., 1985a,b, 1986; Trochimowicz et al., 1988; Heinrich et al., 1995; as cited in IARC, 2010). However, the same report noted that in other studies using different routes of administration, like oral, no excesses in tumour incidence were observed (IARC, 2010).

The Panel noted that there was one carcinogenicity study in rats and one in mice (NCI, 1979), performed with TiO_2 administered via the oral route, and that the outcome of this study was reported to be negative for both mice and rats. These negative findings are supported by negative results from earlier studies reported in the JECFA (1970) evaluation in which similar doses were tested in various animal species but for a shorter duration (~ 56 weeks).

Initiation and promotion studies

In a recent study by Urrutia-Ortega et al. (2016), the authors investigated the effects of intragastric administration of TiO_2 (E 171) in a chemically colitis-associated colorectal cancer (CAC) model in mice. Balb/c male mice (n = 24) were divided in the following 4 groups: (a) control; (b) 5 mg/kg bw food grade TiO_2 (E 171; 99% pure) by gavage, 5 days/week for 10 weeks; (c) the chemically colitis-associated cancer (CAC) group received a single i.p. dose of 12.5 mg/kg bw azoxymethane (AOM) and 2% dextran sulfate sodium (DSS) in the third, sixth and ninth week in water *ad libitum*; (d) the CAC + TiO_2 (E 171) group: AOM, DSS and TiO_2 (E 171). After 11 weeks, mice were necropsied and colon, kidneys, liver, spleen and lungs were collected. TiO_2 (E 171) in combination with the initiator increased the expression of markers of tumour progression including COX2, Ki67 and β-catenin. TiO_2 (E 171) alone did not show any enhancing effect on tumour markers. The Panel noted that further research is needed and that the study cannot be used for risk assessment of TiO_2 (E 171) as a food additive.

3.2.5. Reproductive and developmental toxicity

3.2.5.1. Reproduction toxicity studies

No reproductive (one- or two-generation toxicity) studies with TiO_2 (as the food additive, micro- or nanosized) performed according to the OECD guidelines were available for evaluation.

Jia et al. (2014) studied the effects of TiO_2 (crystal anatase; size 25 nm) in mice. Four-week-old male mice (n = 15/group) were daily administered by gavage with vehicle (phosphate-buffered saline with 0.5% Tween 80), or nano-TiO_2 solution at a dose of 10, 50 or 250 mg/kg bw for 42 days. There was a decrease in body weight gain in the 250 mg/kg bw group (only presented in a graph, body weight values not presented). Sperm abnormalities were increased in the mid- and high-dose groups (mean ~ 21 and 29 vs 13 in the control group). However, it should be noted that the number of abnormalities in the control group was also high. The figures were given for between six and nine animals. No differences in sperm counts were observed. Mean serum testosterone was decreased in all treated groups. The figures were given for between five and seven animals. Testes from the control and the 10 mg/kg groups showed no histopathological changes. Vacuoles were observed in the seminiferous tubules of mice treated with 50 and 250 mg TiO_2/kg bw per day. In the high-dose group, decreased layers of spermatogenic cells were observed. Two randomly selected animals per group were used for this examination and the number of abnormalities was not presented. Real-time quantitative polymerase chain reaction analysis (n = 3) and western blot analysis (n = 4–5) showed differences in the testis messenger RNA expression levels and protein expression levels of the 50 and/or 250 mg/kg bw groups. The results showed downregulation of CYP17 and 17βHSD and upregulation of CYP19 both in gene and protein expression, which may explain the found decreased testosterone levels (Jia et al., 2014).

The results of this study (Jia et al., 2014) pointed to an effect of nanosized TiO_2 on the reproductive system. However, it is not known whether the indicated effects are induced by the nanoparticles themselves or to the TiO_2. In addition, contradictory results on testosterone levels were reported by Tassinari et al. (2014) as described below. The Panel noted that, further research is needed and, this study cannot be used for risk assessment of TiO_2 (E 171) as a food additive.

Tassinari et al. (2014) (described in Section 3.1.2) investigated the possible reproductive and endocrine effects of short-term (5 days) oral exposure to anatase TiO_2 particles (0, 1, and 2 mg/kg bw per day) in Sprague–Dawley rats (n = 7/sex per group). Particles were characterised by SEM and TEM (average particle diameter 284 ± 43 nm, with 10% particles < 100 nm, 48% of particles between 100 and 300 nm, and 87% of particles between 30 and 900 nm). Most of the particles were agglomerates up to 1.6 μm in diameter. TEM analysis showed two different shapes for primary nanoparticles: spherules of 20–60 nm and irregular shapes of 40–60 nm. Their presence in spleen, a target organ for bioaccumulation, was investigated using single-particle inductively coupled plasma mass spectrometry and SEM/energy-dispersive X-ray analysis. Analyses included serum hormone levels (testosterone, 17β-oestradiol and triiodothyronine) and histopathology of thyroid, adrenals, ovary, uterus, testis and spleen. In addition, the spleen was examined by electron microscopy (SEM/energy-dispersive X-ray analysis) for the deposition of TiO_2 nanoparticles. In males from the 2 mg/kg bw per day group, feed intake was significantly decreased. Increased total titanium tissue levels were found in spleen and ovaries. Sex-related histological alterations were observed at both dose levels (i.e. 1 and 2 mg/kg bw per day) in thyroid, adrenal medulla, adrenal cortex (females) and ovarian granulosae, without general

toxicity. Altered thyroid function was indicated by reduced triiodothyronine (T3) (males). Testosterone levels increased in high-dose males and decreased in females. Estradiol levels were not affected by treatment. In the spleen of treated animals, TiO_2 aggregates and increased white pulp (high-dose females) were detected, even though titanium levels in tissue remained low, reflecting the low doses and short exposure time. The authors suggested that their results should prompt a comprehensive assessment of endocrine and reproductive effects of nanomaterials. The Panel agreed that further research is necessary preferentially following OECD guidelines considering the low levels of exposure (1 and 2 mg/kg bw/day) at which effects were reported in this study.

3.2.5.2. Developmental toxicity studies

Mohammadipour et al. (2014) exposed pregnant Wistar rats (n = 6) by gavage to 0 or 100 mg TiO_2 nanoparticles (particle size 10 nm, area > 150 m^2/g, purity 99%, suspended in distilled water) from gestation day 2 to gestation day 21. On post-natal day 1, pups were killed and brains were collected. The titanium content in the hippocampus of the pups in the test group was increased. In addition, reduced cell proliferation was observed in the hippocampus. On post-natal day 60, learning and memory was tested in 12 male pups per group and was found to be impaired in the test group. Although the results of the study point to effects on hippocampus and learning and memory, the Panel noted the limitations of the study such as small group size (only six females per group were used), only one dose level tested, and no information on the (random) selection of the pups. Therefore, according to the Panel, further research is needed before the results of this study can be used for risk assessment.

Warheit et al. (2015a) evaluated three pigment-grade (pg-1, pg-2 and pg-3) and three ultrafine (uf-1, uf-2 and uf-3)/nanoscale (anatase and/or rutile) TiO_2 particulates in prenatal developmental toxicity studies in pregnant rats, according to OECD TG 414. All six test particles contained > 95 wt % TiO_2. Primary particle sizes and surface were characterised as follows: pg-1, pg-2, pg-3 (d_{50} = 153–213 nm and Brunauer–Emmett–Teller = 50–82 m^2/g) and uf-1, uf-2, uf-3 (d_{50} = 43–47 nm and Brunauer–Emmett–Teller = 7–17 m^2/g). The test substances were formulated in sterile water. In three studies, time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n = 22/group) were exposed to TiO_2 particulates (uf-1, uf-3 and pg-1) by oral gavage daily on gestation days 6–20. In three additional studies, pregnant Wistar rats (n = 22–23/group) were exposed to TiO_2 particulates (uf-2, pg-2 and pg-3) by oral gavage daily from gestation days 5–19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day. Clinical signs were recorded at least daily. Body weight and feed intake were measured at regular intervals. Sprague–Dawley rats were killed for a caesarean section on gestation day 21 and Wistar rats on gestation day 20. Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to detect abnormalities. At 1,000 mg uf-1/kg per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. The mean number of male fetuses was 7.2 compared with 5.5 male fetuses for the concurrent control group; the test facility historical control group data ranges from 5.2 to 7.4. The mean number of female fetuses was 4.8 compared with 6.7 for the concurrent control group; the test facility historical control group data ranges from 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mg uf-1/kg bw per day group was 60% (males/females) compared with a sex ratio of 46% in the concurrent control group; the test facility historical control group data ranges from 43% to 53%. Apart from some incidental changes in body weight and feed intake, no other changes were observed in the dams or the fetuses in these studies. The authors concluded that there were no significant toxicological or developmental effects in females or fetuses at any of the dose levels or compounds tested, and considered the NOAEL for each compound to be 1,000 mg/kg bw per day. The Panel agreed with this conclusion.

