Bundesinstitut für Risikobewertung

DOI 10.17590/20220601-121425

Re-evaluation of titanium dioxide: BfR draws similar conclusions as the European Food Safety Authority

BfR Opinion No. 038/2021 of 8 December 2021

The European Food Safety Authority (EFSA) has re-evaluated health risks of titanium dioxide as food additive E 171 and published the result on 6 May 2021. After a systematic literature search, studies on possible (genotoxic) effects of titanium dioxide were evaluated.

EFSA came to the conclusion that genotoxic effects of titanium dioxide cannot be excluded with sufficient certainty. Therefore, titanium dioxide can no longer be considered safe as a food additive. Since no safe dose has yet been determined for genotoxic substances, no Acceptable Daily Intake (ADI) could be derived for the substance.

The BfR has looked at the genotoxicity data considered by the EFSA and draws mostly the same conclusions. However, the BfR points out that there are still gaps in knowledge for a conclusive assessment. For example, it is still unclear to what extent and in what way titanium dioxide can damage the genetic material. What role do the particle properties, their size, shape, crystalline nature play? Is there a cancer risk? Answers to these questions still need to be found.

See also: https://www.bfr.bund.de/cm/349/food-additive-titanium-dioxide-e-171-under-scrutiny.pdf

1 EFSA assessments on the use of titanium dioxide as a food additive

In 2016, the use of titanium dioxide as a food additive (E 171) was assessed by the European Food Safety Authority (EFSA) within the scope of the programme for the reassessment of authorised food additives in accordance with Article 32 of Regulation (EC) No 1333/2008 and Regulation (EU) No 257/2010. In doing so, EFSA assumed that the food additive E 171 contained less than 3.2% nanoparticles (by mass) and therefore attributed limited relevance to the studies with titanium dioxide nanoparticles. In its assessment, EFSA came to the conclusion that up to 2016 on the basis of the available information on genotoxicity, as well as the data on the absorption, distribution and excretion of titanium dioxide nanoparticles and microparticles, a mutagenic potential of orally ingested titanium dioxide (in nano- and microform) is unlikely *in vivo*. Overall, EFSA concluded that there was no evidence of health risks with regard to the oral intake of titanium dioxide (E 171) with food (EFSA 2016). However, EFSA pointed out data gaps on reproductive toxicity and on the characterisation of the food additive E 171.

On behalf of the European Commission, EFSA had assessed four new studies (Heringa *et al.* 2016; Bettini *et al.* 2017; Guo *et al.* 2017; Proquin *et al.* 2017) on the potential toxicity of titanium dioxide as food additive E 171 in June 2018. This data gave EFSA no reason to revise the assessment of 2016 (EFSA 2018).

In 2019, EFSA had commented on an opinion from the French Agency for Food Safety, Environment and Occupational Health (ANSES). EFSA concluded that the report published by ANSES in April 2019 contains no essentially new findings which could cast doubt on the conclusions of the two previous scientific opinions published by EFSA on the use of titanium dioxide as food additive E 171 (EFSA 2019a).

In its 2016 opinion, EFSA had recommended adding particle size distribution as an additional parameter in the specification for titanium dioxide (E171). In June 2019, EFSA had prepared a proposal on this. Specifically, it was recommended to include a median of the minimum external dimension of more than 100 nm, as determined by electron microscopy, as an additional parameter in the specification for titanium dioxide (E171) in the Annex to Regulation (EU) No 231/2012. This corresponds to less than 50% of particles with a minimum external dimension of less than 100 nm (EFSA 2019b). However, so far the specification in the Annex to Regulation (EU) No. 231/2012 has not been amended.

In March 2020, the European Commission asked EFSA to evaluate the reproductive toxicity studies and characterisation studies of food additive E 171 recommended by EFSA in 2016 and requested by the European Commission in January 2017 by March 2021. EFSA completed the relevant evaluation in March 2021 and published its opinion on 6 May 2021 (EFSA 2021). In this opinion, EFSA re-evaluated possible health risks associated with the use of titanium dioxide as a food additive (E 171) based on all currently available relevant scientific evidence.

2 Data basis of the toxicological endpoints relevant for the assessment of possible genotoxic effects

Within the context of the current EFSA opinion, a new animal study investigating possible reproductive toxic effects was taken into account and a systematic literature search was carried out. The re-evaluation also focused on concerns regarding possible mutagenic effects of titanium dioxide. Based on new findings on the particle size distribution of titanium dioxide (E 171), the numerous studies with titanium dioxide nanoparticles were classified as relevant for the assessment for the first time. For the assessment of possible genotoxic effects, publications were identified and evaluated as potentially relevant by EFSA according to a systematic methodology. The studies were reviewed by EFSA in a very detailed procedure with regard to their relevance, reliability and quality (EFSA 2021).

