The five studies by the oral route were given higher weight. All these studies entailed repeated gavage administration of TiO_2 NPs (from 14 to 90 daily treatments): of these, four were evaluated as positive for the induction of MN (Shukla et al., 2014; Grissa et al., 2015; Chakrabarti et al., 2019) or structural CAs (Manivannan et al., 2020) in mice. One (Chen et al., 2014) tested negative in the rat bone marrow MN assay, although some evidence of bone marrow exposure was provided by the concurrent analysis of H2AX foci.

Supporting evidence was provided by studies via intraperitoneal and intravenous injection.

Seven studies using the intraperitoneal route were considered. Increased incidences of MN in mouse bone marrow were observed after single (Lotfi et al., 2016) or repeated (El-Ghor et al., 2014; Fadoju et al., 2019) injections of TiO_2 NPs. Negative results were reported in a MN assay in mouse peripheral blood after three daily injections of TiO_2 NPs (Sadiq et al., 2012), and in a CA assay in bone marrow cells of mice after single injection of TiO_2 (> 100 nm) (Shelby and Witt, 1995). Equivocal results were reported in two studies (Shelby et al., 1993; Zirak et al., 2016).

Out of three intravenous studies, sufficiently reliable for consideration, one study was positive. The study was performed in rats, with single administration of TiO_2 NPs. Increased incidence of MN was observed in bone marrow polychromatic erythrocytes (but not in reticulocytes) (Dobrzynska et al., 2014). Two other studies in mice, with two daily (Louro et al., 2014) or four weekly injection (Suzuki et al., 2016) did not report any significant increase of MN in peripheral blood reticulocytes.

In summary, the in vivo studies – one of them of high relevance and the others of limited relevance – were predominantly positive, independently of the route of exposure. Discrepant results were reported in some studies using comparable dose ranges, species and endpoint, which cannot be traced to size or other specificities of the test material. Rather, it is possible that differences in handling of TiO_2 NPs, and dispersion protocols, which were insufficiently reported for most studies, were important variability factors.

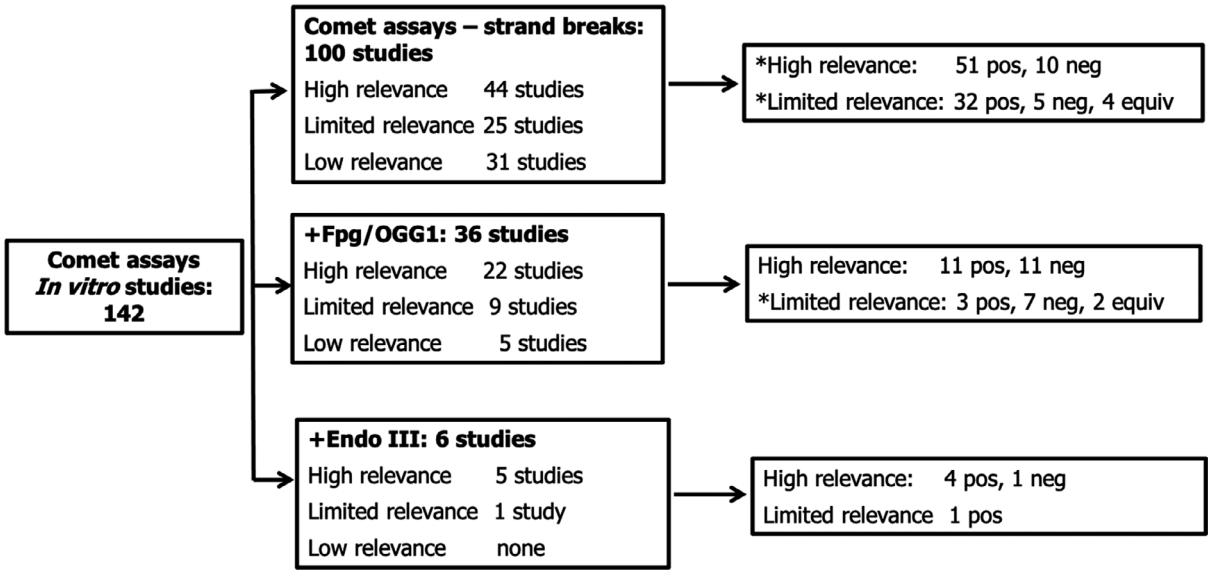
Concluding remarks

Overall, based on the available lines of evidence, the Panel considered that - on balance - TiO_2 NPs have the potential to induce MN/CA. The Panel noted that a significant portion of the studies was performed using TiO_2 NPs < 30 nm, however some positive results were observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in MN/CA assay was observed.

4.3.3. *In vitro* and *in vivo* Comet assay

***In vitro* Comet assay**

One hundred and forty-two *in vitro* studies (reported in 68 publications) using Comet assays in different cell lines were available for the evaluation, including data provided by the NANOGENOTOX Project (Appendices J, L, N). The NANOGENOTOX report summarises results from several laboratories who investigated TiO_2 NPs in various cell lines ((NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7, 8 and 10)). One hundred and six studies (reported in 43 publications plus NANOGENOTOX project, 2013) were classified as of high or limited relevance and further considered in the assessment (Figure 3). The range of TiO_2 particle size tested was from 2.3 nm to 5 μm.



*: Some of the studies used more than one test material and in these cases, the results are reported separately.

Figure 3: *In vitro* Comet assays – Summary of study results from 142 studies reported in 68 publications

Of all the studies analysed, the great majority were performed on human cell lines originating from colon epithelium (Caco-2, HT-29 or a co-culture of both), blood or lung tissue. Other human cells used were derived from liver, lymphoid, endothelial, epidermal, kidney or macrophage cell lines. Rodent cells from rat, mouse or hamster were also used.

The majority of studies ((NANOGENOTOX project, 2013 Documentation provided to EFSA No 7, 8 and 10); Zijno et al., 2015; Proquin et al., 2017; Schneider et al., 2017; Garcia-Rodriguez et al., 2018; Brown et al., 2019; Murugadoss et al., 2020) were positive on Colon cancer cells (Caco-2, HT-29 alone or in co-culture) showing an increase in DNA damage, i.e. strand breaks or strand breaks and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites (Fpg detects oxidised purines). Some of the studies have been found to be negative (Dorier et al., 2019), or equivocal (Vila et al., 2018).

All five studies performed on human peripheral blood mononuclear cells (PBMC) were positive, most of them for strand breaks (Demir et al., 2013; Cowie et al., 2015; Kurzawa-Zegota et al., 2017; Andreoli et al., 2018; Kazimirova et al., 2019) and also for Fpg- and Endo III-sensitive sites (Demir et al., 2013). One from these studies showed a negative response in some donors (Kazimirova et al., 2019).

Two studies performed with human lymphoblastoid TK6 cells, showed DNA damage after exposure to TiO_2 particles (Cowie et al., 2015; El Yamani et al., 2017) and two studies were negative (Magdolenova et al., 2012; Woodruff et al., 2012).