Overall, the Panel noted that prenatal developmental studies with three pigment-grade (pg-1, pg-2 and pg-3) and three ultrafine (uf-1, uf-2 and uf-3)/nanoscale (anatase and/or rutile) TiO_2 particulates performed according to the OECD guidelines (TG 414) did not give concern for maternal or developmental toxicity up to the highest dose tested (1,000 mg/kg bw per day). However, the Panel noted that reproductive toxicity studies performed according to the OECD guidelines using TiO_2, meeting the food additive specifications were not available. Furthermore, the Panel noted that results from other reproductive and developmental studies with titanium nanoparticles (Jia et al., 2014 and Tassinari et al., 2014) showed contradictory results in the change in hormone levels. Because of

deficiencies in the study designs and inadequate data reporting, the Panel considered that the relevance of these findings is currently uncertain for the risk assessment of TiO_2 as a food additive.

3.2.6. Hypersensitivity, allergenicity, intolerance

Numerous studies are available on the effects of TiO_2 nanoparticles on the immune system. Some have been reviewed recently (Smith et al., 2014; Lappas, 2015; Luo et al., 2015).

3.2.6.1. Immunotoxicity

In vitro

Nuuja et al. (1982) investigated the effects of six different TiO_2 pigments (particle sizes not given) on the phagocytic capacity of mouse peritoneal macrophages. Male NMRI mice (4–6 weeks old) were given a single intraperitoneal injection TiO_2 (called TiO_2 pigments by the authors) in 1 mL of 0.9% aqueous NaCl solution. Compared with controls, the phagocytotic activity of mouse peritoneal cells treated with TiO_2 (98%) was reported to increase by < 10% within 2 days after intraperitoneal administration, but in a second set of experiments, the increase was up to 30% at days 7 and 15.

Kumazawa et al. (2002) studied the effect of soluble and particulate titanium (particle sizes 1–3 and 10 μm, 99.9% pure) on the function, morphology and cytotoxicity of human neutrophils. Neutrophils were mixed with titanium in Hanks' balanced salt solution (2 and 10 mg/kg) and incubated at 37°C for 30 min. Compared with the control (Hanks' balanced salt solution), there was no effect of titanium particles on cell survival (2 and 10 mg titanium/kg) or lactate dehydrogenase release (10 mg titanium/kg), but there was a significant effect of 2 mg titanium/kg (1–3 μm particle size) on superoxide anion production ($p < 0.05$), and an effect on tumour necrosis factor (TNF)-α production (1–3 μm particle size). In addition, 1–3 μm titanium particles were inserted subcutaneously into the abdominal cavity of Wistar rats aged between 11 and 12 weeks. The rats were killed 8 weeks later and the tissue section was found to contain phagocytised titanium particles and numerous inflammatory cells. The authors concluded that the increase in inflammatory cells was probably due to the increased productions of superoxide anion and TNF-α production in the presence of titanium.

Kang et al. (2008) investigated the effects of fine (primary particle size 1,000 nm) and ultrafine (primary particle size 21 nm) TiO_2 particles on ROS generation and pro-inflammatory cellular cascades. Fine and ultrafine TiO_2 particles incubated with a mouse peritoneal macrophage cell line (RAW 264.7) for 24 h, at concentrations in the range of 0.5–200 μg/mL did not significantly affect cell viability, as measured by lactate dehydrogenase activity leakage. ROS generation was greater for ultrafine than fine TiO_2 particles at all concentrations tested in the range of 0.5–100 μg/mL at 4 h of incubation. At 24 h of incubation, ROS levels varied less with respect to particle size and were falling to control levels. Compared with controls, only ultrafine TiO_2 particles (0.5 μg/mL for 20 min) induced extracellular signal-regulated kinase-1/2 phosphorylation in a concentration-dependent manner in RAW 264.7 cells, whereas fine TiO_2 induced only minimal changes. Ultrafine TiO_2 (0.5–200 μg/mL) significantly increased TNF-α and macrophage inflammatory protein-2 (MIP-2) secretions in a concentration-dependent manner, compared with control, with peak responses at 200 μg/mL; 6.6-fold TNF-α and 5.8-fold MIP-2. The authors concluded that the effects of fine particles on increases in TNF-α and MIP-2 secretions were less pronounced at each concentration tested with peak responses at 200 μg/mL; 1.4-fold TNF-α and 3.1-fold MIP-2.

Morishige et al. (2010) investigated the effect of anatase and rutile TiO_2 particles of different sizes (anatase: 10 to < 50,000 nm; rutile: 40 to < 5,000 nm) on interleukin-1β (IL-1β) production in macrophage-like human THP-1 cells (acute monocytic leukaemia cell line). Differentiated cells were stimulated with 20, 100 or 500 μg TiO_2/mL for 24 h in the presence or absence of lipopolysaccharide as a THP-1 cell activator. At all concentrations, rutile TiO_2 induced greater IL-1β production than anatase TiO_2. Smaller anatase (compared with larger anatase particles) and larger rutile particles (compared with smaller rutile particles) provoked greater IL-1β production in differentiated THP-1 cells exposed for 6 h at all concentrations. At 20 and 100 μg/mL, spicula (needle-shaped) rutile particles also induced greater IL-1β production than similarly sized and structurally identical, but spherical rutile particles.

Becker et al. (2012) reported that following incubation with TiO_2 nanoparticles, macrophage-like cells readily take up TiO_2 after 6 h, and particles were also found intracellularly in intestinal cells. Incubation of cells with TiO_2 resulted in secretion of IL-1β and IL-8. According to the authors, this may aggravate inflammation in susceptible individuals.

Mice

Larsen et al. (2009) reported that nanosized TiO_2 may have an adjuvant effect after intraperitoneal injection into mice together with ovalbumin.

In mice receiving an intratracheal instillation of 0.5–50 mg/kg of TiO_2 nanoparticles, the levels of the proinflammatory cytokines, IL-1, TNF-α and IL-6, were significantly elevated in a dose-dependent manner 24 h after administration, and remained elevated for up to 14 days. Levels of the TH1 cytokines, IL-12 and interferon-gamma, and the TH2 cytokines, IL-4, IL-5 and IL-10, were also elevated dose dependently at day 1 and remain elevated for up to 14 days after instillation. Increased numbers of B lymphocytes were observed in both spleen and in blood, as well as increased immunoglobulin E production in BAL fluid and serum (Park et al., 2009).

In mice administered via intragastric gavage, TiO_2 nanoparticles caused congestion and proliferation of spleen tissue, with accompanying increases ROS in spleen tissue. The elevated ROS levels in spleens led to lipid peroxidation and upregulation of haem oxygenase expression, suggesting that TiO_2 nanoparticle accumulation in lymphoid organs may exert cytotoxic effects through the induction of oxidative stress (Wang et al., 2011).

Administration of 2.5, 5 or 10 mg/kg bw per day TiO_2 nanoparticles to mice via gavage for 6 months resulted in an accumulation of titanium in the liver and accompanying reductions in body weight, increases in liver damage indices, liver dysfunction, infiltration of inflammatory cells, and hepatocyte apoptosis and necrosis. Additionally, hepatic inflammation was increased, as measured by the upregulation of IL-4, IL-5, IL-12, interferon-gamma, GATA3, GATA4, T-bet, RORgt, STAT3, STAT6, eotaxin, MCP-1 and MIP-2. This indicated that prolonged exposure to TiO_2 nanoparticles may affect the cells and tissues of the lymphoid system, as well as peripheral organs including the liver, in which nanoparticle accumulation results in hepatic inflammation and toxicity (Hong et al., 2014).