Taking these data into account, EFSA concludes that titanium dioxide particles have the potential to cause DNA strand breaks and chromosome damage, but not gene mutations. However, no clear correlation could be derived between the physicochemical properties of titanium dioxide particles (e.g. crystalline shape, particle size, shape, agglomeration state) and the results in the assessed *in vitro* or *in vivo* genotoxicity studies. However, the assessed studies were almost exclusively conducted with approximately spherical particles, which limits the assessment of a correlation with regard to particle shape.

Overall, EFSA comes to the following conclusion: "*…Based on all the evidence available, a concern for genotoxicity could not be ruled out, and given the many uncertainties, the Panel concluded that E 171 can no longer be considered as safe when used as a food additive. …"*

2.1 Potential to induce genetic mutations

Overview of the studies considered by EFSA

EFSA assessed eight bacterial *in vitro* gene mutation tests, all of which were negative. However, the data described therein were not considered meaningful by EFSA due to the limited permeability of the bacterial cell wall to (nano)particles.

EFSA also identified 14 *in vitro* gene mutation tests on mammalian cells, of which seven studies were assessed as "highly relevant" or "of limited relevance" and used for further evaluation. Positive findings (i.e. gene mutation inducing effects) were observed in two HPRT assays and one *spi* gene mutation assay, while no significant effects in terms of gene mutation

induction could be detected in two other HPRT assays and two mouse lymphoma assays. The results of another mouse lymphoma assay in CHO-K1 cells were considered equivocal.

The data from six *in vivo* gene mutation assays in rodents using titanium dioxide nanoparticles < 30 nm were considered by EFSA to be of "high relevance" or "limited relevance" and were taken into account. One study with oral application showed a positive result with regard to the induction of major deletions, while five other studies after intraperitoneal, intravenous or intratracheal application showed a negative result with regard to the induction of gene mutations and minor deletions.

Overall, EFSA concludes that the indications of a potential for induction of gene mutations from *in vitro* gene mutation tests on mammalian cells are not confirmed by the data from *in vivo* studies (with TiO_2 NPs < 30 nm).

Assessment of the data situation by BfR

This assessment by EFSA is comprehensible for BfR.

2.2 Potential for inducing micronuclei and chromosomal aberrations

Overview of the studies considered by EFSA

To assess the potential of titanium dioxide to induce micronuclei or chromosomal aberrations, EFSA identified and evaluated 82 potentially relevant studies. The database on the *in vitro* studies comprised a total of 56 studies, of which 43 studies with 67 individual experiments were considered relevant (high or limited relevance) by EFSA for the assessment. Of the 67 individual experiments considered, 26 reported a positive test result. Twelve of the single experiments considered with a positive result were considered by EFSA to have a high relevance to the assessment. The majority of the studies considered dealt with nanoparticles of a size < 30 nm. One study explicitly addressed E171 and reported a positive result.

In addition, a total of 26 *in vivo* studies were assessed, of which 15 studies were considered by EFSA to be relevant for the assessment (high or limited relevance). Of the 15 *in vivo* studies considered, eight studies reported a positive test result. However, only one of the *in vivo* studies considered was assessed by EFSA as having high relevance to the assessment. This study by Shukla *et al.* reported a positive result (Shukla *et al.* 2014). With regard to the oral route of administration, which is considered particularly relevant within the context of the use of titanium dioxide as a food additive, four out of five studies considered reported a positive test result. A total of 12 studies dealt with nanoparticles of small (< 30 nm) and/or medium (30-60 nm) size and reported both positive and negative results after oral, intraperitoneal and intravenous administration. One study reported a negative result after oral administration of nanoscale particles with a size of approximately 75 nm. Two studies reported one inconclusive and one negative result after intraperitoneal application of larger particles (> 100 nm).

Based on the studies described above, EFSA concluded that titanium dioxide nanoparticles have the potential to induce micronuclei or chromosomal aberrations. According to EFSA's assessment, no clear correlation between particle size and the occurrence of positive results in the micronucleus test or chromosome aberration test was observed.

Assessment of the data situation by BfR

Based on the overall view of the available information, which refers in particular to the *in vivo* micronucleus tests with oral administration and is also supported by further positive results from *in vivo* studies with other routes of administration or *in vitro* studies, the assessment of

EFSA with regard to the existing potential of nanoparticulate titanium dioxide for the induction of chromosomal aberrations appears plausible in the view of BfR. However, it is noted that the *in vivo* studies listed are only considered to be of limited reliability when considered alone due to existing documentation gaps or deviations from OECD test guidelines in the original literature cited.

