Annex B to TOX/2023/16

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment

Review of Efsa Opinion on the Reproductive Toxicity of Titanium Dioxide as a Food Additive

Introduction

1. The information presented in this annex should be read in conjunction with the main draft statement on the EFSA opinion on the reproductive toxicity of titanium dioxide as a food additive. It contains a summary of the data contained in the relevant literature considered by EFSA to support the opinion and discussion of the categorisation used for papers obtained by the literature search.

Categorisation of Papers

2. The advice elaborated on the nanoscale considerations (NSC) and adaptations related to specific aspects of study design with TiO_2 which are adequate for a hazard identification and hazard characterisation of small particles, including nanoparticles.

3. Toxicokinetic and toxicity studies were scored for NSC (dispersion and/or confirmation of internal exposure). The confidence for assessing the toxicological effects of the fraction of small particles, including nanoparticles was as follows:

- Scoring 1 for NSC*: the study is suitable.
- Scoring 2 for NSC: the study has some limitations.
- Scoring 3 for NSC: the relevance of the results cannot be verified.
- Scoring 4 for NSC: the relevance of the results is low.

*Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure).

Table of Additional Papers

Immunotoxicity

Paper	Test System	Exposure	Characterisation of test substance	Result	EFSA's evaluation
Urrutia-Ortega et al. (2016)	Mice	Qualitative analysis in tissues, methodology reliable with some limitations.	E 171, particles below and above 100 nm, crystalline form not reported.	Results obtained indicate that E 171 alone was unable to induce tumour formation, but dysplastic alterations were observed in the distal colon with a statistical significant enhancement of tumour formation in CAC + E 171 group vs. CAC group ($p < 0.01$). Some E 171 particles were internalised in colonic cells of the E 171 and CAC + E 171 groups, and both groups showed a decrease in goblet cells in the distal colon. CAC tumour progression markers including COX2, Ki67 and b-catenin indicated that E 171 exacerbates tumour progression and p65 NF-κB, a key regulator of inflammation was also induced by E 171 administration in CAC group. In the E 171 group, despite the absence of tumour formation, a slight statistical significant increase in COX2, Ki67	Score: 1 The Panel considered that the results suggest that while E 171 (5 mg/kg bw per day) alone administered for 10 weeks has no effect on tumour formation, it can potentiate intestinal tumour formation in mice exposed to AOM/DSS.

				and b-catenin, and p65 NF-κB were observed. Regarding cytokines in colon tissue, no changes, despite enhanced the p65-NF-κB expression, were found in the E 171 alone and CAC groups. Furthermore, a statistically significant decreases of IL-2, TNF-α, INF-g and IL-10 were observed in the CAC + E 171 group when compared to the E 171 group, probably as a consequence of colon tissue damage.	
Talamini et al. (2019)	Mice	Quantitative analysis in tissues with methodology reliable with some limitations.	E 171 (35% nano), anatase, 201 nm in suspension (NTA).	This study aimed to investigate whether TiO2 is deposited in the digestive system and internal organs and whether there are any molecular and cellular alterations associated with an inflammatory response. Neither overt structural and morphological histological alterations nor significant recruitment of monocytes/macrophages were observed in the stomach and whole intestine in E 171-treated animals. Neither disruption of crypt structure nor atypical epithelial cell proliferation in colon was observed. No changes in spleen histology were mentioned.	Score: 1 The Panel considered that analyses, which were limited to few animals (reduced power/data attrition), showed some evidence for modest inflammation which cannot be clearly identified as adverse.