Auttachoat et al. (2014) reported that after 28 days of oral gavage, TiO_2 nanoparticles (1.25–250 mg/kg in 0.5% methylcellulose) had no significant effects on innate, humoral or cell-mediated immune functions in female B6C3F1 mice. There were no effects on the weights of selected organs (spleen, thymus, liver, lung and kidneys). Following dermal exposure on the ears for 3 days, TiO_2 nanoparticles (2.5–10% w/v in 4:1 acetone/olive oil) did not affect auricular lymph node cell proliferation. Dermal sensitisation (2.5–10%) on the back and subsequent challenge (10%) on the right ear with TiO_2 nanoparticles produced no significant effects on percentage ear swelling in the mouse ear-swelling test. However, when TiO_2 nanoparticles were injected subcutaneously along the midline on top of the head at 125–250 mg/kg (in 0.5% methylcellulose), significant increases in auricular lymph node cell proliferation resulted. The authors concluded that immune effects of TiO_2 nanoparticle exposure are dependent on the route of exposure, and that hypersensitivity responses may occur following parenteral exposure or dermal administration of TiO_2 nanoparticles to compromised skin.

Rat studies

TiO_2 nanoparticles were shown to accumulate in the spleen of Sprague–Dawley rats after intravenous administration (5 mg/kg bw), with levels peaking at 24 h and decreasing slightly by days 14 and 28 (Fabian et al., 2008). The Panel noted that the dose injected was very high.

In the study by Olmedo et al. (2008), male Wistar rats were injected intraperitoneally with a suspension of TiO_2 rutile powder at the dose of 1.60 g/100 g bw. After 6 months, the presence of titanium was assessed in serum, blood cells, liver, spleen and lung. Titanium was found in phagocytic mononuclear cells, serum and in the parenchyma of all the organs tested. According to the authors, TiO_2-rutile generated an increase in the percentage of reactive cells, which was smaller than that previously reported with TiO_2-anatase, suggesting that TiO_2-rutile is less reactive than TiO_2-anatase. The Panel noted that both the very high dose injected and the route of injection were not representative of the use of TiO_2 as a food additive.

As reported by Liu et al. (2010), 42 rats were instilled intratracheally with 0.5, 5 or 50 mg/kg bw of nano- (NP-1) and microsized (F-1) TiO_2 particles with a median size of 5 and 200 nm, respectively. Exposure to NP-1 TiO_2 decreased the chemotactic ability of the macrophages and the expression of Fc receptors and major histocompatibility complex class II on their surface. According to the authors, the mechanism responsible for these changes was mediated via altering nitric oxide (NO) and TNF-α expression by the porcine alveolar macrophages (PAMs). The amount of nitric oxide and TNF-α secreted by macrophages gradually increased as the dose of TiO_2 nanoparticles increased. Contrary to the 200 nm TiO_2 particles, 50 nm TiO_2 nanoparticles elicited strong nitric oxide and TNF-α production.

Sprague–Dawley rats were instilled intratracheally with TiO_2 nanoparticles (21 nm) at doses of 0.5, 4 and 32 mg/kg bw, or 32 mg/kg bw TiO_2 microparticles (1–2 μm) twice a week, for four consecutive weeks. Immune function response was characterised by increased proliferation of T cells and B cells following mitogen stimulation and enhanced natural killer cell killing activity in spleen, accompanying by an increased number of B cells in blood. No significant changes of Th1-type cytokines (IL-2 and interferon-gamma) and Th2-type cytokines (TNF-α and IL-6) were observed (Fu et al., 2014).

The Panel noted that in most of these studies, the administered doses used were very high.

3.2.6.2. Hypersensitivity

Humans

The SCCNFP (2000) evaluation reported that five sunscreen formulations were tested in 76 human volunteers (males and females), three forms containing 40% TiO_2 and two forms containing 10% TiO_2. The Shelanski repeated insult patch test method was used. The formulations were applied for 24 h on 2 × 2 cm patches on the lateral surface of the upper arm. Each subject had the same material applied to the same site throughout. Patches were applied 3 days a week for the first 3 weeks. Fourteen days later, challenge patches were applied to both arms, on one side to the original sites, and on the other to previously untreated sites. Scoring was at 48 and 96 h. Some mild erythematous reactions during the induction phase of the trial were recorded. There were no reactions to the challenge and the materials tested were judged not to cause sensitisation.

The SCCNFP (2000) evaluation also reported that a 5% preparation of TiO_2 in petrolatum was used to test 918 patients with various skin diseases (the occluded contact time was 48 h), including a group of 290 dermatitis patients (BIBRA, 1990). TiO_2 was reported not to cause any reaction. The same researchers also reported testing TiO_2 in 50 healthy volunteers and no reaction was observed (no further information) (SCCNFP, 2000).

Overall, the Panel noted that most of the published studies reporting effects of TiO_2 on the immune system have been carried out using nanosized TiO_2 and high doses of administration. However, an adequate characterisation of the size and the nature (rutile or anatase) were rarely provided and it was not clear to what extent the material used was representative of the food grade TiO_2. Finally, the route of administration (intratracheal or intraperitoneal) was often not representative of the use of TiO_2 as a food additive.

- *In vitro*, TiO_2 nanoparticles were readily internalised by immune system cells and might influence multiple manifestations of immune cell activity including cytokine production, proliferation, inflammation, ROS production and adhesion molecule expression, among others.
- *In vivo*, administration of TiO_2 nanoparticles has been reported to have multiple immunomodulatory effects, characterised by nanoparticle accumulation in local (Peyer's patches) and peripheral lymphoid organs, alterations in immune cell number, viability and function. In a few studies, microsized TiO_2 also induced some effects but only at high doses. Although ambiguity remains surrounding the specific immunomodulatory and inflammatory effects resulting from *in vivo* TiO_2 nanoparticle exposure, it seems clear that whereas TiO_2 nanoparticles have such a potential, TiO_2 particles with a larger size, over 100 nm, that is closer to food grade, are less active.

3.2.6.3. Other studies

The greatest number of studies on TiO_2 addressed the consequences of the exposure via inhalation and, in particular, the impact of particle size on the observed effects. The studies performed on pulmonary exposure to TiO_2 showed that toxicity was primarily dictated by particle size and crystal structure, whereby decreasing particle size and anatase as the crystalline form of TiO_2 enhanced particle toxicity (Ferin et al., 1992; Wang et al., 2008a,b).

Although the results of such studies cannot simply be used as basis for the safety evaluation of TiO_2 when taken orally, the studies give an indication on potential biological effects resulting from particles size when exposed by inhalation.

4. Discussion

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that had become available since then and the data available following

public calls for data. The Panel noted that not all original studies on which previous evaluations were based were available.

TiO_2 is a food colour authorised as a food additive in the EU. It was previously evaluated by the SCF in 1975 and 1977, by JECFA in 1969 (JECFA, 1970) and by EFSA in 2004. It has also been reviewed by TemaNord in 2002. In 1969, JECFA allocated an ADI 'not limited except for good manufacturing practice'. In 1975, the SCF did not establish an ADI for TiO_2, whereas in 1977, the SCF included TiO_2 in the category 'colours for which an ADI was not established but which could be used in food'. In 2002, TemaNord concluded that 'the inertness of the substance and the lack of absorption and tissue storage does not warrant further testing or a re-evaluation of the safety in use of this compound'. In 2004, the EFSA AFC Panel assessed the safety of platelet forms of rutile TiO_2 as an alternative to the permitted anatase form, and concluded that 'the bioavailability of these forms was essentially the same. The toxicological database would, therefore, be applicable to either form and that the platelet forms of rutile TiO_2 could be used to replace anatase TiO_2 in any of its current applications'.

The Panel is aware that the ECHA is carrying out an evaluation for a proposal for CLH on TiO_2, for which ANSES is the Rapporteur on behalf of the French Member State Competent Authority. ANSES prepared a report in which concluded that TiO_2 should be considered as being potentially carcinogenic to humans when inhaled and thus be classified Carc. Cat 1B – H350i. However, it also concluded that there was no carcinogenic concern after oral or dermal administration. A public consultation on this report is currently underway.[13]

In nature, TiO_2 exists in different crystalline forms, anatase and rutile being the two most important natural forms. The food additive TiO_2 (E 171) is a white to slightly coloured powder and it is insoluble in water and organic solvents (Commission Regulation (EU) No 231/2012).