Fourteen studies (nine with high and five with limited relevance) used a lung model (cell lines A549, BEAS-2B, 16HBE14o, HBE). The majority of these studies showed positive results regarding strand breaks (Falck et al., 2009; Karlsson et al., 2009; Jugan et al., 2012; NANOGENOTOX project, 2013 Documentation provided to EFSA No 7, 8 and 10; Prasad et al., 2013; Cowie et al., 2015; Wang et al., 2015; Biola-Clier et al., 2017; El Yamani et al., 2017; Stoccoro et al., 2017; Murugadoss et al., 2020; Zijno et al., 2020)) as well as oxidised DNA lesions (Di Bucchianico et al., 2017; El Yamani et al., 2017; Stoccoro et al., 2017; Zijno et al., 2020). Only two studies were negative for strand breaks (Vales et al., 2015; Di Bucchianico et al., 2017).

A number of other Comet assay studies were performed with various human cell types such as HepG2, THP-1, BeWo b30 placenta, HEK293, cerebral endothelial cells, HeLa, HUVECs, THP-1, TH-1, GM07492, MCF-7, L-02 human fetus hepatocytes, NHEK normal keratinocytes, HEp-2 derived from HeLa, A431 keratinocytes, EUE human embryonic epithelial cells. The majority of these studies showed positive results (Osman et al., 2010; Shukla et al., 2011, 2013; Demir et al., 2013; NANOGENOTOX project, 2013 Documentation provided to EFSA No 7, 8 and 10); Cowie et al., 2015, Shi et al., 2015; Ferraro et al., 2016; Brown et al., 2019; Liao et al., 2019; Murugadoss et al., 2020; Kumar et al., 2020); however,

some of them demonstrated negative results (Woodruff et al., 2012; Franchi et al., 2015; Sramkova et al., 2019; Elje et al., 2020) or were equivocal (Magdolenova et al., 2012; Brzicova et al., 2019).

The Panel also evaluated *in vitro* comet assay studies that were conducted on cells from monkey, rat, mouse or hamster origin showing a similar pattern of response, the majority of studies were positive (Nakagawa et al., 1997; Barillet et al., 2010; Guichard et al., 2012; Cowie et al., 2015; Stoccoro et al., 2016; Jain et al., 2017; Brown et al., 2019; Chakrabarti et al., 2019). Three from four different types of TiO_2 tested in mouse lymphoma L5178Y cells by Nakagawa et al. (1997) were negative (anatase 21 nm, rutile 255 nm and rutile 420 nm) and one was positive (anatase 255 nm). In a study of Brown et al. (2019), E 171 was positive for strand breaks in all studied cell lines, and positive for oxidised DNA lesions only in one of them (HepG2) (Brown et al., 2019).

The Panel noted that around 57% of the available results were obtained with TiO_2 NPs < 30 nm. No clear dependence of the positive effects on the particle size in the comet assay was observed. The majority of *in vitro* comet assay gave positive results, regardless of the size of the tested particles (87% positive findings for TiO_2 particles > 30 nm and 78% positive findings for TiO_2 NPs < 30 nm). Five studies of high or limited relevance investigated, by the *in vitro* Comet assay, the effect of E 171 treatment; 4 studies were positive for strand breaks and 1 negative.

In vivo Comet assay

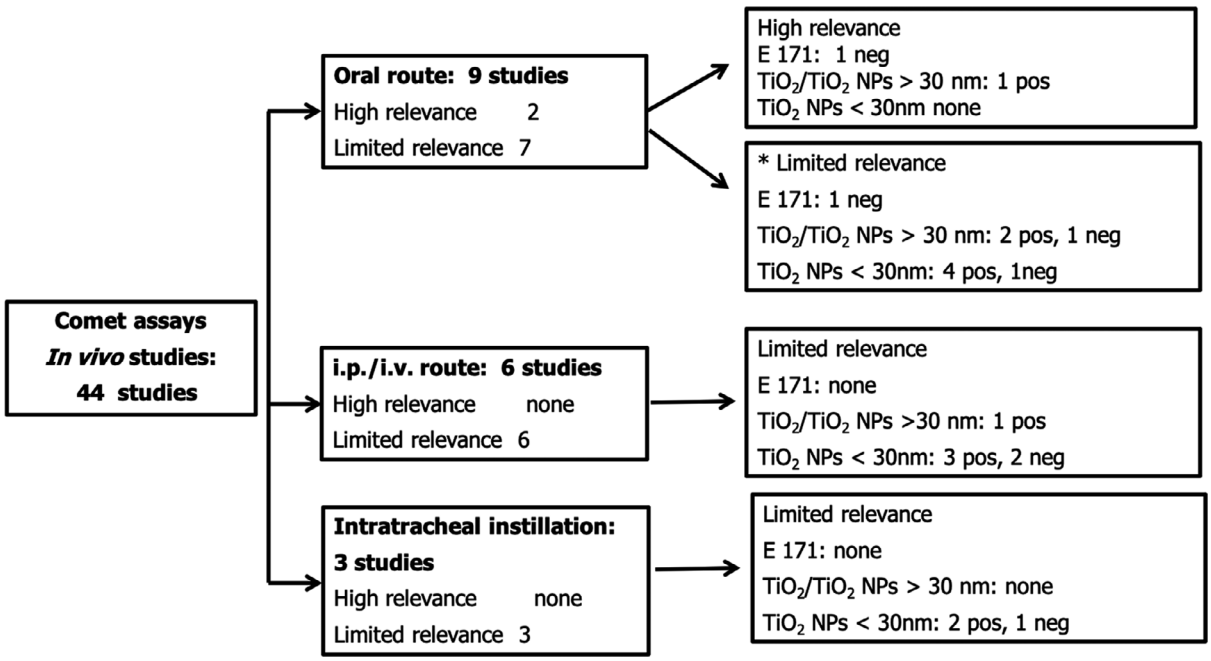
The ability of TiO_2 to induce single-strand breaks (SSBs) and Fpg-sensitive sites has been investigated by the *in vivo* comet assay. Thirty-four studies published between 2015 and 2020, in addition to 10 previously considered by the ANS Panel (EFSA ANS Panel, 2016), have been evaluated in the current assessment. Eighteen studies out of 44 were classified as of high or limited relevance (Appendices K, M, Table 9 and Figure 4).