The data on which the EFSA opinion is based do not allow an assessment of a correlation between the physicochemical properties (particle size and other particle properties such as particle shape, crystal structure or agglomeration state) and the occurrence of positive results in the micronucleus test or chromosome aberration test. The available *in vivo* studies after oral administration indicate in total a genotoxic potential especially for nanoparticles of small or medium size (5-58 nm). However, the potential with regard to the induction of micronuclei or chromosomal aberrations for larger particles (especially > 100 nm) after oral administration is not sufficiently characterised by the studies on which the EFSA assessment is based.

2.3 Potential to induce DNA damage

Overview of the studies considered by EFSA

In its opinion, EFSA identified a total of 142 *in vitro* studies and 44 *in vivo* studies that investigated the genotoxic potential of titanium dioxide particles using the comet assay.

With regard to the *in vitro* studies, 106 studies with 142 individual experiments were assessed by EFSA as "highly relevant" or "of limited relevance". The majority of the *in vitro* studies considered reported a positive test result.

Of the 44 *in vivo* studies, 18 studies were considered "highly relevant" or "of limited relevance" for the oral uptake pathway. Of these, nine studies dealt with the oral uptake pathway, which is the main focus for the evaluation of titanium dioxide as a food additive. The EFSA opinion states that strand breaks were detected in six of the nine studies with oral application. The reticulo-endothelial system was mostly identified as the target organ. The data from animal experiments are consistent with the findings from *in vitro* experiments. In individual studies, a correlation of several endpoints to genotoxicity could also be observed. For example, Grissa *et al*. reported positive findings in the comet assay in leukocytes, micronuclei in bone marrow and micronuclei in red blood cells (Grissa *et al.* 2015).

Based on the study data, EFSA concludes that titanium dioxide particles can induce DNA damage. However, it notes that a majority of the positive studies were conducted with particles < 30 nm, but DNA strand breaks were also induced with particles > 30 nm. Furthermore, EFSA points out that on the basis of the studies considered, no clear dependence between the induction of DNA damage in the comet assay and particle size can be derived.

Assessment of the data situation by BfR

Overall, EFSA's presentation and interpretation of the main study results considered in the opinion are comprehensible.

Statements on a possible dependence between positive results and the physicochemical properties of the test material are fraught with uncertainty based on the data considered. One reason is that the EFSA opinion does not provide sufficiently robust data for a clear separation between nano- and microscale material with regard to the *in vivo* studies.

2.4 Investigation of interactions with DNA

Overview of the studies considered by EFSA

The EFSA opinion describes five *in vitro* studies and two *in vivo* studies in which the interaction between titanium dioxide nanoparticles and DNA was investigated. In two animal studies, binding of the particles to DNA isolated from liver cells of the test animals was detected after intraperitoneal and intranasal application of titanium dioxide nanoparticles (Li *et al.* 2010; Jin *et al.* 2013). The binding led to changes in the DNA conformation. The results of other *in vitro* studies listed have also shown an interaction between the nanoparticles and the DNA. In contrast, no binding to DNA was observed when microparticles in the rutile crystal form (diameter < 5 µm) were examined.

Based on the results, both electrostatic interactions between the titanium dioxide nanoparticles and the DNA and an intercalation of the nanoparticles into the DNA were considered possible by the study authors. In addition, some studies also postulated the possibility of a covalent bond between the nanoparticles and the DNA. In its opinion, EFSA concluded that, based on the techniques used, it is not possible to make a conclusive assessment of whether the interactions described also involve covalent binding to DNA.

Assessment of the data situation by BfR

The presentation and interpretation of the main study results by EFSA is comprehensible and is shared by BfR. From BfR's point of view, however, there is a need for further research (see section 3.5).

In detail, it should be noted that the studies were predominantly conducted with titanium dioxide nanoparticles with a size of less than 30 nm. In two studies conducted by Patel *et al*., the particle size was indeed specified as < 100 nm; however, according to the original publication from 2017, a characterisation of the particles revealed an average size of about 20 nm (Patel *et al.* 2017), so that a relevant proportion of small particles with a size below 30 nm must also be assumed here.