				In the liver, increased size of necroinflammatory foci infiltrated with F4/80 monocytes/macrophages was noted three days after the last E 171 dose (n = 4) vs. controls (n = 2), while no changes were observed in stomach or intestine of treated animals. Liver histological findings are not supported by ROS production data or cytokine levels. An increased ROS production (superoxide anion) was observed in the stomach and in the intestine, with no changes in the liver (n = 4), of treated animals. Regarding cytokine mRNAs, as assessed by quantitative real-time PCR in stomach, whole intestine and liver, data were not striking: a very modest increase of IL-1b was observed in stomach and whole intestine of animals exposed to E 171, a decrease in TNF-α was observed in the whole intestine but in the stomach, with no changes in liver. A slight decrease in IL-10 was observed in the liver, which was not associated with an increase in pro-inflammatory cytokines (IL-1b or TNF-α). mRNA data were provided for n = 10 for IL-1b and TNF-α, n = 5 for IL-10. The levels of serum cytokines	

				were not consistent with tissue mRNA expression. As an increase in IL-6 and SDF1, with no changes in TNF-α or IL-1b, were observed in treated animals (n = 5).	
Riedle et al. (2020)	Mice	Qualitative analysis in tissues, methodology reliable with some limitations.	E 171, anatase, 119 nm (TEM).	Peyer's patches from 18 weeks feeding were examined by confocal microscopy and SEM with EDX analysis. Remaining Peyer's patches were collected and enzymatically digested to form single-cell suspensions for analysis by flow cytometry after immunostaining for CD4, CD45R and CD8a for lymphocytes or CD11b, CD11c and CD8a for myelocytes.	Score: 1 The Panel considered that this study demonstrates that particles in E 171 administered via the diet are taken up by basal cells of intestinal lymphoid follicles. The Panel considered that the parameters investigated did not show an effect on the immune system or inflammation.
Pinget et al. (2019)	Mice	Internal exposure not examined	E 171, anatase, 30–300 nm (SEM).	Five to six male C67Bl/6JAusb mice were exposed by drinking water to E 171 (0, 2, 10, 50 mg/kg bw per day) for 4 weeks. Histological analysis of the gut revealed a reduction of colonic crypt length. An increase in colon macrophages and CD8 cells were observed by FACS analysis in cell suspensions prepared from colon, and increased mRNA encoding	Score: 2

				for IL-10, TNF-α and IL-6 was detected by RT-PCR in RNA extracted form colon tissue.	
Bettini et al. (2017)	Rats	Qualitative measurement in tissues, methodology reliable with some limitations.	1) E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles < 100 nm; 2) TiO2 NPs (NM-105), anatase/rutile, 15–24 nm.	Titanium was detected in the immune cells of Peyer's patches. Effects were not noted in the spleen, but in Peyer's patches dendritic cell percentage were increased, as measured by flow cytometry of cells isolated from tissue samples. This effect was transient, as it was observed 7 days after exposure, but not at 100 days. The percentage of regulatory T cells and T-helper (Th) cells were significantly decreased at both time points in E 171 exposed animals. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T-helper (Th)-1 IFN-γ secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated in vitro with anti CD3/CD28 antibodies. Regarding the effects of TiO2 NP, similar to E 171 an increase in the percentage of dendritic cells in Peyer's patches was observed with no decrease in the percentage of Tregs. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T	Score: 1 Panel considers that these data indicate that E 171 has pro-inflammatory potential at the systemic level, paralleled by the development of an inflammatory microenvironment in the intestinal mucosa and that E 171 alone at a dose of 10 mg/kg bw per day may induce development of ACF in male rats. The Panel also noted that E 171 at a dose of 10 mg/kg bw per day increased the number of ACF initiated by a genotoxic carcinogen.