Table 9: Summary table of test results *in vivo* Comet assay. Studies within the same route of exposure are ordered by the size of the tested material

Study design	Test material	Results	Reliability/ Relevance	Reference
Oral				
Rat; 50 and 500 mg/kg bw; once/week, 10 weeks; analysis: 24 h	NSC: 2, E 171 Anatase, three size groups of particles: 135, 305, 900 nm (TEM image)	**Negative** liver, lung	1/high	Jensen et al. (2019)
Mice; 40 and 200 mg/kg bw per day; 7 days; analysis 24 h after the last dose	NSC: 3, TiO_2, Anatase **160 nm**	**Positive** BM **Negative** liver, brain	2/limited	Sycheva et al. (2011)
Rat; 10 mg/kg bw per day; 7 days	NSC: 1, E 171: **118 nm** Anatase (range 20–340 nm) and TiO_2 NPs NM-105) **15–24 nm**	**Negative** Peyer's patch cells (both materials)	2/limited	Bettini et al. (2017)
Mice; 10, 50, 250 μg/mouse; analysis: 3 days after treatment	NSC: 1, TiO_2, Anatase **117 nm;** and TiO_2 NPs, anatase **17 nm**	**Positive** Blood leucocytes	2/limited	Murugadoss et al. (2020)
Mice; 10, 50, 100 mg/kg bw per day; 14 days; analysis: 24 h	NSC: 1, TiO_2 NPs, Anatase, **20–50 nm**	**Positive** Liver	1/high	Shukla et al. (2014)
Rat; 0.5 mg/kg bw per day; 45 days; analysis: immediate	NSC: 1, TiO_2 NPs, crystalline form unknown, **42 nm**	**Negative** Blood and liver	2/limited	Martins et al. (2017)
Mice; 0.2, 0.4, 0.8 mg/kg bw per day; 28 days; analysis: immediate	NSC: 2, TiO_2 NPs, Rutile, **25 nm**	**Positive** Liver, BM, spleen, thymus, lymph nodes	2/limited	Manivannan et al. (2020)
Mice; 500, 1,000, 2,000 mg/kg bw per day; 7 days; analysis: day 8	NSC: 4, TiO_2 NPs, Anatase, **10–25 nm**	**Positive** Liver and kidney	2/limited	Shi et al. (2015)

Study design	Test material	Results	Reliability/ Relevance	Reference
Rat; 50, 100, 200 mg/kg bw per day; 60 days; analysis: 24 h	NSC: 2, TiO_2 NPs, Anatase, **5–12 nm**	**Positive** Leucocytes	2/limited	Grissa et al. (2015)
Intraperitoneal injection				
Mice; 500, 1,000, 2,000 mg/kg bw per day 5 days; analysis: 24 h	NSC: 1; TiO_2 NPs, Anatase/ rutile **44 nm**	**Positive** BM, liver, brain	2/limited	El-Ghor et al. (2014)
Mice; 50 mg/kg bw per day; 3 days; analysis: immediate	NSC: 1; TiO_2 NPs Anatase **8.9–15.3 nm**	**Positive** Liver +OGG1/ EndoIII: lung and liver	2/limited	Li et al. (2017b)
Intravenous injection				
Rat; 5 mg/kg bw; single dose; analysis: 24 h, 1 and 2 weeks	NSC: 2, TiO_2 NPs (NM-105), **15–24 nm**	**Negative** Leucocytes (BM)	2/limited	Dobrzynska et al. (2014)
Mice; 2,10, 50 mg/kg bw per week; 4 weeks; analysis: 3 days	NSC: 1, TiO_2 NPs (P25), **15–24 nm**	**Negative** Liver	2/limited	Suzuki et al. (2016)
Rats; 0.59 mg/kg bw (single); analysis: 24 h, 1, 2, 4 weeks	NSC: 1, TiO_2 NPs (NM-105), **15–24 nm**	**Positive** PBMC (only at 24 h)	2/limited	Kazimirova et al. (2019)
Rat; 5, 25, 50 mg/kg bw per week; once/week, 4 weeks; analysis: immediate	NSC: 1, TiO_2 NPs, Anatase **10–20 nm**	**Positive** Brain	2/limited	Meena et al. (2015b)
Intratracheal instillation				
Rat; 0.5, 2.5, 10 mg/kg bw (3 times every 4 days, over 8 days); analysis 2 h and 35 days	NSC: 1, TiO_2 NPs (P25), **15–24 nm**	**Positive** Lung (2 h, 35 days), liver (2 h, 35 days), blood (35 days)	2/limited	Relier et al. (2017)
Mice; 18, 54, 162 μg/mouse (single); analysis: 1, 3 and 28 days	NSC: 1, TiO_2 NPs, Rutile, **10 nm**	**Positive,** Lung (28 days), liver (3–28 days); (negative: BAL)	2/limited	Wallin et al. (2017)
Rat; 1, 5 mg/kg bw (single); 0.2, 1 mg/kg bw (once/week) x 5 weeks; analysis: 3 and 24 h	NSC: 1, TiO_2 NPs, Anatase, **5 nm**	**Negative** Lung	1/limited	Naya et al. (2012b)

BAL: bronchoalveolar lavage cells; NSC: nanoscale considerations, NP: nanoparticle; PBMC: peripheral blood mononuclear cells.



*: Some of the studies used more than one test material and, in these cases, the results are reported separately.

Figure 4: *In vivo* Comet assays – Summary of test results

Among the nine gavage studies selected for the assessment, six were positive (Sycheva et al., 2011; Shukla et al., 2014; Grissa et al., 2015; Shi et al., 2015; Manivannan et al., 2020; Murugadoss et al., 2020) and three were negative (Bettini et al., 2017; Martins et al., 2017; Jensen et al., 2019) including the two studies performed with E 171 (Bettini et al., 2017; Jensen et al., 2019). To identify possible factors responsible for the different outcomes of the assays, the Panel took into consideration physico-chemical characteristics of TiO_2 NPs (crystalline form, size of constituent particles, shape and agglomeration state), time of exposure, doses and target tissues.

The crystalline form, the size and the agglomeration state of TiO_2 NPs have all been hypothesised to play a role in the genotoxic potential. The majority of the comet assays (in which the crystalline form is indicated) contained > 90% TiO_2 in the anatase form. Only one study used pure rutile (Manivannan et al., 2020). No obvious correlation could be identified between specific physico-chemical properties of the TiO_2 particles and the outcome of the assays.

Neither the time of exposure, which ranged from a few days (3–7 days) up to several weeks (4–10 weeks) nor the administered TiO_2 particles doses discriminated positive from negative results. In addition, the Panel calculated a cumulative dose by integrating dose and time of treatment (Table 9). This factor alone, however, appeared not to be the main determinant of the assay results.

Comet assays were performed on several target tissues. Negative results were reported for the lung (Jensen et al., 2019) and in the immune cells of the jejunal and ileal Peyer's patches (Bettini et al., 2017) and brain (Sycheva et al., 2011). Contradictory results were reported for leucocytes (Grissa et al., 2015; Martins et al., 2017; Murugadoss et al., 2020) and liver (Sycheva et al., 2011; Shukla et al., 2014; Shi et al., 2015; Martins et al., 2017; Jensen et al., 2019). Positive results were observed for bone marrow (Sycheva et al., 2011; Manivannan et al., 2020), thymus and afferent lymph node (Manivannan et al., 2020) as well as in the kidney (Shi et al., 2015) and spleen (Manivannan et al., 2020). The Panel noted that the majority of the positive results were obtained from organs of the reticulo-endothelial system.

Additional information was provided by four studies with intravenous administration of TiO_2 NPs (anatase or anatase/rutile being anatase > 80%, < 30 nm size). Negative results were reported in liver with TiO_2 NPs (P25) (Suzuki et al., 2016), while positive ones were reported in the brain with TiO_2 NPs (anatase, 10–20 nm) (Meena et al., 2015b). Using the same test item (NM-105, a mixture of anatase and rutile), experimental conditions and harvest time, positive results were observed from peripheral blood leucocytes (Kazimirova et al., 2019) whereas leucocytes from the bone marrow were negative (Dobrzynska et al., 2014).