2.5 Occurrence of *aberrant crypts* (AC)

Overview of the studies considered by EFSA

EFSA considered a total of three studies on the occurrence of aberrant crypts. The study on reproductive toxicity according to OECD Test Guideline 443 (*Extended One Generation Reproductive Toxicity Study*; EOGRTS) requested by EFSA in 2016 was assessed as a new study. In addition, two other studies were considered that investigated this endpoint (Bettini *et al.* 2017; Blevins *et al.* 2019).

The study by Bettini *et al*. (2017) was previously assessed by the ANS Panel and limitations were discussed in detail (EFSA 2018). In the study, a clustered occurrence of *aberrant cryptic foci* (ACF) was observed in adult male Wistar rats at a dose of 10 mg/kg body weight per day. The study by Blevins *et al*. (2019) and the newly submitted reproductive toxicity study assessed by EFSA did not confirm these findings. EFSA assesses the latter two studies as less informative because the exposure of the test animals to titanium dioxide nanoparticles is unclear here.

Assessment of the data situation by BfR

Aberrant crypts and foci with a clustered occurrence of this atypical to dysplastic morphology are considered a precursor of colorectal polyps and thus a preneoplastic lesion. A frequent

occurrence can regularly be observed in the intestinal mucosa of experimental animals after treatment with corresponding genotoxic carcinogens. In this case, the ACF develops within two weeks. With regard to the number and severity of the changes, a dose dependency can be observed with genotoxic carcinogens.

In its opinion, EFSA states that although the findings described by Bettini *et al*. (2017) could not be confirmed in two further studies (Extended One-Generation Reproductive Toxicity Study and Blevins *et al*. 2019) the exposure of the test animals to titanium dioxide nanoparticles was unclear for these two studies. Because the exposure to titanium dioxide nanoparticles was unclear in the other two studies, EFSA gave less weight to these studies than to the study by Bettini *et al*. (2017).

BfR cannot fully accept the weighting given in the opinion to the studies by Bettini *et. al*. (2017) in comparison to the study by Blevins *et al*. (2019), whose difference is primarily based on the preparation and administration of the test substance. The BfR notes that the doses used in the study by Bettini *et. al*. (2017) with only one dose (10 mg/kg bw/day) can be considered marginal at best in terms of frequency and severity. In the study by Blevins *et al*. (2019), in which a range up to approx. 300 mg/kg bw/day was investigated in accordance with the OECD recommendations with four dose groups, the findings on colorectal preneoplastic lesions in the form of ACF could not be confirmed despite an increased group size. At present, BfR does not have sufficient information on the new study conducted according to OECD Test Guideline 443 to assess the study results.

2.6 Findings from other studies

Overview of the studies considered by EFSA

For the assessment of genotoxic properties of E 171, results from studies on the phosphorylation of histone H2AX (γH2AX), studies on the formation of oxidised DNA bases (8-oxo-dG) and on the formation of reactive oxygen species, on the influence on the DNA methylation pattern for the assessment of epigenetic effects as well as on the possible induction of cell transformation in the frame were additionally considered.

To assess the potential of titanium dioxide particles to induce phosphorylation of H2AX, EFSA assessed four *in vitro* studies and two *in vivo* studies, with an increase in γH2AX detected in two of the four *in vitro* studies and in both *in vivo* studies. EFSA considers these studies to be of limited value.

Five *in vitro* studies and one *in vivo* study were assessed to evaluate the formation of oxidised DNA bases (8-oxo-dG) in response to the formation of reactive oxygen species. In four *in vitro* studies and in the *in vivo* study, a positive result was observed in each case after treatment with titanium dioxide nanoparticles and microparticles. Based on the data described here, EFSA concludes that titanium dioxide nanoparticles and microparticles have the potential to damage DNA. However, only limited significance is attributed to these studies.

To assess the formation of reactive oxygen species (ROS), EFSA considered *in vitro* and *in vivo* studies in which the induction of ROS was partly investigated in parallel with the induction of micronuclei and DNA damage. In the majority of the *in vitro* studies, induction of ROS after exposure to titanium dioxide particles could be demonstrated. In the *in vivo* studies considered, in addition to the induction of ROS by titanium dioxide particles, the induction of the expression of marker genes for apoptosis was also detected. The ROS induction was observed independently of the mode of application.

To assess possible epigenetic effects, five *in vitro* studies were evaluated by EFSA. These showed a change in promoter methylation of specific genes or in the global cellular DNA methylation pattern after exposure to titanium dioxide nanoparticles. EFSA concludes that the studies may be suitable for detecting epigenetic changes after titanium dioxide particle application. They are considered by EFSA as supporting information.