Interested parties provided analytical data on the particle size characteristics of TiO_2 (E 171; anatase or rutile) used as a food/feed additive and additional information was available from public literature. The Panel noted that determination of the fraction of TiO_2 nanoparticles in the food additive (E 171) is method dependent. The Panel also noted that, according to the data provided by industries and from the literature, TiO_2 (E 171) as a food additive would not be considered as a nanomaterial according to the EU Recommendation on the definition of a nanomaterial.[8]

The Panel noted that there are no set limits for the particle size of TiO_2 in the EU specifications, and therefore characterisation of the particle size in the food additive E 171 should be included among the specifications.

The Panel noted that the manufacturing process for powdered or particulate food additives resulted in material with a range of sizes. Although the median size of the particles is generally significantly greater than 100 nm, a small fraction will always be, and has been, with at least one dimension below 100 nm. The material used for toxicological testing would have contained this nano fraction. The test requirements stipulated in current EFSA guidance documents and European Commission guidelines for the intended use in the food/feed area apply in principle to unintended nano forms, as well as to engineered nanomaterials. Therefore, the Panel considers that, in principle, for a specific food additive containing a fraction of particles with at least one dimension below 100 nm, adequately conducted toxicity tests should be able to detect hazards associated with this food additive, including its nanoparticulate fraction. The Panel considers that for the re-evaluation of food additives, this procedure would be sufficient for evaluating constituent nanoform fraction in accordance with the recommendation of the EFSA Nano Network in 2014 (EFSA, 2015). In addition, the Panel noted analytical data provided by interested parties on the particle size distribution of food-grade TiO_2, which confirmed the small percentage in the nanoscale (< 100 nm), but that actual values depended on the method used. From this information, a percentage value of 3.2% of nanoparticles by mass, was considered by the Panel to be reasonable to address in a conservative way a preliminary content estimate in the food additive TiO_2 (E 171).

The Panel was provided with the unpublished results of a number of RIVM studies on TiO_2 nanoparticles. These studies were evaluated along with the published literature and they did not affect the Panel's conclusions drawn from the whole dataset. The Panel recommends that, once publicly available, further information on the RIVM studies should be published as an addendum to this Opinion.

In absorption, distribution and excretion studies in animals (rat and mice), differences in the observed results appear to be dependent on study design and duration.

The Panel concluded that:

- the absorption of orally administered TiO_2 is extremely low,
- the bioavailability of TiO_2 (measured either as particles or as titanium) is low,
- the bioavailability measured as titanium appeared to be independent of particle size,
- the vast majority of an oral dose of TiO_2 is eliminated unchanged in faeces,
- a small amount (maximum of 0.1%) of orally ingested TiO_2 was absorbed by the GALT and subsequently distributed to various organs and elimination rates from these organs were variable,
- there were significant and highly variable background (basal) levels of titanium in animals and humans, which presented challenges in the analysis at the low levels of titanium uptake reported and could complicate interpretation of the reported findings in some studies.

The acute oral toxicity of TiO_2 is very low, with oral LD_{50} values > 10 g/kg bw per day for mice and > 25 g/kg bw per day for rats.

Overall, the Panel noted that there was rather limited information available on the short-term and subchronic toxicity of the food additive TiO_2 (E 171). In a well-performed 28-day gavage study in rats with non-coated pigment-grade TiO_2 (rutile form; d_{50} 173 nm) at a dose of 24,000 mg TiO_2/kg bw, no treatment-related adverse effects were observed. Occurrence of particles in intestinal lymphoid tissue was not regarded as adverse. The NOAEL for the study was 24,000 mg/kg bw per day. Although the study was not performed using the food additive TiO_2 (E 171), the Panel considered the results useful as supporting evidence in the assessment of the use of TiO_2 as a food colour. In a 90-day study, doses up to 16,900 mg TiO_2/kg bw per day for male mice and up to 8,100 mg TiO_2/kg bw per day for male rats did not result in differences in body weight or in relevant gross or microscopic pathology as compared with the control. However, no haematological parameters and no biochemical parameters in urine and blood were measured.

The Panel concluded that the available mixed results provided some evidence of *in vitro* genotoxicity for TiO_2 micro- and nanoparticles. The Panel noted that most positive results have been reported under experimental conditions associated with the induction of oxidative stress, and that the genotoxic effects observed mainly concern indicator assays, which in some studies were shown not to be associated with permanent chromosome damage.

In vivo, overall negative results were obtained in genotoxicity studies with microsized TiO_2 pigment. Limited evidence of genotoxicity, if any, was provided by studies with orally administered TiO_2 nanoparticles. Limited or no indication of genotoxicity of TiO_2 nanoparticles was also provided by studies using the intravenous route of administration, which allowed maximum exposure of target tissues.

The Panel concluded that the use of TiO_2 as a food additive does not raise a concern with respect to genotoxicity.

Two carcinogenicity studies, performed with TiO_2 administered to mice and rats via the oral route were available and the outcome of these studies was reported to be negative for both mice and rats. Based on these data, and on earlier data reported in the JECFA (1970) evaluation, the Panel concluded that TiO_2 is not carcinogenic after oral administration. This is in line with the recent assessment performed by ANSES for the ECHA evaluation in which it is concluded that there was no carcinogenic concern after oral or dermal administration. The Panel identified a NOAEL of 2,250 mg TiO_2/kg bw per day, the highest dose tested, from a chronic toxicity and carcinogenicity study in rats.

No reproductive (one- or two-generation toxicity) studies with TiO_2 (as the food additive, micro- or nanosized) performed according to the OECD guidelines were available for evaluation. However, the Panel noted that in the NCI (1979) chronic toxicity and carcinogenicity study, no histopathological changes in the male and female reproductive organs were reported at the highest doses tested of 6,500 and 8,350 mg/kg bw per day for male and female mice, respectively, and at the highest doses tested of 2,250 and 2,900 mg/kg bw per day for male and female rats, respectively.

Overall, the Panel noted that prenatal developmental studies with three pigment-grade (pg-1, pg-2, pg-3) and three ultrafine (uf-1, uf-2, uf-3)/nanoscale (anatase and/or rutile) TiO_2 particulates performed according to the OECD guidelines (TG 414) did not give concern for maternal or developmental toxicity up to the highest dose tested (1,000 mg/kg bw per day). However, the Panel noted that reproductive toxicity studies performed according to the OECD guidelines using TiO_2, meeting the food additive specifications were not available. Furthermore, the Panel noted that results from other reproductive and developmental studies with TiO_2 nanoparticles (Jia et al., 2014; Tassinari et al., 2014) indicating effects on the reproductive system, showed contradictory results in the change

in hormone levels. Because of deficiencies in the study designs and inadequate data reporting, the Panel considered that the relevance of these findings is currently uncertain for the risk assessment of TiO_2 as a food additive.

For the safety assessment of TiO_2 used as a food additive, based on information reported in the examined literature and information supplied following calls for data taking into account the following considerations:

- the food additive E 171 mainly consists of microsized TiO_2 particles, with a nanosized (< 100 nm) fraction less than 3.2% by mass;
- the absorption of orally administered TiO_2 particles (micro- and nanosized) in the gastrointestinal tract is negligible, estimated at most as 0.02–0.1% of the administered dose;
- no difference is observed in the absorption, distribution, and excretion of orally administered microsized and nanosized TiO_2 particles;
- no adverse effect resulting from the eventual accumulation of the absorbed particles is expected based on the results of long-term studies which did not highlight any toxicity up to the highest administered dose;
- the uncertainties in the toxicological database arising from limitations in the available reproductive toxicity studies;

the Panel considered that an ADI should not be established, and that a margin of safety (MoS) approach would be appropriate (EFSA ANS Panel, 2012).

As regards hypersensitivity, the Panel noted that the available studies on the effects of TiO_2 (nano)particles on the immune systems pointed to different outcomes. However, they indicated that the reported effects were dependent on the core composition, size and concentration of the particles, and on the duration and route of exposure. The Panel considered that, given the absence of clear characterisation of the material used, the difference in effects observed following various routes of administration and the diversity in the effects reported, a conclusion on the possible immunotoxic effects of the food additive TiO_2 cannot be reached. However, the Panel noted that the larger the TiO_2 particles, the lower their potential to induce effects, and that from animal data it appeared that the route of injection influences the response, TiO_2 particles being less reactive after oral administration.

To assess the dietary exposure to TiO_2 (E 171) from its use as a food additive, the exposure was calculated based on: maximum levels of data provided to EFSA (defined as the *maximum level exposure assessment scenario*) and reported use levels (defined as the *refined exposure assessment scenario*) as provided by industry and Member States.