Intraperitoneal administration of TiO_2 NPs induced SSBs in the liver, lung, brain and bone marrow in a large range of doses and a relatively short time of exposure (50–2,000 mg/kg bw for 3–5 days) (El-Ghor et al., 2014; Li et al., 2017b).

Two studies using intratracheal instillation were negative with no DNA damage observed in bronchoalveolar lavage (BAL) (Wallin et al., 2017) and lung (Naya et al., 2012) (single and chronic exposures up to 5 weeks in the range of 1–5 mg/kg bw). In contrast, Wallin et al. (2017) and Relier et al. (2017) reported positive results in liver and lung. The induction of DNA damage in liver following intra-tracheal instillation demonstrates a systemic effect which is possibly triggered by an inflammatory response observed in the lung.

Concluding remarks

Based on the results of the *in vitro* and *in vivo* comet assays, the Panel concluded that TiO_2 particles have the potential to induce DNA damage. The Panel noted that a significant portion of the studies were performed using TiO_2 NPs < 30 nm, however some positive results were also observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in Comet assay was observed.

4.3.4. DNA Binding

The ability of TiO_2 NPs (anatase, 5 nm) to bind DNA *in vivo* was investigated by Li et al., 2010. Binding to genomic DNA prepared from livers of TiO_2 NPs-treated ICR mice (by i.p. 5, 10, 50, 100 and 150 mg/kg bw per day for 14 days) was investigated by UV–Vis absorption spectroscopy, circular dichroism (CD), extended X-ray absorption fine structure (EXAFS) spectroscopy and gel electrophoresis. A dose-dependent increase in the content of TiO_2 NPs in liver DNA was identified by ICP-MS. CD spectroscopy indicates that changes in the DNA conformation occur in the 50–150 mg/kg bw per day dose range. In addition, EXAFS spectroscopy indicates that nano-anatase TiO_2 NPs can be bound with the oxygen or phosphorus atoms of the nucleotide and nitrogen atoms of base pairs in DNA.

After intranasal administration (300 μg/rat per day for 45 days), the interaction between TiO_2 particles and liver DNA extracted from Sprague–Dawley rats was investigated by UV–Vis absorption spectrometry, atomic force microscopy (AFM), TEM, micro-synchrotron radiation X-ray fluorescence (m-SRXRF) and gel electrophoresis (Jin et al., 2013). The analysed TiO_2 particles were a) nano-anatase (d < 25 nm); b) micro-rutile (d < 5 μm); c) a mixture of 5–10% rutile and 90–95% anatase (d < 100 nm). DNA binding was observed with the TiO_2 NPs anatase a) and TiO_2 NPs rutile/anatase mixture c) but not with micro rutile b). According to the authors TiO_2 NPs anatase can insert itself between DNA base pairs or covalently bind to DNA nucleotide via P–O–Ti–O bond. The Panel noted that evidence for covalent binding was not provided since a decrease in UV absorption can also be explained by non-covalent interactions.

Assessment of *in vitro* DNA binding capacity of TiO_2 NPs (< 100 nm) by UV–Vis spectroscopy identified an hyperchromic effect, probably due to strong stacking interactions between human genomic DNA and TiO_2 NPs (Patel et al., 2016, 2017). In addition, fluorescence emission spectra of intercalated ethidium bromide and human genomic DNA incubated with increasing concentrations of TiO_2 NPs indicate that these NPs also intercalate DNA strands. The authors suggest that all these results are due to electrostatic interactions between DNA and TiO_2 NPs leading to conformational changes in DNA. A strong binding affinity of TiO_2 NPs with human genomic DNA was identified by fluorescence spectroscopy (binding constant: 4.1×10^6 M^{-1}). TiO_2 NPs (14 nm) interaction with calf thymus DNA was studied by UV–Vis spectroscopy and molecular docking analysis (Ali et al., 2018). The hyperchromic behaviour observed by UV–Vis spectrometry confirms unwinding of double-stranded DNA. The DNA binding constant was found to be 5.4×10^3 M^{-1}. Molecular docking analysis revealed a selective binding of TiO_2 NPs with A-T bases in minor groove of DNA.

Hekmat et al. (2013) investigated the structural changes in calf thymus DNA caused by the combined exposure to TiO_2 NPs anatase (< 10 nm) and doxorubicin (DOX) as well as by the single components. UV–Vis and CD spectrometry, thermal denaturation and fluorescence emission spectra demonstrated that there is an interaction of TiO_2 NPs with DNA leading to changes in the secondary structure of the DNA helix.

By using a similar approach, the same group investigated the structural changes in calf thymus DNA induced by a combined treatment of TiO_2 NPs anatase (< 10 nm) + paclitaxel (PTX) in comparison to single exposures to either compound. Upon addition of TiO_2 NPs to the solution of DNA,

hyperchromism was observed, indicating the formation of a complex between DNA and TiO_2 NPs (Hekmat et al., 2020).

The ability of TiO_2 NPs (21 nm) to interact with DNA (from salmon sperm) was confirmed by other analytical techniques (capillary electrophoresis coupled with UV and Fourier transform infrared spectroscopy). Electrostatic interactions of TiO_2 NPs via the sugar-phosphate backbone were demonstrated both with double-stranded and single-stranded DNA, with the last one showing stronger interactions (Alsudir and Lai, 2017).

The Panel noted that the interaction between TiO_2 NPs and DNA resulted in spectrally contrasting effects when examined by UV–VIS spectrometry *in vitro* (hyperchromicity) or after exposure *in vivo* (hypochromicity).

In conclusion, there is evidence, from both *in vitro* and *in vivo* studies, for interaction(s) of TiO_2 NPs with DNA. However, due to the techniques employed, the precise nature of these interactions, i.e. whether involving covalent or non-covalent binding, could not be established.

4.3.5. Other studies

γH2AX foci and other markers of DNA Damage

The induction of γH2AX foci, a marker of DNA double-strand breaks, by TiO_2 particles was investigated in four *in vitro* and two *in vivo* studies.

Kathawala et al. (2015) reported a slight but statistically significant increase in the percentage of cells with γH2AX foci in human primary epidermal keratinocytes and a concomitant increase in the intracellular ROS. Toyooka et al. (2012) reported a concentration-dependent increase in the induction of γH2AX foci in A549 human lung carcinoma cells both with micro- and (more pronounced) with nanoparticles and a concomitant induction of double-strand breaks, as detected by biased sinusoidal field gel electrophoresis (BSFGE). On the contrary, Jugan et al. (2012) in the same cell line observed no similar effect on the induction of γH2AX foci. Exposure to TiO_2 particles (12, 24 and 142 nm) did not significantly increase the amount of γH2AX foci in the nuclei of NRK-52E rat kidney cells (Barillet et al., 2010).

In vivo, a dose-dependent increase in cells with γH2AX foci was reported in the bone marrow of C57BL/6Jp^{un}/p^{un} mice by Trouiller (Trouiller et al., 2009). Another study (Chen et al., 2014) also reported a similar result in the bone marrow of Sprague–Dawley male rats, in the absence of MN induction.