				helper (Th)-1 IFN-γ secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated in vitro with anti CD3/CD28 antibodies. Regarding intestinal mucosal inflammation, E 171 for one week did not initiate intestinal inflammation, but a 100-day E 171 treatment promoted colon microinflammation evidenced by significantly increased IL-1β, IL-8 and TNF-α expression in the colon. In the same samples, increased IL-10 was also observed.	
Blevins et al. (2019)	Rats Six-week-old male Wistar Han IGS (Crl:WI (Han)) rats	Internal exposure not examined	E 171, anatase, 110–115 nm (SEM), 36% particles < 100 nm.	Collectively, these results suggest that E 171 consumption does not alter T-cell-mediated mechanisms of immune control, either promoting inflammatory CD4+T helper cell activation or in reducing the percentage of anti-inflammatory Treg cells. Regarding the effects on cytokines, data presented suggest that dietary E 171 does not induce inflammation peripherally or in the GI tract at both time points. A modest increase in the relative spleen weight in 22.4 mg E 171/kg bw per day + DMH compared to not	Score: 3 Panel noted a considerable variability in the results, which may mask possible effects. Furthermore, the Panel noted that the examination for presence of ACF and ABC was not performed on the whole colon but was limited to three 2 cm long samples (one from the proximal, mid-portion and the distal parts). Dietary E 171, with or without treatment with DMH,

				initiated animals, an increase in IL-17A in colon (22.4 mg E 171/kg bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects, were observed. There were no changes in spleen cellularity across any of the treated groups. No changes were observed in the percentage of CD103+ DC, CD4+ T helper cells or total or activated Treg in peripheral blood, spleen or Peyer's patches in animals exposed to E 171 + DMH compared to animals treated with only DMH. there were no treatment related histopathological changes in the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E 171. Rats that were initiated with DMH only and those which received 171 in the diet after the initiation displayed several histopathological abnormalities Regarding colonic ACF and ABC, according to the authors, a technical issue was encountered in that much of the epithelial surface of the sampled colon (proximal, middle and distal) was obscured when observed by light microscopy. The authors'	had no effect on the length of the colonic glands examined or the number of goblet cells/unit. Overall, the Panel considered that this study indicates that acute and subchronic dietary intake of E 171 resulted in no significant effects on either peripheral or GI tract immune homeostasis as evidenced by immune cell phenotyping or inflammatory cytokine analysis. Limitations in the pathological examination for ABC and ACF (sampled colon area limited; delayed fixation) preclude a conclusion on potential for ABC and ACF formation.

				reported that they were unable to examine the entire surface of the colon samples No change in the number of ACF and ABC were observed due to E 171 exposure alone.	
Han et al. (2020a)	Sprague – Dawley rats	Quantitative analysis in tissues; methodology not reliable.	E 171, anatase, 150 nm (DLS).	There were no changes in the weight of immune organs or their histology. The proportion of lymphocytes slightly decreased (by 9%) in male but not female rats administered the highest E 171 dose, without an apparent dose response relationship. According to the study authors, the level of GM-CSF was reduced by 41% in females only at the highest dose of 1,000 mg/kg bw per day. In males there was also a decrement in GM-CSF level (approximately by 30%), that was slightly less pronounced than in females, and due to a higher variability in the controls, did not gain statistical significance. A reduction in the levels of IgM was observed in both sexes at the highest dose tested, i.e. by 12% in females and by 9% in males, but there were no clear dose response relationships. Transcriptomics also showed that immune response-related microRNAs were most strongly affected by E 171	Score: 2 The Panel noted the lack of a dose response, the magnitude of the effect was small that did not allow a firm conclusion given the natural variability in the parameters measured.