Based on the available dataset, the Panel calculated two refined exposure estimates based on different assumptions: a *brand-loyal consumer scenario*, in which it is assumed that the population is exposed over a long period of time to the food additive present at the maximum reported use/analytical levels for one food category and to a mean reported use/analytical level for the remaining food categories; and a *non-brand-loyal scenario*, in which it is assumed that the population is exposed over a long period of time to the food additive present at the mean reported use/analytical levels in all relevant food categories.

The Panel considered that the refined exposure assessment approach was a more realistic scenario, because it was based on the range of usage and analytical data, assumed that the processed foods and beverages contain the additive at the mean concentration level for all products (non-brand-loyal consumer scenario) and considers one product containing TiO_2 at the maximum concentration level (brand-loyal consumer scenario). However, the Panel noted that due to the low amount of data provided to EFSA (reported use levels or analytical data), only 14 food categories were taken into account, representing between 60% and 80% of food (by weight) authorised to contain TiO_2 according to annex II.

The Panel noted that the refined exposure estimates will not cover future changes in the level of use of TiO_2.

For the *maximum level exposure assessment scenario*, at the mean, the exposure estimates ranged from 0.4 mg/kg bw per day for infants and the elderly to 10.4 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 1.2 mg/kg bw per day for the elderly to 32.4 mg/kg bw per day for children.

For the *refined estimated exposure scenario*, in the *brand-loyal scenario*, the exposure estimates ranged, at the mean, from 0.4 mg/kg bw per day for infants and the elderly to 8.8 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 1.1 mg/kg bw per day for the elderly to 30.2 mg/kg bw per day for children. In the *non-brand-loyal scenario*, the exposure estimates

ranged, at the mean, from 0.2 mg/kg bw per day for infants and the elderly to 5.5 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.5 mg/kg bw per day for the elderly to 14.8 mg/kg bw per day for children.

In the case of TiO_2, the Panel did not identify brand loyalty to a specific food category and therefore the Panel considered that the non-brand-loyal scenario covering the general population was the more appropriate and realistic scenario for risk characterisation because it is assumed that the population would probably be exposed long-term to food additives present at the mean reported use/analytical levels in processed food.

Based on a NOAEL of 2,250 mg TiO_2/kg bw per day and the exposure data for the non-brand loyal scenario, the Panel calculated the MoS values for the different population groups (Table 16).

Table 16: MoS values calculated based on the exposure estimated through the non-brand loyal scenario estimates as presented in Table 8, in six population groups (min–max across the dietary surveys)

Population groups	MoS calculation based on exposure to the non-brand loyal scenario	
	Mean	p95
Infants	2,800–11,000	600–3,200
Toddlers	500–3,800	350–1,200
Children	400–2,500	150–950
Adolescents	550–5,700	200–1,800
Adults	550–7,500	250–2,100
The elderly	800–11,000	300–4,500

MoS: margin of safety.

The Panel noted that the lowest MoS calculated from the NOAEL of 2,250 mg TiO_2/kg bw per day identified in the available toxicological data and exposure data obtained from the reported use/analytical levels of TiO_2 (E 171) considered in this opinion is above 100. In the Guidance for submission of food additives (EFSA ANS Panel, 2012), the Panel considered that, for non-genotoxic and non-carcinogenic compounds "a MoS of 100 or more between a NOAEL or BMDL and the anticipated exposure would be sufficient to account for uncertainty factors for extrapolating between individuals and species". Consequently, the Panel considered that the reported use/analytical levels of TiO_2 (E 171) considered in this opinion would not be of safety concern.

The Panel considered that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI).

For the purpose of providing an indicative estimate of exposure to nanoparticles of titanium dioxide from the use of TiO_2 as a food additive, the Panel considered that the highest reported weight percentage value of 3.2% of nanoparticles by mass could reasonably be used in a conservative way to address this issue.

Based on this maximum reported level of 3.2% of nanoparticles by mass in all foods categories considered in the exposure assessment from the use of E 171 as a food additive, the Panel noted that indicative estimates of exposure to nanoparticles of titanium dioxide coming from TiO_2 (E 171) ranged for the maximum level exposure assessment scenario, at the mean, from 0.01 mg/kg bw per day for infants and the elderly to 0.33 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.04 mg/kg bw per day for infants and the elderly to 1.04 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the brand-loyal scenario, the exposure estimates ranged at the mean from 0.01 mg/kg bw per day for infants and the elderly to 0.28 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.03 mg/kg bw per day for the elderly to 0.97 mg/kg bw per day for children.

For the refined estimated exposure scenario, in the non-brand-loyal scenario, the exposure estimates ranged at the mean from 0.01 mg/kg bw per day for infants, adolescents, adults and the elderly to 0.18 mg/kg bw per day for children. At the 95th percentile, exposure estimates ranged from 0.02 mg/kg bw per day for infants and the elderly to 0.47 mg/kg bw per day for children.

The Panel noted that from its indicative estimates of exposure to nanoparticles that could be present in the food additive TiO_2, the uncertainties identified could result in an overestimation if all

food categories considered in its exposure assessment had nanoparticles present at the maximum reported percentage value by mass (3.2%).

Conclusions

From the available data on absorption, distribution and excretion, the Panel concluded that:

- the absorption of orally administered TiO_2 is extremely low;
- the bioavailability of TiO_2 (measured either as particles or as titanium) is low;
- the bioavailability measured as titanium appeared to be independent of particle size;
- the vast majority of an oral dose of TiO_2 is eliminated unchanged in the faeces;
- a small amount (maximum of 0.1%) of orally ingested TiO_2 was absorbed by the gut-associated lymphoid tissue (GALT) and subsequently distributed to various organs and elimination rates from these organs were variable.

The Panel further concluded that there were significant and highly variable background levels of titanium in animals and humans, which presented challenges in the analysis at the low levels of titanium uptake reported and could complicate interpretation of the reported findings.

The Panel 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*.

The Panel noted that possible adverse effects in the reproductive system were identified in some studies conducted with material which was either non-food-grade or inadequately characterised nanomaterial (i.e. not E 171). There were no such indications in the available, albeit limited, database on reproductive endpoints for the food additive (E 171). The Panel was unable to reach a definitive conclusion on this endpoint due to the lack of an extended 90-day study as in the Guidance for submission of food additives (EFSA ANS Panel, 2012) or a multigeneration or extended-one generation reproduction toxicity study with the food additive (E 171). Therefore, the Panel did not establish an ADI.

From a carcinogenicity study with TiO_2 in mice and in rats, the Panel chose the lowest NOAEL reported which was 2,250 mg TiO_2/kg bw per day for males from the rat study, the highest dose tested in this species and sex.

The Panel considered that on the database currently available and the considerations on the absorption of TiO_2 the margins of safety calculated from the NOAEL of 2,250 mg TiO_2/kg bw per day identified in the toxicological data available and exposure data obtained from the reported use/analytical levels of TiO_2 (E 171) considered in this opinion would not be of concern.

The Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI).

Recommendations

The Panel recommended that:

- In order to enable the Panel to establish a health-based guidance value (ADI) for the food additive TiO_2 (E 171), additional testing could be performed. An extended 90-day study or a multigeneration or extended-one generation reproduction toxicity study according to the current OECD guidelines could be considered. Such studies should be performed with TiO_2 (E 171) complying with the EU specifications and additionally including a characterisation of the particle size distribution of the test material. However, in deciding on actual testing, considerations of animal welfare need to be balanced against the improvement in the toxicological database within a tiered testing approach.
- The EU specifications for TiO_2 (E 171) should include a characterisation of particle size distribution using appropriate statistical descriptors (e.g. range, median, quartiles) as well as the percentage (in number and by mass) of particles in the nanoscale (with at least one dimension < 100 nm) present in TiO_2 (E 171) used as a food additive. The measuring methodology applied should comply with the EFSA Guidance document (EFSA Scientific Committee, 2011).
- The maximum limits for the impurities of the toxic elements (arsenic, lead, mercury and cadmium) in the EU specification for TiO_2 (E 171) should be revised in order to ensure that TiO_2 (E 171) as a food additive will not be a significant source of exposure to those toxic elements in foods.