Overall, the induction of γH2AX foci, a marker of DNA double-strand breaks, was observed in two out of four *in vitro* studies and was also reported in two *in vivo* studies.

A ToxTracker assay, that includes the analysis of the expression of genes responsive to DNA damage and oxidative stress, was negative for all the endpoints in primary mouse embryonic fibroblasts (Brown et al., 2019).

Limited relevance was assigned to all of these studies.

Oxidised DNA bases

The induction of 8-oxodG (as a marker of oxidation-induced DNA damage) by TiO_2 particles was investigated in five *in vitro* studies (to which limited relevance was assigned), four of which were positive.

Significant increases of 8-oxodG in DNA (measured by HPLC with electrochemical detection) were reported in human peripheral blood mononuclear cells (PBMC, mixed population of lymphocytes and monocytes) after treatment with TiO_2 NPs and TiO_2 particles covering the nano and micro range (anatase, rutile and a mixture of anatase and rutile) (Andreoli et al., 2018). Also in human colon carcinoma Caco-2 cells a significant increase in basal levels of 8-oxodG compared to control was reported after treatment with anatase NPs (20–60 nm) (Zijno et al., 2015).

A treatment with TiO_2 NPs (21 $\pm$ 9 nm) increased the level of 8-oxodG lesions (HPLC–MS/MS) in two different human cell lines: BEAS-2B normal bronchial lung cells and A549 alveolar carcinoma lung cells (Biola-Clier et al., 2017). In another study using A549 alveolar carcinoma lung cells, the only oxidised base detected in cells exposed to TiO_2 NPs (anatase 12 nm, P25 and rutile 21 nm) was 8-oxodG whereas other lesions such as thymidine glycols, 5-(hydroxymethyl)-2′-deoxyuridine, 5-formyl-2′-deoxyuridine or 8-oxo-7,8-dihydro-2′-deoxyadenosine were either below the detection limit or were not produced in higher frequency upon treatment. No induction of oxidised bases were observed with TiO_2 (anatase, 140 nm) in this study (Jugan et al., 2012)

In contrast, no significant increase of the 8-oxodG (HPLC–MS/MS) level was reported in a study (Dorier et al., 2019) in which a co-culture of Caco-2 colon and HT-29 human colon cancer cells was treated with the additive E 171 and two different TiO_2 NPs (12 ± 3 nm and 24 ± 6 nm).

In an *in vivo* study, oxidation-induced DNA damage was investigated by measuring the level of 8-oxodG in DNA isolated from livers of TiO_2 NP-treated and untreated mice (Trouiller et al., 2009). Administration of TiO_2 NPs (P25) with drinking water at a dose of 100 mg/kg bw per day for 5 days resulted in a 1.5-fold increase of the 8-oxodG level in NP-treated mice, compared to the control group.

Another *in vivo* study on the induction of 8-oxodG (Rehn et al., 2003) was not considered relevant for this assessment because it was based on the analysis of lung cells after intratracheal instillation.

Overall, based on the available *in vitro* studies, nano- and microparticles of anatase and rutile seem to have a potential to induce oxidation of DNA resulting in 8-oxodG.

Reactive oxygen species

The induction of ROS, such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, was investigated in many *in vitro* and *in vivo* studies in parallel with investigation of DNA damage by comet and MN assays.

There are several *in vitro* studies in which the induction of ROS by TiO_2 particles has been observed in different cell lines (Kang et al., 2008; Xu et al., 2009; Shukla et al., 2011, 2013; Wang et al., 2011, 2019; Guichard et al., 2012; Jugan et al., 2012; Saquib et al., 2012; Kathawala et al., 2015; Khan et al., 2015; Shi et al., 2015; Tomankova et al., 2015; Jain et al., 2017; Dorier et al., 2019; Liao et al., 2019; Santonastaso et al., 2019). In some of these studies, it was shown that the generation of ROS was inhibited by the addition of antioxidants (Kang et al., 2008; Xu et al., 2009) or SOD (e.g. Wang et al., 2011). In the study performed by Guichard et al. (2012), the induction of ROS in SHE cells was higher with TiO_2 NPs (anatase and rutile < 100 nm) than with TiO_2 anatase particles of 160 nm size and rutile particles of 530 nm size.

In contrast, no induction of intracellular ROS (evaluated by flow cytometry) was observed in an *in vitro* study in BEAS-2B normal bronchial lung cells after one week of exposure to anatase TiO_2 NPs (NM-102) (Vales et al., 2015) and no significant increase of ROS was observed with E 171 at concentrations of 0.143 or 1.43 μg/cm^2 in human colon carcinoma Caco-2 cells (Proquin et al., 2017).

In vivo, increased levels of ROS, NO, MDA, IFN-γ, TNF-α and activation of NF-κB and decreased levels of SOD and GSH-Px as well as expression of apoptosis markers (p53, Bax, Bcl-2 and cyto c) were observed in rats exposed to TiO_2 NPs (anatase) by intravenous injection at doses up to 50 mg/kg bw once a week for 4 weeks (Meena et al., 2015b). TiO_2 NPs induced increased ROS levels also after intratracheal instillation of mice (Danielsen et al., 2020). Likewise, exposure of mice to TiO_2 NPs (anatase), administered by gavage at doses of up to 100 mg/kg bw per day for 14 days, resulted in increased levels of ROS (Shukla et al., 2014). In a study in which TiO_2 NPs (anatase) were administered by gavage to mice at doses of 500, 1,000 and 2,000 mg/kg bw per day for 7 days, increased levels of ROS were observed at 1,000 and 2,000 mg/kg bw in a dose-dependent manner in liver and kidney (Shi et al., 2015). However, no changes in the ROS levels were observed in these organs at 500 mg/kg bw, compared to the control group. The administration of TiO_2 NPs at the dose of 1,000 mg/kg bw to Nrf2$^{(-/-)}$ mice (in which Nrf2, a regulator for the expression of antioxidant genes, was knocked out) resulted in higher increases of the ROS levels in liver and kidney than the administration of the same dose to wild type mice (Shi et al., 2015).

Epigenetic DNA methylation

There are some studies in which epigenetic DNA methylation has been investigated.

A decrease of global DNA methylation was observed with TiO_2 NPs (P25) in several mammalian cell cultures, whereas promotor methylation of several specific genes was increased in a study performed by Pogribna et al. (2020). In this study, altered expression levels of several genes involved in the regulation of DNA methylation were also observed (Pogribna et al., 2020). In a human lung carcinoma cell line A549, TiO_2 NPs (P25) induced a statistically significant demethylation after 72 h of exposure while no effect was observed after 48 h (Stoccoro et al., 2017). A significant reduction of genomic DNA methylation levels after treatment with TiO_2 NPs (60 nm) was observed in the human lung carcinoma cell line A549 and a human bronchial epithelial cell line in a study by Ma et al. (Ma et al., 2017). Bidirectional changes in methylation of some specific loci were observed *in vitro* by (Emi et al., 2020) with TiO_2 particles (1 μm).