In addition, the influence of titanium dioxide particles on cell transformation was assessed by EFSA under the item "Further studies", whereby four *in vitro* studies were considered here. These show increased colony formation and "*anchorage independent growth*" after titanium dioxide nanoparticle application. One study showed an increase in colony number as well as colony enlargement. EFSA considers that the *in vitro* studies provide evidence for the potential of titanium dioxide nanoparticles to induce cell transformation processes as an initial step in carcinogenesis. Overall, EFSA concludes that these results have limited relevance for the assessment of genotoxicity and carcinogenicity.

Assessment of the data situation by BfR

In summary, the results of the other studies assessed by EFSA are presented in a comprehensible manner. The conclusions drawn from them are shared by BfR with reservations.

The studies on the phosphorylation of H2AX have only limited significance. γH2AX is not a specific marker for DNA double strand breaks, but indicates a general DNA damage that can also occur as a result of a cellular stress response or apoptotic processes. In this respect, the biological relevance of these data is difficult to assess.

The data on epigenetic changes after titanium dioxide particle application are considered by EFSA as limited supporting information. In the EFSA opinion, among other things, changes in the DNA methylation pattern in the epigenetic control regions ("*CpG islands*") of genes encoding DNA repair proteins are considered as a possible mechanism of action of the titanium dioxide-mediated genotoxic effects. The BfR is of the opinion that the biological relevance of the data on effects on the epigenome cannot yet be assessed.

The data considered under "Other studies" also have only limited relevance for the assessment of genotoxicity and carcinogenicity.

3 Conclusion from the BfR's point of view

3.1 Evaluation of the methodological approach

In total, EFSA researched almost 12,000 publications. After applying a systematic methodology, more than 200 publications were identified and evaluated among them in which possible genotoxic effects by titanium dioxide were investigated.

The strategy for researching the literature considered for the assessment of possible genotoxic effects as well as the evaluation of the studies in terms of robustness and relevance following the internationally established scientific criteria is presented transparently in the appendices. In the compilation of the literature used (chapter "*References*"), a number of references with incomplete bibliographic information can be found. Some of the studies mentioned in *Appendix F* are not included in the reference list.

3.2 Considerations on the mechanism of action of titanium dioxide-mediated genotoxic effects

Several *in vitro* and *in vivo* studies indicate that titanium dioxide particles cause DNA strand breaks, oxidatively generated DNA damage and chromosome damage. Several mechanisms, possibly acting in parallel, are considered for the observed genotoxic effects. In addition to the titanium dioxide-mediated formation of reactive intermediates (reactive oxygen or nitrogen species (ROS/RNS)), the EFSA opinion discusses a direct interaction of titanium dioxide nanoparticles with DNA.

With regard to the formation of reactive intermediates, it is discussed that this could be due to an intrinsic property of titanium dioxide nanoparticles, proceed via a titanium dioxide particle-mediated inflammatory reaction or be mediated by effects of titanium dioxide nanoparticles on mitochondria (Ghosh *et al.* 2013; Louro *et al.* 2014; Barkhade *et al.* 2019).

According to the EFSA opinion, there is evidence that titanium dioxide nanoparticles may enter the nucleus and mitochondria (Louro *et al.* 2014). Most studies showing uptake into the cell nucleus on the basis of electron microscopy images were carried out with titanium dioxide nanoparticles smaller than 30 nm. These are contrasted with publications that were also partly carried out with titanium dioxide nanoparticles smaller than 30 nm, but which do not show uptake into the cell nucleus.

In vitro studies with isolated DNA showed an interaction with titanium dioxide. In proteomics, titanium dioxide is used to purify phosphorylated proteins (Larsen *et al.* 2005). Therefore, it is understandable that titanium dioxide can interact with the phosphate backbone of isolated DNA. Whether the findings can be transferred to *in vivo* conditions cannot be judged from the data presented. Under *in vivo* conditions, particles are surrounded by a protein corona, so it is unclear whether the particles could interact with DNA. In the EFSA opinion, two *in vivo* studies from the same research group are cited to demonstrate an interaction of titanium dioxide nanoparticles with DNA using UV VIS spectroscopy. Overall, the data known so far do not provide robust evidence for the ability of titanium dioxide nanoparticles to covalently bind with DNA *in vivo*.

The findings summarised by EFSA do not provide robust evidence for a direct genotoxic mechanism of action by titanium dioxide particles. The indirect genotoxic mechanism of action, via the formation of reactive intermediates by titanium dioxide, is plausible. The studies considered in the "*Other endpoints*" section have only limited significance for the assessment of the genotoxic potential.