Documentation provided to EFSA

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Abbreviations

8-OH-dG	8-hydroxy 2′-deoxyguanosine
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AECD	area equivalent circular diameter
AESGP	Association of the European Self-Medication Industry
AFC	EFSA Former Panel on Additives, Flavourings, Processing Aids and Materials in Contact with Food
AOM	azoxymethane
ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
ANSES	French Agency for Food, Environmental and Occupational Health and Safety
AUC	area under the curve
BIBRA	British Industrial Biological Research Association
bw	body weight
BMDL	benchmark dose modelling
CAC	colitis-associated cancer
CAS	Chemical Abstracts Service
CEFIC	European Chemical Industry Council
CHO	Chinese hamster ovary
C.I.	Colour Index
CIAA	Confederation of the Food and Drink Industries of the EU
CLH	harmonised classification and labelling
d_{50}	median particle size
DLS	dynamic light scattering
DSS	dextran sulfate sodium
ECHA	European Chemical Agency
EINECS	European Inventory of Existing Commercial Chemical Substances
FCS	Food Categorisation System
FDA	Food and Drug Administration
FDE	Food Drink Europe
FSANZ	Food Standard Agency of New Zealand
GALT	gut-associated lymphoid tissue
GNPD	Global New Products Database
HD	hydrodynamic diameter
hOGG	human 8-hydroxyguanine DNA-glycosylase
IACM	International Association of Colour Manufacturers
IARC	International Agency for Research in Cancer
ICGA	International Chewing Gum Association
ICRP	International Commission on Radiological Protection
IL	interleukin
IPCS	International Programme on Chemical Safety
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee of Food Additives
KEM	keratinocyte growth medium

LC	left-censored
LD_{50}	median lethal dose
LOD	limit of detection
LOQ	limit of quantification
MIP-2	macrophage inflammatory protein-2
MoS	margin of safety
MPL	maximum permitted level
MS	Member State
MTT	methyl tetrazolium cytotoxicity
NCE	normochromatic erythrocytes
NCI	National Cancer Institute
N.F.	National Formulary
NIOSH	National Institute for Occupational Safety and Health
NOAEL	no observable adverse effect level
NSF	US National Sanitation Foundation
NTP	National Toxicological Programme
OECD TG	Organisation for Economic Co-operation and Development Testing Guidelines
PALS	phase analysis light scattering
PAMs	porcine alveolar macrophages
PCE	polychromatic erythrocytes
QS	quantum satis
RIVM	National Institute for Public Health and the Environment
ROS	reactive oxygen species
SCCNFP	Scientific Committee on Cosmetics and Non-Food Products
SCCS	Scientific Committee on Consumer Safety
SCE	sister chromatid exchange
SCF	Scientific Committee on Food
SEM	scanning electron microscopy
SHE	Syrian hamster embryo
STT	short-term test
TBIL	total bilirubin
TDMA	Titanium Dioxide Manufacturers Association
TEM	transmission electron microscopy
TK	thymidine kinase
TNF	tumour necrosis factor
UF	ultrafine
UV	ultraviolet
XSDC	X-ray disc centrifugation

Appendix A – Summary of reported use levels (mg/kg) of TiO_2 (E 171) provided by industry

Food category number	Food category name	MPL	Restriction/ exceptions	Total number of data	Reported use levels from industry			Information provided by	Comments
					Number of data	Typical mean (range)	Highest maximum level		
01.8	Dairy analogues, including beverage whiteners	QS		1	1	125	125	FDE	
03	Edible ices	QS		2	2	428	857	FDE	
05.2	Other confectionery, including breath-refreshening microsweets	QS		5	5	1,074	4,500	FDE	
05.3	Chewing gum	QS		2	1	3,400	3,800	FDE	
					1	2,829	16,000	ICGA	
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	QS		13	13	1,296	20,000	FDE	
07.2	Fine bakery wares	QS		2	2	179	555	FDE	
08.2.3	Casings and coatings and decorations for meat	QS	Except edible external coating of pasturmas	2	2	18	35	FDE	
12.5	Soups and broths	QS		1	1	193	193	FDE	
12.6	Sauces	QS	Excluding tomato-based sauces	5	5	1,646	4,000	FDE	
12.7	Salads and savoury-based sandwich spreads	QS		1	1	2,500	3,000	FDE	
14.1.4	Flavoured drinks	QS	Excluding chocolate milk, malt products	6	6	28	70	FDE	
15.2	Processed nuts	QS		4	4	3,775	7,000	FDE	
16	Desserts, excluding products covered in category 1, 3 and 4	QS		1	1	140	200	FDE	
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms, excluding chewable forms	QS		16	15	2,801	12,000	AESGP	
					1	2	4	Capsugel	Empty gelatin capsule

QS: quantum satis; FDE: FoodDrinkEurope; ICGA: International Chewing Gum Association; AESGP: Association of the European Self-Medication Industry.

Appendix B – Summary of analytical results (mg/kg) of TiO_2 (E 171) provided by Members States

Food category number	Food category name	MPL	N	No LC	Range		All data				Positive values				
					LOD	LOQ	Min	Median	Mean	Max	Number of positive values	Min	Median	Mean	Max
13	Foods intended for particular nutritional uses as defined by Directive 2009/39/EC	QS	2	0	2,000	5,000	16,950	18,150	18,150	19,350	2	16,950	18,150	18,150	19,350
17	Food supplements as defined in Directive 2002/46/EC of the European Parliament and of the Council, excluding food supplements for infants and young children	QS	26	4	2,000	5,000	1,000	15,150	14,438	26,950	22	6,000	16,900	16,745	26,950

LC: left-censored data; LOD: limit of detection; LOQ: limit of quantification; Max: maximum; Min: minimum; MPL: maximum permitted level; N: number of analytical results.

Appendix C – Number and percentage of food products labelled with TiO_2 (E 171) out of the total number of food products present in Mintel GNPD per food subcategory between 2011 and 2015

Mintel sub-category[a]	Total number of products	Products labelled with TiO_2 (E 171)	
		Number	%
Gum	1,262	642	50.9
Sticks, Liquids & Sprays	88	22	25.0
Mixed Assortments	271	56	20.7
Pastilles, Gums, Jellies & Chews	3,346	345	10.3
Lollipops	341	34	10.0
Liquorice	690	54	7.8
Other Sugar Confectionery	950	66	6.9
Yeast Extracts	15	1	6.7
Non-Individually Wrapped Chocolate Pieces	4,687	312	6.7
Standard & Power Mints	787	44	5.6
Creamers	182	10	5.5
Other Frozen Desserts	1,396	76	5.4
Seasonal Chocolate	4,962	219	4.4
Boiled Sweets	858	35	4.1
Beverage Mixes	767	26	3.4
Marshmallows	431	14	3.2
Cakes, Pastries & Sweet Goods	11,877	385	3.2
Baking Ingredients & Mixes	8,031	234	2.9
Mayonnaise	802	21	2.6
Dairy-Based Frozen Products	7,001	174	2.5
Dessert Toppings	573	12	2.1
Toffees, Caramels & Nougat	1,738	30	1.7
Medicated Confectionery	891	14	1.6
Other Chocolate Confectionery	263	4	1.5
Beverage Concentrates	2,097	23	1.1
Sweet Biscuits/Cookies	15,483	162	1.0
Chilled Desserts	5,583	54	1.0
Chocolate Spreads	979	9	0.9
Dressings & Vinegar	3,035	27	0.9
Chocolate Tablets	7,344	64	0.9
Instant Rice	120	1	0.8
Shelf-Stable Desserts	2,945	21	0.7
Individually Wrapped Chocolate Pieces	2,296	14	0.6
Spoonable Yoghurt	8,752	49	0.6
Processed Cheese	1,875	10	0.5
Nuts	4,018	21	0.5
Instant Noodles	995	5	0.5
Sandwiches/Wraps	2,406	12	0.5
Snack Mixes	1,273	6	0.5
Eggs & Egg Products	1,298	6	0.5
Chocolate Countlines	2,059	9	0.4
Caramel & Cream Spreads	243	1	0.4
Nectars	3,581	12	0.3
Table Sauces	5,376	17	0.3