Epigenetic modifications, particularly in gene promoter regions, have been shown to result in changes in the expression of DNA repair genes. Biola-Clier et al. (2017) studied overall DNA

methylation and specific methylation of DNA repair gene promoters after TiO_2 NPs (21 nm) exposure. They found that 31 out of 44 upstream DNA regulators for genes involved in several DNA repair pathways including nucleotide excision repair, base excision repair, mismatch repair and double-strand break repair were downregulated.

These studies might be worth to address epigenetic endpoints. They were considered as supporting information for the evaluation of genotoxicity.

Cell transformation

Statistically significant increases in the frequency of morphologically transformed Balb/c 3T3 cells (mouse embryo fibroblasts) were observed after treatment with TiO_2 NPs (P25) (Stoccoro et al., 2016). In a further cell transformation assay with the same cell line, a significant induction of transformed colonies (foci type III) was observed with rutile (micro- and nanosized), whereas no significant effects were observed with anatase (micro and nanosized) (Uboldi et al., 2016). Increased number of colonies growing in soft agar has been observed with TiO_2 NPs (anatase, 21 and 50 nm) in human embryonic kidney cells and in mouse embryonic fibroblasts (NIH/3T3). The effect was concentration-related and statistically significant at 1,000 μg/ml but not at 10 and 100 μg/mL and also not with microparticles (Demir et al., 2015). In another study, a statistically significant concentration-dependent increase in the number of colonies growing on soft-agar in human bronchial epithelial cells (BEAS-2B cells) was shown for both the total of colonies and medium-large size colonies after exposure to TiO_2 NPs (anatase, 22 nm) at concentrations up to 20 μg/mL (Vales et al., 2015).

The cell transformation assays provide information on initial steps of carcinogenesis that may include both genotoxic and non-genotoxic events. Results from cell transformation assays are considered to be of limited relevance for the evaluation of genotoxicity. Their relevance for the assessment of carcinogenicity is also limited.

4.3.6. Mode of Action

Numerous studies, both *in vitro* and *in vivo*, indicate that TiO_2 particles, over a wide range of sizes, induce DNA strand breaks, oxidatively generated DNA lesions and chromosomal damage**.**

Several mechanisms have been proposed to explain the genotoxicity associated with TiO_2 NPs exposure. They invoke DNA damage by reactive oxygen and nitrogen species (ROS/RNS) which may be triggered by inflammation, by intrinsic ability of TiO_2 NPs or via induction of mitochondrial dysfunction. There is also some evidence that TiO_2 NPs:DNA binding, possibly via electrostatic interactions, may alter DNA secondary structure.

In vivo exposure to TiO_2 NPs can be associated with an inflammatory response. This occurs independently of the route of administration (Saber et al., 2012; El-Ghor et al., 2014; Shukla et al., 2014; Meena et al., 2015b; Shi et al., 2015; Relier et al., 2017; Wallin et al., 2017; Murugadoss et al., 2020). This response is characterised by increased macrophage and neutrophil infiltration, the release of inflammatory mediators (chemokines, cytokines) and the increased production of ROS and oxidative stress markers (Dankovic et al., 2007; Olmedo et al., 2008; Chen et al., 2009). Following oral administration, the induction of DNA strand breaks was associated with an inflammatory response (Shukla et al., 2014). Along the same lines, intravenous administration of TiO_2 NPs induced an increase in MN frequency associated with an inflammatory response (Kumar et al., 2016).

Co-administration of TiO_2 NPs and chlorophillin, a free-radical scavenger, was associated with a reduction in DNA strand breaks and chromosomal damage (El-Ghor et al., 2014). In addition, TiO_2 NPs induced more DNA strand breaks in mice defective in the Nrf2 transcription factor ($Nrf2^{-/-}$) than in their wild type counterparts. Since Nrf2 regulates antioxidant and inflammatory responses (Shi et al., 2015), both sets of observations are consistent with a role of ROS/RNS and inflammation in TiO_2 NPs-associated DNA damage, which is reflected in positive findings in several genotoxicity studies. The Panel noted, however, that there is no simple correlation between the extent of TiO_2 NP-induced inflammation and DNA damage since an inflammatory response without genotoxic effects can also be observed (e.g. Saber et al., 2012).

An alternative mechanism invokes the intrinsic ability of TiO_2 NPs to generate ROS/RNS, i.e. to generate reactive radicals also in an acellular system (Knaapen et al., 2004). This hypothesis is supported by experimental evidence coming from investigations with acellular systems (Gilmour et al., 1997; Fenoglio et al., 2009). Intracellular superoxide can also be formed by interaction of TiO_2 NPs with proteins to form a TiO_2 NPs corona (Jayaram and Payne, 2020). Additional potential sources of ROS include activation of cytoplasmic NADPH oxidases (Bedard and Krause, 2007) and damage to the

mitochondrial membrane (Ghosh et al., 2013). With regard to the latter possibility, TiO_2 NPs (< 100 nm) have been shown to be localised in liver mitochondria (Louro et al., 2014) and to induce mitochondrial swelling, promote membrane fluidity and increase ROS generation (Kathawala et al., 2015; Barkhade et al., 2019).

In vitro studies in different experimental models using blood, GI tract, liver, lung and other organs and tissues, showed an association between TiO_2 induced DNA strand breaks/chromosome damage and oxidative stress, measured as increased ROS level and/or decreased level of antioxidants (Turkez and Geyikoglu, 2007; Shukla et al., 2011, 2013; Prasad et al., 2013; Srivastava et al., 2013; Proquin et al., 2017; Stoccoro et al., 2017; Liao et al., 2019; Tables on *in vitro* comet assay in Appendices J, L, N)

Additionally, *in vitro* studies indicated that TiO_2 NPs exposure induced the well-known premutagenic DNA lesion produced by ROS, i.e. 8-oxodG (Shukla et al., 2011, 2013; Jugan et al., 2012; Demir et al., 2013; Stoccoro et al., 2016, 2017; Di Bucchianico et al., 2017; El Yamani et al., 2017; Schneider et al., 2017; Andreoli et al., 2018; Zijno et al., 2020). However, a lack of association between DNA strand breaks and DNA 8-oxodG levels has also been reported. Indeed, increased DNA 8-oxodG levels in Caco-2 cells (Zijno et al., 2015) and human lung cells (Bhattacharya et al., 2009) were not associated with increased DNA strand breaks. Moreover, no firm conclusions can be drawn from the few *in vivo* studies investigating the level of TiO_2 NPs-induced oxidatively damaged DNA. No changes in the levels of liver fpg-sensitive sites were found in intravenous-treated $Ogg1^{-/-}$ mice that are defective in the repair of DNA 8-oxodG (Asare et al., 2016), whereas oral exposure increased levels of oxidatively damaged liver DNA (Trouiller et al., 2009; Shukla et al., 2014).