3.3 Correlation between observed effects and physicochemical properties of the tested titanium dioxide material

The data on the genotoxicity of titanium dioxide on which EFSA's opinion is based give an inconsistent picture, which could be due, among other things, to the use of test materials with different physicochemical properties and to the different study design.

Nanomaterials were used for a large proportion of the studies. There are no internationally harmonised test methods for nanomaterials so far. The lack of standardisation of test methods for nanomaterials makes it difficult to compare the studies. In addition, it is pointed out that experiences from EU projects (NANOGENOTOX, NANoREG) show that in interlaboratory tests on *in vitro* genotoxicity tests with titanium dioxide the results in the different laboratories were not reproducible in all laboratories despite the use of the same material, the same cell line and the same SOP protocol (unpublished data). Currently, a guideline is being developed at OECD level to address the specific testing requirements of nanomaterials.

Nanomaterials can vary in their physicochemical properties (e.g. crystalline form, particle size, shape and agglomeration state). It is possible that these influence the toxic properties of nanomaterials. Titanium dioxide, for example, occurs in different crystal forms: Rutile, Anatase and Brookite. Basically, the scientific literature on studies investigating a link between the toxicity of titanium dioxide particles and the crystal form is inconsistent.

In its assessment, EFSA assumes that a nanoscale content in E 171 cannot be ruled out. The particle size distribution of the food additive E 171 is polydisperse and very diverse between manufacturers – with nanoscale fractions (≤ 100 nm), which in individual cases can even be more than 50% of the particle number (Verleysen *et al.* 2020; Verleysen *et al.* 2021). Most of the *in vivo* studies on the genotoxicity of titanium dioxide cited in the EFSA opinion have been conducted with nanoparticles. The mean particle size in most studies was < 30 nm. The proportion of studies with particles larger than 100 nm is significantly lower in comparison.

Nanoparticles are generally assumed to be more reactive than larger particles due to their larger specific surface area (surface-to-volume ratio). Nanoparticles can also cross some body barriers more easily than non-nanoscale materials due to their small size and therefore have different toxicokinetics (absorption, distribution and excretion).

However, studies on toxicokinetics do not show a generally preferential uptake of particles with a certain particle size for the small intestine. Consequently, taking into account the current technical possibilities for particle size, no threshold value can be derived for the intestinal uptake of titanium dioxide particles, neither for small nor for large particles (nano- to micrometre range).

As most studies on genotoxicity were conducted with nanoparticles of titanium dioxide and only few robust data are available on larger particles only, no clear statement on the dependence of toxicity on particle size can be made at present.

EFSA writes in its conclusion that no clear correlation was observed between the effects shown in the *in vitro or in vivo* genotoxicity tests and the physicochemical properties of the titanium dioxide particles. For the comprehensibility of this conclusion, it would have been helpful to prepare the data according to the different physicochemical parameters of the test materials used (e.g. particle size as well as other particle properties such as particle shape, crystal structure or agglomeration state) in the studies considered relevant by EFSA, provided that these parameters were reported in the original publications on the peer-reviewed studies.

Overall, the data on which the EFSA opinion is based do not allow any statement regarding a correlation between the physicochemical properties and the occurrence of positive results in genotoxicity tests. From the BfR's point of view, targeted studies under standardised test conditions are required in order to be able to draw a clear conclusion on a possible correlation with the physicochemical properties of titanium dioxide particles.

3.4 Consequences for the derivation of a health-based guideline value

Although several mechanisms, possibly acting in parallel, are considered for the observed genotoxic effects, above all the titanium dioxide-mediated formation of reactive oxygen species (ROS) is considered important. It is discussed whether ROS formation is due to an intrinsic property of titanium dioxide nanoparticles, proceeds via titanium dioxide particle-mediated inflammatory reactions or is mediated by effects of titanium dioxide nanoparticles on mitochondria. The relative contribution of the overall mechanisms of action discussed to titanium dioxide-mediated genotoxicity cannot be conclusively assessed from the available data.

The *in vivo* genotoxicity studies considered by EFSA do not allow a clear conclusion with regard to a possible association between physicochemical properties of titanium dioxide particles and a genotoxic effect.

Overall, there is uncertainty as to whether a threshold value for the possible genotoxic effects due to exposure to the food additive E 171 can be assumed.

It is therefore understandable for BfR that EFSA has not derived a health-based guideline value (HBGV) following international guidelines.

3.5 Data gaps and need for research from BfR's point of view

EFSA's assessment that the concerns regarding possible genotoxic effects of E 171 cannot be dispelled and thus the suspicion regarding a mutagenic effect cannot be invalidated is understandable on the basis of the available data.