				exposure, which may support the effect observed at the high dose.	
TiO_2 or TiO_2 NPs	**Test System**	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**
Mohamed (2015)	Male Swiss Webster mice, aged 10–12 weeks	Quantitative measurement in tissues, methodology with important flaws	TiO_2 NPs, rutile/anatase (77/22%), 47 nm (TEM).	According to the authors, routine histopathology showed dose-dependent effects in the stomach. At 5 mg/kg, submucosal oedema was noted after 24 h that developed into ulcerations and mucosal necrosis after one and two weeks, respectively. After exposure to 50 mg/kg bw per day, submucosal vasculitis, massively degenerated glands and both mucosal and submucosal necrosis was evident after 24 h, 7 days and 14 days, respectively. Submucosal congested blood vessels, focal areas of leucocytic cell infiltrations and necrotic glands with mononuclear cell infiltrations (i.e. the highest grade of damage) were seen with TiO_2 NPs at 500 mg/kg bw per day at all time points.	Score: 2 The Panel noted that histopathological changes were not sufficient reported as no incidences and/or severity score were provided. The Panel considered that these data suggest an inflammatory response in the stomach after short-term bolus exposure to TiO_2 NPs (47 nm).
Li et al. (2019)	Male C57BL/6 mice	Quantitative analysis in tissues,	(1) TiO_2 NPs, anatase, 25 nm; (2)	Results indicate that short-term ingestion of TiO_2 NPs (25 nm) (1 mg/kg bw per day for 7 days) led to	Score: 2 The Panel considered that this study provides no

	(8 weeks old)	methodology with important flaws.	TiO_2 NPs, anatase, 50 nm; (3) TiO_2 NPs, anatase, 80 nm. Purity not reported for none of them.	colonic epithelial injury, reduced expression levels of tight junction proteins and reduced thickness of the 'luminal mucus layer'. This was associated with altered gut microbiota composition, with reduction in number of Bifidobacterium compared with controls. Regarding immunological organs, histological findings were not reported.	relevant information on immunological effects of TiO_2 NPs.
Hashem et al. (2020)	Adult male Wistar rats	Internal exposure not examined.	TiO_2 (from Sigma, no information on constituent particle size distribution nor crystalline form).	At the end of treatment, the body weight of low- and high-dose groups was 25% lower than that of the control group and there was a statistically significantly dose-dependent leucopenia and thrombocytopenia, as well as eosinophilia and neutrophilia . Statistically significantly dose-dependent spleen alterations were observed that included lymphoid necrosis, white pulp expansion and increased numbers of macrophages. A marked increase in $CD4^+$ and $CD8^+$ immunolabelling was noted in the spleen. In addition, IgG and IgM measured by ELISA were statistically	Score: 3 The Panel concluded that these findings taken together indicate that TiO_2-induced haematological and immunological alterations after exposure for 90 days at all dose tested.

				significantly elevated in TiO_2-treated rats. Phagocytic activity measured by a modified colorimetric nitro blue tetrazolium assay as well as lysozyme expression and nitric oxide levels measured by ELISA were significantly reduced following TiO_2 exposure. Lymphocyte proliferation in response to PHA, measured by a lymphocyte transformation assay and using MTT as a proxy for cell number was statistically significantly reduced.	
TiO_2 NPs < 30 nm	**Test System**	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**
Yu et al. (2016)	CD-1 (ICR) female mice	Internal exposure not examined.	TiO2 NPs, anatase, 5–6 nm	Inflammatory lesions and tissue damage were seen histopathologically, and were more pronounced at the mid and high doses. It was not reported whether the histopathology was performed blinded, but the results were corroborated with objective measures. The expression of NF-κB, and of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IFN-γ expression were increased in a dose-	Score: 2 The Panel considered that these data indicate an effect of TiO_2 NPs (5–6 nm) exposure at all dose levels tested, as evidenced by histopathological lesions, corroborated by intermediate endpoints indicating disturbance of intracellular ion homeostasis that were adrenergic