Mintel sub-category[a]	Total number of products	Products labelled with TiO_2 (E 171)	
		Number	%
Meat Substitutes	1,908	6	0.3
Soft Cheese & Semi-Soft Cheese	4,995	15	0.3
Salads	2,337	7	0.3
Meat Pastes & Pates	2,776	8	0.3
Water-Based Frozen Desserts	1,072	3	0.3
Meal Kits	1,809	5	0.3
Snack/Cereal/Energy Bars	4,232	11	0.3
Fish Products	10,920	26	0.2
Soft Cheese Desserts	1,364	3	0.2
Noodles	482	1	0.2
Sucrose	975	2	0.2
Meal Replacements & Other Drinks	990	2	0.2
Instant Pasta	549	1	0.2
Cooking Sauces	4,446	7	0.2
Prepared Meals	9,894	14	0.1
Hors d'oeuvres/Canapes	3,631	5	0.1
Energy Drinks	1,484	2	0.1
Poultry Products	5,483	7	0.1
Fresh Cheese & Cream Cheese	2,457	3	0.1
Flavoured Alcoholic Beverages	1,800	2	0.1
Sandwich Fillers/Spreads	901	1	0.1
Malt & Other Hot Beverages	921	1	0.1
Popcorn	981	1	0.1
Dips	1,282	1	0.1
Potato Snacks	4,388	3	0.1
Rice	2,932	2	0.1
Liqueur	1,467	1	0.1
Hard Cheese & Semi-Hard Cheese	5,903	4	0.1
Wheat & Other Grain-Based Snacks	1,689	1	0.1
Corn-Based Snacks	1,955	1	0.1
Pasta	8,874	4	0.0
Fruit/Flavoured Still Drinks	2,590	1	0.0
Meat Products	13,984	4	0.0
Seasonings	8,423	2	0.0
Savoury Biscuits/Crackers	4,214	1	0.0
Vegetables	9,283	2	0.0
Cold Cereals	5,472	1	0.0
Juice	6,949	1	0.0
Bread & Bread Products	8,926	1	0.0
Total sample	278,705	3,516	1.3

(a): According to Mintel food categorisation.

Appendix D – Concentration levels of TiO_2 (E 171) used in the refined exposure scenarios (mg/kg)

FCS category no.	FCS food category	MPL	Concentration levels used in the refined exposure assessment		Data source/comments
			Mean	Max	
01.4	Flavoured fermented milk products, including heat-treated products	QS			Not taken into account (no concentration data)
01.5	Dehydrated milk as defined by Directive 2001/114/EC	QS			Not taken into account (no concentration data)
01.6.3	Other creams	QS			Not taken into account (no concentration data)
01.7.1	Unripened cheese, excluding products falling in category 16 (except mozzarella and unflavoured live fermented unripened cheese)	QS			Not taken into account (no concentration data)
01.7.3	Edible cheese rind	QS			Not taken into account (no corresponding FoodEx code/ no concentration data)
01.7.4	Whey cheese	QS			Not taken into account (no concentration data)
01.7.5	Processed cheese	QS			Not taken into account (no concentration data)
01.7.6	Cheese products	QS			Not taken into account (no concentration data)
01.8	Dairy analogues, including beverage whiteners	QS	125	125	Reported use levels
03	Edible ices	QS	429	857	Reported use levels
04.2.4.1	Fruit and vegetable preparations excluding compote – only mostarda di frutta	QS			Not taken into account (no corresponding FoodEx code/ no concentration data)
04.2.4.1	Fruit and vegetable preparations excluding compote – only seaweed-based fish analogues	QS			Not taken into account (no corresponding FoodEx code/ no concentration data)
04.2.5.3	Other similar fruit or vegetable spreads, except crème de pruneaux	QS			Not taken into account (no concentration data)
05.2	Other confectionery, including breath-refreshening microsweets	QS	1,074	4,500	Reported use levels
05.3	Chewing gum	QS	3,115	16,000	Reported use levels
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	QS			Not taken into account (no corresponding FoodEx code)
06.3	Breakfast cereals	QS			Not taken into account (no concentration data)
06.5	Noodles	QS			Not taken into account (no concentration data)
06.6	Batters	QS			Not taken into account (no corresponding FoodEx code/ no concentration data)
06.7	Pre-cooked or processed cereals	QS			Not taken into account (no corresponding FoodEx code/ no concentration data)

FCS category no.	FCS food category	MPL	Concentration levels used in the refined exposure assessment		Data source/comments
			Mean	Max	
07.2	Fine bakery wares	QS	160	318	Reported use levels
08.2.3	Casings and coatings and decorations for meat	QS			Not taken into account (no corresponding FoodEx code)
09.2	Processed fish and fishery products, including molluscs and crustaceans – only surimi and similar products and salmon substitutes	QS			Not taken into account (no concentration data)
09.2	Processed fish and fishery products, including molluscs and crustaceans – only fish paste and crustacean paste	QS			Not taken into account (no concentration data)
09.2	Processed fish and fishery products, including molluscs and crustaceans – only precooked crustacean	QS			Not taken into account (no concentration data)
09.2	Processed fish and fishery products, including molluscs and crustaceans – only smoked fish	QS			Not taken into account (no concentration data)
09.3	Fish roe – only processed fish roe	QS			Not taken into account (no concentration data)
12.2.2	Seasonings and condiments	QS			Not taken into account (no concentration data)
12.4	Mustard	QS			Not taken into account (no concentration data)
12.5	Soups and broths	QS	193	193	Reported use levels
12.6	Sauces	QS	1,433	4,000	Reported use levels
12.7	Salads and savoury-based sandwich spreads	QS	2,500	3,000	Reported use levels
12.9	Protein products, excluding products covered in category 1.8	QS			Not taken into account (no concentration data)
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC	QS			Not taken into account (no concentration data)
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal	QS			Not taken into account (no concentration data)
13.4	Foods suitable for people intolerant to gluten as defined by Regulation	QS			Not taken into account (no concentration data)
14.1.4	Flavoured drinks	QS	39	70	Reported use levels
14.2.3	Cider and perry	QS			Not taken into account (no concentration data)
14.2.4	Fruit wine and made wine	QS			Not taken into account (no corresponding FoodEx code/ no data provided)
14.2.5	Mead	QS			Not taken into account (no corresponding FoodEx code/ no data provided)

FCS category no.	FCS food category	MPL	Concentration levels used in the refined exposure assessment		Data source/comments
			Mean	Max	
14.2.6	Spirit drinks as defined in Regulation (except whisky or whiskey)	QS			Not taken into account (no concentration data)
14.2.7.1	Aromatised wines	QS			Not taken into account (no concentration data)
14.2.7.2	Aromatised wine-based drinks	QS			Not taken into account (no concentration data)
14.2.7.3	Aromatised wine-product cocktails	QS			Not taken into account (no concentration data)
14.2.8	Other alcoholic drinks, including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol and	QS			Not taken into account (no concentration data)
15.1	Potato-, cereal-, flour- or starch-based snacks	QS			Not taken into account (no concentration data)
15.2	Processed nuts	QS	3,775	7,000	Reported use levels
16	Desserts, excluding products covered in category 1, 3 and 4	QS	140	200	Reported use levels
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms, excluding chewable forms	QS	14,438	26,950	Analytical data
17.2	Food supplements supplied in a liquid form	QS			
17.3	Food supplements supplied in a syrup-type or chewable form	QS			

FCS: Food Categorisation System; MPL: maximum permitted level; QS: quantum satis.

Appendix E – Summary of total estimated exposure of TiO_2 (E 171) from its use as a food additive for maximum scenario and refined exposure scenarios per population group and survey: mean and 95th percentile (mg/kg bw per day)