From the BfR's point of view, targeted studies on the following aspects could contribute significantly towards improving the scientific basis for assessment:

(1) *Relationship between physicochemical properties of titanium dioxide particles and genotoxic properties*

Most of the *in vivo* genotoxicity studies cited in the opinion have been conducted with nanoparticles. According to EFSA's presentation, the available data do not currently allow a clear statement to be made on the extent to which the physicochemical properties of titanium dioxide in E 171 have an influence on genotoxicity.

Studies under standardised conditions with titanium dioxide materials of defined size (in particular with materials that do not contain nanoscale components) could therefore be suitable for drawing robust conclusions with regard to a possible relationship between physicochemical properties of titanium dioxide particles and genotoxic properties.

(2) *Studies on possible mechanisms of action*

The titanium dioxide-mediated formation of reactive oxygen species (ROS) is considered to be of particular importance for genotoxicity. If one further assumes that the larger surface-to-volume ratio (specific surface area) of nanoparticles and the resulting higher reactivity in the case of nanoparticles plays a role, it could be examined whether the nanoscale fraction is responsible for the genotoxic findings.

In principle, damage to DNA can result from direct interaction of the substance with DNA or be triggered by indirect mechanisms that do not require direct interaction with DNA. The risk assessment for a DNA-reactive, genotoxic-carcinogenic effect is based on the assumption that it is not possible to determine an exposure level that is entirely without additional risk. For this reason, no health-based guidance value (HBGV) is usually derived.

From the BfR's point of view, targeted studies on possible interactions of titanium dioxide with DNA could improve the data basis for conclusions with regard to a possible mechanism of action. Overall, from the BfR's point of view, there is a need for research on the relationship between particle size and transfer into the cell nucleus, whether the corresponding interaction between titanium dioxide and DNA is dependent on size, and whether covalent bonds can occur in addition to electrostatic interactions and intercalation.

(3) *Investigations on the carcinogenic potential*

In order to be able to conclusively assess the carcinogenic potential of nanoscale titanium dioxide particles in particular, there is a lack of corresponding reliable studies, especially involving the administration of test material with smaller particle sizes down to the range clearly below 100 nm.

Currently, only one carcinogenicity study is available after oral administration in mice and rats (NCI 1979), in which no statistically significant neoplastic effects were observed even in the highest dose group (50 g/kg feed). However, the particle size distribution was not determined in this study, which is why only limited relevance was attributed to this study.

4. References

Barkhade T., Mahapatra S. K., Banerjee I. (2019). Study of mitochondrial swelling, membrane fluidity and ROS production induced by nano-TiO2 and prevented by Fe incorporation. *Toxicology Research* **8:** 711-722.

Bettini S, Boutet-Robinet E., Cartier C., Coméra C., Gaultier E., Dupuy J., Naud N., Taché S., Grysan P., Reguer S., Thieriet N., Réfrégiers M., Thiaudière D., Cravedi J. P., Carrière M., Audinot J. N., Pierre F. H., Guzylack-Piriou L., Houdeau E. (2017). Food-grade TiO_2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. *Scientific reports* **7:** 40373.

Blevins L. K., Crawford R. B., Bach A., Rizzo M. D., Zhou J., Henriquez J. E., Olive Khan D. M. I., Sermet S., Arnold L. L., Pennington K. L., Souza N. P., Cohen S. M., Kaminski N. E. (2019). Evaluation of immunologic and intestinal effects in rats administered an E 171-containing diet, a food grade titanium dioxide (TiO2). *Food Chem Toxicol* **133:** 110793.

EFSA (European Food Safety Auhtority) (2019a). EFSA statement on the review of the risks related to the exposure to the food additive titanium dioxide (E 171) performed by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES). *EFSA Journal* **17(6):** 5714.

EFSA (European Food Safety Auhtority: Panel on Food Additives and Flavourings (FAF)) (2021). Safety assessment of titanium dioxide (E171) as a food additive. *EFSA Journal* **19(5):** 6585.

EFSA (European Food Safety Auhtority: Panel on Food Additives and Nutrient Sources added to Food (ANS)) (2016). Re-evaluation of titanium dioxide (E 171) as a food additive. *EFSA Journal* **14(9):** 4545.

EFSA (European Food Safety Auhtority: Panel on Food Additives and Nutrient Sources added to Food (ANS)) (2018). Evaluation of four new studies on the potential toxicity of titanium dioxide used as a food additive (E 171). *EFSA Journal* **16(7):** 5366.