				dependent fashion (statistically significant increase up to 1.8-fold compared with the control); expression of the NF-κB inhibitor I-κB was decreased in a dose-dependent fashion (statistically significant decrease up to 1.55-fold compared with the control), as evidenced by western blotting.	receptors in the heart. These lesions are accompanied by increases in the expression of intermediate inflammatory endpoints. The Panel noted effects on inflammatory mediators with TiO_2 NPs (5–6 nm) at all doses tested and corroborated by histopathological lesions.
Li et al. (2018)	Male C57BL/6 mice, 8 weeks old	quantitative measurement in tissues, methodology with important flaws.	: 1) TiO_2 NPs, anatase, 20 nm (in water, DLS); 2) TiO_2 NPs, rutile, edged with corners morphology (SEM), 15 nm (in water, DLS).	There were no effects on body weight. Whereas particles were observed in the spleen, examination of H&E-stained samples of the spleen revealed no histopathological changes. No histopathological changes were seen recorded in other tissues examined (the lung, jejunum, kidney, liver or brain). In colon, the increased length of villi was increased and irregularly arranged epithelial cells were reported irregularly arranged after exposure to TiO_2 NPs. Rutile NPs had a more pronounced influence on the gut microbiota than anatase NPs. The most influenced	Score: 4

				phylum was Proteobacteria, which was significantly increased by rutile NPs but not by anatase NPs. At the genus level, Rhodococcus was enriched by rutile NPs, Prevotella was significantly decreased by both the TiO_2 NPs.	
Zhang et al. (2020)	C57BL/6J mice, aged 7 weeks	Internal exposure not examined.	TiO_2 NPs, 21 nm (TEM), crystalline form and purity unknown.	The results show that oral exposure to TiO_2 NPs resulted in significantly changed richness and composition of the gut microbiota. No changes in parameters indicating inflammation (IL-6 and IL-1β) in either intestines or brain were observed.	Score: 4 The Panel considered that exposure to TiO_2 NPs (21 nm) leads to changes in the microbiota composition, but the study does not indicate a local or systemic inflammatory action.
Chen et al. (2015a)	Four-week-old healthy Sprague–Dawley rats	Internal exposure not examined.	TiO_2 NPs, anatase 24 nm (TEM).	Regarding the effects on spleen and white blood cells in animals exposed to TiO_2 NPs alone, no significant histopathological changes were observed in the spleen in all groups. On the contrary, increases in white blood cells parameters (white blood cell counts and granulocytes) were observed in female rats after exposure to TiO_2 NPs 50 mg/kg bw per day for 90 days and among male rats exposed to TiO_2 NPs 50 mg/kg bw per day for 30 (white blood cells counts, lymphocytes, monocytes	The Panel considered that the increase in leucocytes may suggest an inflammatory response induced by TiO_2 NPs (24 nm) at the highest dose tested (50 mg/kg bw per day).

				absolute numbers and in the percentage of lymphocytes and granulocytes) and 90 days (percentage of monocytes); and a decrease in the while blood cells at 90 days in rats exposed to 10 mg/kg bw per day.	
Chen et al. (2019)	Three-week-old male Sprague – Dawley rats	Internal exposure not examined.	TiO_2 NPs, anatase, 29 nm (SEM).	Histopathologically, reduced numbers of goblet cells were found as a result of exposure, as well as inflammatory infiltration, while in serum increased IL-6 expression was observed.	Score: 2
Grissa et al. (2020)	Male Wistar rats	Internal exposure: quantitative in tissues; methodology with important flaws.	TiO_2 NPs, anatase, 5–12 nm (TEM, XRD).	A statistically significant dose-related increase in the level of NO in 100 and 200 mg/kg bw per day TiO_2 NPs groups was observed together with a statistically significant increase in brain TNF-α in 200 mg/kg bw per day TiO_2 NPs group. The increase was dose-related for both parameters.	Score: 2 The Panel noted changes for the above-mentioned inflammatory markers at doses starting from 100 mg TiO_2 NPs (5–12 nm)/kg bw per day.

Reproductive Toxicity Studies - TiO_2 NPs or TiO_2 containing a fraction of nanoparticles.