TiO_2 NPs-induced production of ROS may also trigger the formation of DNA double-strand breaks as measured by γH2AX foci *in vitro* (Kathawala et al., 2015; Wang et al., 2019). This property is reflected in an increase of CA and MN frequency in several *in vitro* studies (Turkez and Geyikoglu, 2007; Osman et al., 2010; Shukla et al., 2011; Prasad et al., 2013; Srivastava et al., 2013; Kurzawa-Zegota et al., 2017; Proquin et al., 2017; Stoccoro et al., 2017; Liao et al., 2019). In some of these studies the increased MN frequency was correlated with DNA damage measured by the comet assay (Osman et al., 2010; Shukla et al., 2011, 2013; Prasad et al., 2013; Kurzawa-Zegota et al., 2017; Proquin et al., 2017; Stoccoro et al., 2017; Liao et al., 2019). In addition, six *in vivo* studies investigated both the induction of DNA strand breaks and chromosomal damage in the same experimental setting. Four of these studies provided concordant positive results for both endpoints (El-Ghor et al., 2014; Shukla et al., 2014; Grissa et al., 2015; Manivannan et al., 2020) indicating a potential clastogenic mode of action.

An indirect confirmation of an association between TiO_2 NPs exposure and induction of DNA double-strand breaks comes from the positive result reported in an *in vivo* DNA deletion assay in the p^{un} locus (eye-spot assay) by Trouiller et al. (2009). The eye-spot assay detects deletions of one copy of a duplicated 70-kb DNA fragment within the p^{un} locus and is considered an experimental method suitable for the detection of homologous recombination events (Karia et al., 2013). Homologous recombination can be associated with the repair of double-strand breaks or the processing of a blocked DNA replication fork. Therefore, a positive result in the eye-spot assay could reflect the ability of TiO_2 NPs to cause DNA breaks, consistently with the findings reported in the comet and in MN/CA studies.

On the other hand, no evidence of induction of gene mutations *in vivo* was reported in four different studies analysing target genes that are considered suitable for detecting a wide range of molecular events, including point mutations, small and large deletions (Pig-a, *gpt*, Spi and *lacZ* mutations).

Studies applying kinetochore staining (Rahman et al., 2002) or FISH analysis (Stoccoro et al., 2017) indicate that MN induction by TiO_2 NPs predominantly occurs via clastogenic events.

TiO_2 NPs have been found in the nucleus either as single particles or as agglomerates (Jugan et al., 2012; Ahlinder et al., 2013; Shukla et al., 2013; Louro et al., 2014; Jain et al., 2017). Some studies failed to demonstrate internalisation of TiO_2 particles into nuclei (Singh et al., 2007; Di Virgilio et al., 2010). In contrast, Ahlinder et al. (2013) demonstrated nuclear uptake by two techniques – TEM and Raman spectroscopy. Most of TiO_2 uptake studies were performed *in vitro* on TiO_2 NPs smaller than 30 nm. Bettini et al. (2017) reported that after administration of E 171, TiO_2 particles could be internalised into nucleus. In addition, TiO_2 particles were identified in the nuclei closely associated with the phosphorus-positive chromatin signal (Bettini et al., 2017).

The mechanisms underlying nuclear internalisation is to a large extent unknown. Generally, NPs may be transported to the cell nucleus through nuclear pores by passive diffusion, or interaction with transport receptors (Panté and Kann, 2002; Terry et al., 2007). They also could enter the nucleus

during the cell division (Kim et al., 2012). According to Ahlinder et al. (2013), their findings provide evidence of possible direct interactions between TiO_2 NPs and DNA as a cause of genotoxicity.

Following nuclear internalisation, TiO_2 NPs might interact with proteins involved in the control of chromosome segregation and the spindle apparatus. Chromosome mis-segregation due to disturbance of mitotic progression has been described in NIH 3T3 cells following long-term exposure to TiO_2 NPs (Huang et al., 2009) and an interaction of TiO_2 particles in E 171 with the centromere region during mitosis has been reported in colon HCT116 cells (Proquin et al., 2017). While aneugenicity seems to be a plausable effect for TiO_2 particles present in the nucleus, the MN studies with centromere staining suggest that clastogenicity is the predominant mode of action.

A limited number of studies indicated that TiO_2 NPs can interact with DNA. The authors of these studies conclude that binding of TiO_2 NPs to DNA occurs *via* electrostatic and/or other kinds of non-covalent interaction leading to minor alterations in DNA conformation and secondary structure (Hekmat et al., 2013; Jin et al., 2013; Patel et al., 2016; Alsudir and Lai, 2017; Ali et al., 2018; 2020). However, the Panel considered that while there is limited understanding of the non-covalent interactions with DNA, there is no evidence of covalent binding to DNA.

4.3.7. Conclusions

Overall, combining the available lines of evidence, the Panel concluded that TiO_2 particles had the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations.

No clear correlation was observed between the physico-chemical properties of TiO_2 NPs, such as crystalline form, size of constituent particles, shape and agglomeration state, and the outcome of either *in vitro* or *in vivo* genotoxicity assays.

There is some evidence for internalisation of TiO_2 nanoparticles in the nucleus and mitochondria.

There is evidence for several modes of action for genotoxicity that may operate in parallel:

- Direct interaction of TiO_2 nanoparticles with DNA (there is no proof for covalent binding).
- Direct formation of reactive (oxygen) species due to intrinsic properties of TiO_2 nanoparticles.
- Reactive (oxygen) species formation via TiO_2 particles-induced inflammation.
- Reactive (oxygen) species formation via interference of TiO_2 nanoparticles with mitochondrial function.

Additionally, there are indications that TiO_2 particles may:

- induce epigenetic modifications affecting the expression of genes involved in the maintenance of genome function (e.g. downregulation of some genes involved in DNA repair pathways).
- interact with proteins involved in the control of chromosome segregation and the spindle apparatus.

The relative contribution of the modes of action mentioned above to the genotoxicity elicited by TiO_2 particles is unknown and there is uncertainty on whether a threshold mode of action could be assumed. Even if it was assumed that all modes of action would be indirect, the available data would not allow identification of a threshold dose. Therefore, the Panel concluded that a concern for genotoxicity of TiO_2 particles that may be present in E 171 cannot be ruled out. A cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified.

4.4. Exposure

4.4.1. Authorised uses and use levels

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

Currently, E 171 is authorised in four food categories at *quantum satis* (*QS*), in addition it is listed among the food colours of Group II authorised at QS in other 44 food categories. Table 10 lists the 48 food categories in which E 171 is authorised.

Table 10: MPLs of E 171 in foods according to the 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	Pre-cooked or processed cereals	Group II		QS
07.2	Fine bakery wares	Group II		QS
08.3.3	Casings and coatings and decorations for meat	Group II	Except edible external coating of *pasturmas*	QS
09.2	Processed fish and fishery products, including molluscs and crustaceans	Group II	only surimi and similar products and salmon substitutes based on *Theragra chalcogramma, Pollachius virens* and *Clupea harengus*	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

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	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
14.2.7.3	Aromatised wine-product cocktails	Group II		QS
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

Food category number	Food category name	E-number/ group	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
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, excluding food supplements for infants and young children	Group II		QS
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children	Group II		QS

MPL: maximum permitted level.

Annex II part B1 lists all authorised food colours and according to Annex II part E, all food colours are authorised for use for the decorative colouring of egg shells, therefore E 171 is also authorised on the shell of eggs (FC 10.1 and 10.2).

E 171 is not authorised according to Annex III to Regulation (EC) No 1333/2008.