	Number of subjects	Maximum scenario		Brand-loyal scenario		Non brand-loyal scenario	
		Mean	p95	Mean	p95	Mean	p95
Infants							
Bulgaria (NUTRICHILD)	659	0.4	1.8	0.4	1.8	0.2	0.9
Germany (VELS)	159	1.4	6.6	1.3	5.3	0.6	2.8
Denmark (IAT 2006_07)	826	0.5	2.3	0.4	1.9	0.2	1.1
Finland (DIPP 2001 2009)	500	0.6	1.4	0.6	1.2	0.3	0.7
United Kingdom (DNSIYC 2011)	1,366	1.9	9.6	1.8	9.2	0.8	3.9
Italy (INRAN_SCAI_2005_06)	12	1.0		0.9		0.7	
Toddlers							
Belgium (Regional Flanders)	36	9.2		7.6		4.6	
Bulgaria (NUTRICHILD)	428	2.3	7.5	2.1	6.7	1.0	2.9
Germany (VELS)	348	7.0	15.0	5.3	12.5	2.8	6.4
Denmark (IAT 2006 07)	917	3.7	10.1	2.9	7.8	1.4	3.6
Spain (enKid)	17	2.3		1.9		1.1	
Finland (DIPP 2001 2009)	500	1.2	4.0	1.1	3.6	0.6	2.0
United Kingdom (NDNS-RollingProgrammeYears1-3)	185	5.9	17.3	5.0	14.2	2.6	6.8
United Kingdom (DNSIYC 2011)	1,314	4.3	14.0	3.8	12.9	1.9	5.9
Italy (INRAN SCAI 2005 06)	36	1.8		1.6		0.9	
Netherlands (VCP kids)	322	7.1	19.3	5.7	14.7	2.9	6.8
Children							
Austria (ASNS Children)	128	4.7	12.2	3.6	10.8	2.4	7.5
Belgium (Regional Flanders)	625	7.3	15.3	6.0	12.7	3.5	7.1
Bulgaria (NUTRICHILD)	433	3.3	9.8	2.8	8.3	1.5	3.9
Czech Republic (SISP04)	389	5.7	18.8	4.7	15.3	2.2	6.4
Germany (EsKiMo)	835	4.3	12.0	3.4	10.0	1.7	4.6
Germany (VELS)	293	8.0	16.7	5.8	12.4	3.1	6.1
Denmark (DANSDA 2005-08)	298	5.5	13.5	3.9	9.8	1.9	4.6
Spain (enKid)	156	4.5	12.5	3.7	10.3	1.8	5.6
Spain (NUT INK05)	399	5.1	14.1	4.4	12.8	2.2	5.9
Finland (DIPP 2001 2009)	750	10.4	32.4	8.8	30.2	3.2	9.2
France (INCA2)	482	4.6	9.5	3.5	7.1	2.0	4.2
United Kingdom (NDNS-RollingProgrammeYears1-3)	651	6.4	15.5	5.1	13.3	2.7	6.2
Greece (Regional Crete)	838	4.4	13.7	3.9	12.7	2.9	10.5
Italy (INRAN SCAI 2005 06)	193	1.8	4.9	1.5	4.1	0.9	2.4
Latvia (EFSA TEST)	187	9.1	23.1	8.0	19.9	5.5	14.8
Netherlands (VCP kids)	957	7.3	16.5	5.6	12.7	2.9	6.3
Netherlands (VCPBasis AVL2007 2010)	447	8.6	17.7	6.2	13.7	3.5	7.1
Sweden (NFA)	1,473	10.4	22.1	8.0	17.0	4.4	9.0

	Number of subjects	Maximum scenario		Brand-loyal scenario		Non brand-loyal scenario	
		Mean	p95	Mean	p95	Mean	p95
Adolescents							
Austria (ASNS Children)	237	2.7	7.7	2.3	7.1	1.6	5.8
Belgium (Diet National 2004)	576	4.6	12.4	3.9	10.5	2.0	5.2
Cyprus (Childhealth)	303	0.8	3.3	0.7	2.7	0.4	1.3
Czech Republic (SISP04)	298	3.7	12.2	3.1	10.7	1.5	4.9
Germany (National Nutrition Survey II)	1,011	4.5	13.4	3.9	11.6	1.8	5.1
Germany (EsKiMo)	393	3.3	9.5	2.6	7.6	1.3	3.6
Denmark (DANSDA 2005-08)	377	3.1	7.6	2.3	5.5	1.1	2.4
Spain (AESAN FIAB)	86	2.3	7.4	2.1	6.5	0.8	2.8
Spain (enKid)	209	3.8	10.4	3.1	8.3	1.5	4.0
Spain (NUT INK05)	651	3.4	8.6	2.9	7.0	1.4	3.5
Finland (NWSSP07 08)	306	6.7	23.5	5.9	21.2	1.9	6.2
France (INCA2)	973	2.7	6.3	2.1	4.8	1.1	2.7
United Kingdom (NDNS-RollingProgrammeYears1-3)	666	3.7	9.4	3.1	8.0	1.5	3.7
Italy (INRAN SCAI 2005 06)	247	1.1	3.1	0.9	2.5	0.6	1.5
Latvia (EFSA TEST)	453	6.5	18.0	5.6	15.0	4.1	10.8
Netherlands (VCPBasis AVL2007 2010)	1,142	5.6	13.9	4.3	10.6	2.3	5.2
Sweden (NFA)	1,018	6.2	14.8	4.9	11.6	2.6	6.0
Adults							
Austria (ASNS Adults)	308	4.5	12.7	3.9	11.0	2.6	7.2
Belgium (Diet National 2004)	1,292	3.3	9.7	2.9	8.7	1.5	4.1
Czech Republic (SISP04)	1,666	1.7	6.1	1.5	5.2	0.9	3.7
Germany (National Nutrition Survey II)	10,419	3.6	10.4	3.2	9.0	1.5	4.0
Denmark (DANSDA 2005-08)	1,739	1.9	5.2	1.4	4.1	0.7	1.8
Spain (AESAN)	410	1.3	4.7	1.2	3.7	0.7	2.1
Spain (AESAN FIAB)	981	1.7	4.4	1.5	3.7	0.7	2.0
Finland (FINDIET2012)	1,295	4.2	15.0	3.6	13.6	1.6	5.2
France (INCA2)	2,276	1.8	4.5	1.5	3.7	0.8	1.9
United Kingdom (NDNS-RollingProgrammeYears1-3)	1266	2.9	7.8	2.5	6.4	1.3	3.4
Hungary (National Repr Surv)	1,074	0.8	3.4	0.7	3.3	0.4	1.8
Ireland (NANS 2012)	1,274	3.2	9.0	2.7	7.8	1.3	3.8
Italy (INRAN SCAI 2005 06)	2,313	0.7	2.4	0.6	2.1	0.4	1.4
Latvia (EFSA TEST)	1,271	4.7	13.0	4.3	11.7	3.2	9.2
Netherlands (VCPBasis AVL2007 2010)	2,057	3.7	9.0	3.0	7.5	1.6	3.9
Romania (Dieta Pilot Adults)	1,254	0.6	2.2	0.5	1.9	0.3	1.1
Sweden (Riksmaten 2010)	1,430	6.8	14.8	5.7	12.4	4.0	9.7
The elderly							
Austria (ASNS Adults)	92	3.6	9.4	3.1	7.4	2.4	6.3
Belgium (Diet National 2004)	1,215	2.2	6.4	2.0	6.0	1.2	2.9
Germany (National Nutrition Survey II)	2,496	1.9	5.7	1.7	5.2	0.9	2.4
Denmark (DANSDA 2005-08)	286	1.0	3.1	0.8	2.5	0.4	1.3
Finland (FINDIET2012)	413	2.4	7.9	2.0	7.2	1.0	3.5

	Number of subjects	Maximum scenario		Brand-loyal scenario		Non brand-loyal scenario	
		Mean	p95	Mean	p95	Mean	p95
France (INCA2)	348	1.4	3.3	1.2	2.8	0.6	1.5
United Kingdom (NDNS-RollingProgrammeYears1-3)	305	2.5	6.5	2.1	5.8	1.2	2.9
Hungary (National Repr Surv)	286	0.6	2.3	0.6	2.2	0.3	1.2
Ireland (NANS 2012)	226	2.3	6.5	2.0	6.2	1.1	2.9
Italy (INRAN SCAI 2005 06)	518	0.5	2.1	0.5	1.9	0.3	1.1
Netherlands (VCPBasis AVL2007 2010)	173	2.7	6.8	2.2	5.4	1.3	3.1
Netherlands (VCP-Elderly)	739	2.9	6.8	2.4	5.9	1.4	3.4
Romania (Dieta Pilot Adults)	128	0.4	1.2	0.4	1.1	0.2	0.5
Sweden (Riksmaten 2010)	367	4.5	10.7	3.9	9.2	2.8	7.0

Appendix F – Toxicological studies with coated TiO_2 nanoparticles considered by the Panel

Brun E, Jugan M-L, Herlin-Boime N, Jaillard D, Fayard B, Flank A-M, Mabondzo A and Carriere M, 2011. Investigation of TiO_2 nanoparticles translocation through a Caco-2 monolayer. Journal of Physics: Conference Series, 304, 012048.

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Warheit DB, Brown SC and Donner EM, 2015b. Acute and subchronic oral toxicity studies in rats with nanoscale and pigment grade titanium dioxide particles. Food and Chemical Toxicology, 84, 208–224.