Paper	**Test System**	**Exposure**	**Characterisation of test substance**	**Result**	**EFSA's evaluation**

Warheit et al. (2015a)	Rats	In three studies, time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22/group) were daily exposed to TiO_2(uf-1, uf-3 and pg-1) by gavage on GDs 6–20. In three additional studies, pregnant Wistar rats (n=22–23/group) were daily exposed to TiO_2(uf-2 and pg-2) by gavage from GDs 5 to19. The dose levels	1) anatase/rutile (89/11%)(uf-1), d50=43 nm (XSDC), d50=23 nm (TEM), irregular; 2) anatase (100% nano) (uf-2),Safety assessment of the food additive titanium dioxide (E 171)www.efsa.europa.eu/efsajournal106 EFSA Journal 2021;19(5):6585d50=42 nm (XSDC), d50=19 nm (TEM), irregular; 3) rutile (100% nano) (uf-3), d50=47 nm (XSDC),d50=	Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to identify any abnormalities. At 1,000 mg uf-1/kg per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. The mean number of male fetuses was 7.2 compared with 5.5 male fetuses for the concurrent control group; the test facility historical control group data ranged at that time from 5.2 to 7.4. The mean number of female fetuses was 4.8 compared with 6.7 for the concurrent control group; the test facility historical control group data ranged at that time from 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mguf-1/kg bw per day group was 60% (males/females) compared with a sex ratio of 46% in the concurrent control group; the test facility historical control group data ranged at that time from 43% to 53%. Apart from some	Score: 4 The authors concluded that there were no significant toxicological or developmental effects in females or fetuses at any of the dose levels or compounds tested and considered the NOAEL for each compound to be 1,000 mg/kg bw per day, the highest dose tested. The Panel agreed with both the author and ANS Panel conclusions

		used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day.	22 nm (TEM), rod-like; 4) anatase (27% nano) (pg-1), d50=153 nm (XSDC), d50=120 nm(TEM), irregular; 5) rutile (11% nano) (pg-2), d50=195 nm (XSDC), d50=165 nm (TEM), irregular.	incidental changes in body weight and feed intake, no other changes were observed in the dams or the fetuses in these studies	
Khorsandi et al. (2016)	Mice	Internal exposure not examined.	TiO_2 NPs < 30 nm	While body weight was unaffected by treatment, the authors reported dose-dependent decreases in testis weight from a dose of 100 mg/kg bw per day. The Panel noted that there is a discrepancy between the statement in the text that a statistically significant decrease was only observed at 300 mg/kg bw per day, and the presentation in Table 1 of the publication that indicates significant differences to the control in both the mid- and high-dose groups. These groups also showed decreases in	Score: 2 The Panel considered that TiO_2 NPs (size unknown) at 100 mg/kg bw per day had an effect on testis weight.

				serum and testicular testosterone levels, the diameter and total volume of seminiferous tubules, the height of the spermatogenic epithelium and total Leydig cell numbers. Contrarily, the total volume of the interstitial tissue was found to be increased.	
Khorsandi et al. (2017)	Mice	Internal exposure not examined.	TiO_2 NPs, 20–30 nm (AFM), crystalline form unknown	The authors reported significant decreases in testis weight, circulating and testicular testosterone, testicular CAT and SOD concentrations, sperm counts and sperm motility. Significant increases were found in the percentage of abnormal or degenerative spermatogenic tubules, germ cell apoptosis, testicular MDA concentration and in the percentage of sperm with abnormal morphology. Body weight in the TiO_2 NPs group was similar to the control at termination.	Score: 2 The Panel considered that testicular toxicity was observed with TiO_2 NPs (20–30 nm) at a dose of 300 mg/kg bw per day, the only dose tested.
Karimipour et al. (2018)	Mice	Internal exposure not examined.	TiO_2 NPs, anatase, 10–25 nm	The authors reported a significantly decreased pregnancy rate (70% vs. 100% in the control group), a 20% decrease in litter size and increases in circulating oestrogen (20%) as well as MDA (25%). They observed degeneration and reduction of follicles, cyst formation and impairment of follicular development in the ovaries of the TiO_2 NPs group	Score: 2 The Panel considered that the study shows an impairment of female fertility at a dose of 100 mg TiO_2 NPs (10–25 nm)/kg bw per day.