4.4.2. Exposure data

Reported use levels or data on analytical levels of E 171

Data on the occurrence of E 171 in food were collected at the time of the re-evaluation of E 171 by the ANS Panel by means of a call for data launched in 2013.[20] In response to this call, 61 use levels and 28 analytical results on E 171 were submitted to EFSA by industry and Member States, respectively (EFSA ANS Panel, 2016). These use levels cover 14 food categories.

Levels of E 171 in food have been reported in scientific publications (Lomer et al., 2000; Chen et al., 2013; Weir et al., 2012; Peters et al., 2014; Rompelberg et al., 2016; Kim et al., 2018; Lim et al., 2018; Taboada-López et al., 2019). Most of these publications are reporting on the development of new methodologies for the analysis of E 171 in food. Foods usually analysed are chewing-gum, coatings, fine bakery wares, beverage whiteners, flavoured drinks and sauces. Most of the analysed foods correspond to food categories already considered in the re-evaluation of E 171 (EFSA ANS Panel, 2016). It has been noted that some of the use levels reported in scientific publications refer to food categories for which E 171 is not authorised in the EU. Since most of the publications are from outside Europe, it is uncertain if products in which E 171 has been analysed, are available on the European market.

In addition, in a report issued by the Dutch National Institute for Public Health and the Environment (RIVM) (Sprong et al., 2015), data on use levels submitted by industry in the Netherlands, as well as analytical data afterwards also published by Rompelberg et al. (2016) are reported.

For the current exposure assessment, the Panel considered, in addition to the data collected during the EFSA call for data in 2013, use levels reported by industry in the Netherlands in order to increase the number of food categories having data from 14 to 16, since two additional food categories were covered (Sprong et al., 2015). Levels used for estimating the dietary exposure assessment of E 171 are presented in Appendix Q.

Summarised data extracted from the Mintel's Global New Products Database

The Mintel's GNPD is an online database which monitors new introductions of packaged goods in the market worldwide. It contains information on over 3.6 million food and beverage products of which more than 1,300,000 are or have been available on the European food market. Mintel started covering EU's food markets in 1996, currently having 24 out of its 27 member countries plus Norway and the UK presented in the Mintel's GNPD.[21]

[20] https://www.efsa.europa.eu/sites/default/files/consultation/callsfordata/130327.pdf
[21] Missing Cyprus, Luxembourg and Malta.

For the purpose of this Scientific Opinion, the Mintel's GNPD[22] was used for checking the labelling of food and beverage products and food supplements for E 171 within the EU's food market as the database contains the compulsory ingredient information on the label.

According to the Mintel's GNPD, E 171 was labelled on more than 13,000 products of which 5,300 products were published in the database between January 2016 and February 2021. Since 2016, the number of food products labelled with E 171 is decreasing each year.

Appendix R lists the percentage of the food products labelled with E 171 out of the total number of food products per food subcategory according to the Mintel's GNPD food classification for the period January 2016–February 2021.[22] The percentages ranged from less than 0.1% in many food subcategories to 52%. The highest percentages are for the subcategories 'Sticks, Liquids & Sprays' (52.2%), 'gum' (39%), 'mixed assortments' (20.9%), 'lollipops' (14.6%), 'vitamins and dietary supplements' (13.9%). The average percentage of food products in the EU labelled as containing E 171 was 1% of all the food products included in the subcategories in Mintel in which E 171 is listed.

As can be seen in Appendix R, some foods are labelled with E 171 because the additive is authorised in an ingredient of the food. This is the case for instance for 'spoonable yoghurt' (FC 01.4) and 'chocolate tablets' (FC 05.1) which contain dragées (i.e. confectionary) authorised to contain titanium dioxide (E 171) as belonging to the FC 05.4. For a few other food categories in which the use of E 171 is not authorised, foods were found labelled as containing E 171 (e.g. nectars, sucrose, tea, bread and bread products). In these categories, the number of foods labelled with titanium dioxide (E 171) is low (approximately 35 products in total).

Food consumption data used for exposure assessment

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.[23] Competent authorities in the 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, 2011).

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons may not be appropriate. 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 includes the currently best available food consumption data across Europe.

Food consumption data from the following population groups were used for the exposure assessment: infants, toddlers, children, adolescents, adults and the elderly. For the present assessment, food consumption data were available from 40 different dietary surveys carried out in 23 European countries (Table 11). Since more dietary surveys are available in the EFSA comprehensive database compared to 2016, more countries are now considered in each population group. As the 95th percentile of exposure was only calculated for those population groups with a sufficiently large sample size (EFSA, 2011), in the present assessment, it was not estimated for infants from Italy and France, for toddlers from Belgium and Italy and for adolescents from Estonia.

Table 11: Population groups considered for the exposure estimates of 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, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, UK
Toddlers[(a)]	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain, UK

[22] http://www.gnpd.com/sinatra/home/ accessed on 8/2/2021.
[23] February 2020 release. Available online: http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm

Population	Age range	Countries with food consumption surveys covering more than 1 day
Children[(b)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, UK
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain, Sweden, UK
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, UK
The elderly[(b)]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, UK

(a): The term 'toddlers' in the Comprehensive Database (EFSA, 2011) corresponds to 'young children' in Regulations (EC) No 1333/2008 and (EU) No 609/2013.
(b): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Comprehensive Database (EFSA, 2011).

Consumption records were codified according to the FoodEx2 classification system (EFSA, 2015). Nomenclature from the FoodEx classification system was linked to the food categorisation system (FCS) as presented in Annex II of Regulation (EC) No 1333/2008, part D, to perform the exposure assessments. In practice, the FoodEx2 food codes were matched to the FCS food categories.

Food categories considered for the exposure assessment of E 171

The food categories for which occurrence data of E 171 are available were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx2 classification system), at the most detailed level possible (EFSA, 2015).

Data on the food supplements (FC 17) do not indicate which is the form (liquid or solid) of the product. Therefore, the levels were assigned to the whole FC 17.

The EFSA Comprehensive European Food Consumption Database considered in the current assessment is different from the one used in the re-evaluation of E 171 (EFSA ANS Panel, 2016) and allows to consider FC 05.4 for which reported use level were available at that time.

16 food categories were taken into account as occurrence data were available (Appendix Q). For relevant food categories, the refinements considering the restrictions/exceptions when as set in Annex II to Regulation No 1333/2008 were applied.

4.4.3. Exposure estimates

Dietary exposure to E 171 from its use as a food additive

The Panel estimated the chronic dietary exposure to E 171 for the following population groups: infants, toddlers, children, adolescents, adults and the elderly. The methodology to estimate dietary exposure to E 171 in the current assessment and the different scenarios – maximum level exposure assessment scenario, refined exposure assessment scenarios (brand-loyal and non-brand-loyal) and food supplements consumers only exposure assessment scenario – are described in the approach for the refined exposure assessment of food additives under re-evaluation (EFSA ANS Panel, 2017).

Table 12 summarises the estimated exposure to E 171 from its use as a food additive in six population groups (Table 11) according to the different exposure scenarios. Detailed results per population group and survey are presented in Appendix S.