EFSA (European Food Safety Authority: Panel on Food Additives and Flavourings (FAF)) (2019b). Scientific opinion on the proposed amendment of the EU specifications for titanium dioxide (E 171) with respect to the inclusion of additional parameters related to its particle size distribution. *EFSA Journal* **17(7):** 5760.

Ghosh M., Chakraborty A., Mukherjee A. (2013). Cytotoxic, genotoxic and the hemolytic effect of titanium dioxide (TiO2) nanoparticles on human erythrocyte and lymphocyte cells in vitro. *J Appl Toxicol* **33:** 1097-1110.

Grissa I., Elghoul J., Ezzi L., Chakroun S., Kerkeni E., Hassine M., El Mir L., Mehdi M., Ben Cheikh H., Haouas Z. (2015). Anemia and genotoxicity induced by sub-chronic

intragastric treatment of rats with titanium dioxide nanoparticles. *Mutation Research Genetic Toxicology and Environmental Mutagenesis* **794:** 25–31.

Guo Z., Martucci N. J., Moreno-Olivas F., Tako E., Mahler G. J. (2017). Titanium dioxide nanoparticle ingestion alters nutrient absorption in an in vitro model of the small intestine. *NanoImpact* **5:** 70-82.

Heringa M. B., Geraets L., van Eijkeren J. C., Vandebriel R. J., de Jong W. H., Oomen A. G. (2016). Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations. *Nanotoxicology* **10:** 1515-1525.

Jin C., Tang Y., Fan X. Y., Ye X. T., Li X. L., Tang K., Zhang Y. F., Li A. G., Yang Y. J. (2013). *In vivo* evaluation of the interaction between titanium dioxide nanoparticle and rat liver DNA. *Toxicol Ind Health* **29:** 235-244.

Larsen M. R., Thingholm T. E., Jensen O. N., Roepstorff P., Jorgensen T. J. D. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Molecular & Cellular Proteomics* **4:** 873-886.

Li N., Ma L. L., Wang J., Zheng L., Liu J., Duan Y. M., Liu H. T., Zhao X. Y., Wang S. S., Wang H., Hong F. S., Xie Y. N. (2010). Interaction between nano-anatase TiO2 and liver DNA from mice *in vivo*. *Nanoscale Research Letters* **5:** 108-115.

Louro H., Tavares A., Vital N., Costa P. M., Alverca E., Zwart E., de Jong W. H., Fessard V., Lavinha J., Silva M. J. (2014). Integrated approach to the in vivo genotoxic effects of a titanium dioxide nanomaterial using lacZ plasmid-based transgenic mice. *Environmental and Molecular Mutagenesis* **55:** 500-509.

NCI (U.S. National Cancer Institute) (1979). Bioassay of titanium dioxide for possible carcinogenicity. *Technical Report Series* **97:** 1-130.

Patel S., Patel P. , Bakshi S.R. (2017). Titanium dioxide nanoparticles: an in vitro study of DNA binding, chromosome aberration assay, and comet assay *Cytotechnology* **69:** 245–263.

Proquin H., Rodríguez-Ibarra C., Moonen C. G., Urrutia Ortega I. M., Briede J. J., de Kok T. M., van Loveren H., Chirino Y. I. (2017). Titanium dioxide food additive (E171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions. *Mutagenesis* **32:** 139-149.

Shukla R. K., Kumar A., Vallabani N. V., Pandey A. K., Dhawan A. (2014). Titanium dioxide nanoparticle-induced oxidative stress triggers DNA damage and hepatic injury in mice. *Nanomedicine (London)* **9:** 1423-1434.

Verleysen E., Waegeneers N., Brassinne F., De Vos S., Jimenez I. O., Mathioudaki S., Mast J. (2020). Physicochemical characterization of the pristine E171 food additive by standardized and validated methods. *Nanomaterials (Basel)* **10:** 592.

Verleysen E., Waegeneers N., De Vos S., Brassinne F., Ledecq M., Van Steen F., Andjelkovic M., Janssens R., Mathioudaki S., Delfosse L., Machiels R., Cheyns K., Mast J. (2021). Physicochemical characterization of nanoparticles in food additives in the context of risk identification. *EFSA Supporting publication* **2021:EN-9992**

Further information on the topic of additives is available from the BfR website:
https://www.bfr.bund.de/de/bewertung_von_lebensmittelzusatzstoffen-2274.html

About the BfR

The German Federal Institute for Risk Assessment (BfR) is a scientifically independent institution within the portfolio of the German Federal Ministry of Food and Agriculture (BMEL). BfR advises the German federal government and federal states on questions of food, chemical and product safety. BfR conducts its own research on topics that are closely linked to its assessment tasks.