				but presented no quantitative data. The in vivo findings were supported by a lower number of oocytes isolated from the exposed group and a higher percentage of developmental arrest before the blastocyst stage after in vitro fertilisation. The authors suggest that the observed effects could be the consequence of an indirect effect of TiO_2 NPs through the generation of increased ROS levels.	
Karimi et al. (2019)	6- to 8-week-old male NMRI mice	Internal exposure not examined.	TiO_2 NPs, 68 nm (DLS; no direct information on constituent particle size, thePanel considered the majority of constituent particles to be below 30 nm), crystalline form and purity unknown	TiO_2 NPs at 50 mg/kg significantly reduced testis weight in the presence of a non-significant trend towards lower body weight, accompanied by reduced serum testosterone to around 30% of control levels. Similarly, seminiferous tubule diameter and epithelium height were affected by TiO_2 NPs treatment. TiO_2 NPs treatment significantly reduced the maturity of the germinal epithelium, as determined by the Johnsen's scoring system. Similar significant adverse findings were observed in reduced sperm counts, increased sperm abnormalities and reduced sperm motility.	Score: 2 The Panel noted that 50 mg TiO_2 NPs (< 30 nm)/kg bw per day resulted in adverse effects on the testis.

Lu et al. (2020)	ICR mice, age 6–8 weeks	Internal exposure not examined.	TiO_2 NPs, anatase, 7 nm (TEM)	Through TEM, the authors report tight junction damage in the BTB at 50 and 100 mg/kg bw per day, though the histopathological pictures provided are hard to interpret. BTB-related proteins F-actin, ZO-1 and claudin-11 were dose-relatedly significantly increased up to 2-fold at the high dose, with no significant changes observed in connexin-43 and occludin. F-Actin was significantly increased at all doses tested. As to elements of MAPK signalling pathways, ERK and JNK mRNA expression was slightly but significantly increased in testis tissue at the high dose only. No effect was found on p38 mRNA expression. Serum testosterone was 50% decreased at the two highest doses tested, accompanied by a slight but significant reduction in oestradiol at the same doses. Sperm counts were unaffected by exposure, but sperm motility was dose-relatedly reduced from around 70% in controls to around 50% at the high dose, accompanied by increased sperm malformation rates from 3% in controls to 8% at the high dose, both	Score: 3 The Panel considered that TiO_2 NPs (7 nm), at 50 or 100 mg/kg bw per day, resulted in a dose-related reduction of sperm motility and increased sperm malformations, accompanied by histological observations in the testis, changes in BTB-related protein levels, changes in MAPK-related mRNA levels and reduced circulating testosterone concentrations.

				effects being statistically significant at the two highest doses.	
Lee et al. (2019)	Sprague – Dawley rats (12 females per group	Internal exposure examined: quantitative analysis in blood/tissues; methodology reliable with some limitations.	TiO_2 NPs (the Panel noted that from the description of the tested material, it corresponds to P25 (15–24 nm))	There were no statistically significant differences in general clinical signs, body weight, organ weights (absolute and relative to body weight), macroscopic findings, apart from a statistically significant decrease in food intake of the females of the high-dose group. The authors considered that this decrease did not have toxicological significance since it was minimal and there was no correlated decreased body weight or body weight gain during the study period. The Panel agreed with this conclusion. Caesarean section parameters and fetal external and visceral examinations did not reveal any statistically significant differences. The only difference seen was a small but statistically significant increase (4%) in the number of ossification centres in the metatarsals of both hindlimbs of the fetuses of 100 mg/kg bw per day group. The Panel agreed with the authors that this could be considered as an incidental finding as	Score: 3 The Panel considered that no adverse effects were reported with TiO_2 NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.

				no other related effects were observed.	