F1 generation cohort 1B

According to the authors, there were no test substance-related differences in serum oestradiol, oestrone and testosterone between F1 cohort 1B control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 cohort 1B at sacrifice on about PND 90.

Mean oestradiol levels were lower than controls in mid- and high-dose males (−18.1% and −14.8%, respectively), statistically significant for the mid-dose group only. The study authors considered this finding as incidental because in cohort 1B control mean oestradiol was anomalously high; the mean values in cohort 1B mid- and high-dose males were actually higher (not lower) than the normal circadian range in F0, cohort 1A and cohort 2A. The finding was therefore attributed to increased concentrations of oestradiol in the control group of cohort 1B and was therefore considered to be incidental and of no toxicological relevance.

Mean testosterone levels were higher than controls in mid- and high-dose cohort 1B females (+ 20.4% and + 12.3% compared to control for 300 and 1,000 mg/kg bw per day, respectively), reaching statistical significance for the high-dose group only. The authors considered this finding to be incidental because all the values in the mid- and high-dose females of cohort 1 B were within or only slightly above the normal circadian range. The authors further noted that female testosterone levels in cohort 1B controls were slightly lower than those in F0 and cohorts 1A and 2A controls.

The Panel agreed with the study authors that these are isolated findings, not related to treatment, and that there were no test substance-related effects on serum oestradiol, oestrone and testosterone in F1 cohort 1B.

F1 generation cohort 2A

According to the authors, there were no test substance-related differences in serum oestradiol, oestrone and testosterone) between control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 cohort 2A at sacrifice on PND 84–90.

Non-statistically significant differences were noted by the study authors for mean serum oestrone and testosterone levels in F1 cohort 2A males and oestradiol in females. Some variations in hormone levels were observed, but these variations were within the normal circadian range (with the exception of oestrone, whose circadian variation was not measured).

The Panel agreed with the study authors that these are isolated findings, not related to treatment, and that there were no test substance-related effects on serum oestradiol, oestrone and testosterone in F1 cohort 2A.

F1 generation cohort 2B

According to the study authors, there were no test substance-related differences in serum oestradiol, oestrone and testosterone between control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 cohort 2B at sacrifice on PND 21–23.

Mean serum testosterone levels were significantly lower than controls in mid-dose females (−31%). Lower mean testosterone levels, albeit non-statistically significant, were also seen in mid- and high-dose males and in low- and high-dose females, without a dose–response relationship. Some of the individual values in all dose groups were below the circadian range determined in satellite animals during the study. The Panel considered that these findings were incidental.

It is noted that lower testosterone levels, albeit non-statistically significant, were also seen in treated cohort 2A females, but again without a dose–response relationship. The Panel therefore considered that this finding was incidental, and that there were no test substance-related effects on serum oestradiol, oestrone and testosterone in F1 cohort 2B.

Overall conclusions on effects on hormone levels

Overall, the Panel concluded that, although there were occasional statistically significant differences between control and treated groups, there were no consistent treatment-related effects on T4, T3, TSH, oestradiol, oestrone and testosterone levels in F0 or F1 animals, and that therefore there was no evidence for a treatment-related effect on thyroid or sex hormone levels.

4.2.5. Pathology

All or part of the animals – depending on the cohort to which they were assigned – were subjected to an extensive macroscopic and microscopic examination, in accordance with OECD TG 443. Necropsy of the F0 generation (dams and males) was scheduled (shortly) after weaning of the F1 animals;

necropsy of the F1 generation at the end of the dosing period (PND 86–96, cohort 1A), after behavioural testing (PND 84–90, cohort 2A) or on PND 21/22/23 (cohort 2B). At necropsy, animals were sacrificed under CO_2 anaesthesia and by exsanguination from abdominal aorta and were further dissected and examined macroscopically for any abnormalities or pathological changes, with special attention given to the reproductive system and GI tract. Organ weights were recorded.

Pathology examination was performed in accordance with OECD TG 443.[18] This guideline indicates that in principle the control and high-dose groups must be examined and, when adverse effects are observed in the high-dose group, then the mid- and low-dose groups should additionally be examined. In males, qualitative stages of spermatogenesis and histopathology of interstitial testicular structure were studied on one testicle and one epididymis. In females, primordial and small growing follicles were evaluated quantitatively together with the corpora lutea on one ovary. Fresh bone marrow was obtained from the femur (3 air-dried smears/animal: all groups).

Tissues examined for neurohistopathology were selected from F1 cohorts 2A and 2B (control and high-dose group), i.e. brain [olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, mid-brain (tectum, tegmentum and cerebral peduncles), brainstem and cerebellum]. From the F1 cohort 2A, the eye with optic nerve and retina, muscle (skeletal), nerve (sciatic) and spinal cord (3 sections) were also studied. Neurohistopathology evaluation included a qualitative and semi-quantitative microscopic inspection of the selected nervous tissues focussing on: (i) alterations in gross size or shape of olfactory bulbs, cerebrum or cerebellum; (ii) in the relative size of various brain regions (resulting, e.g. from loss or persistence of normally transient populations of cells or axonal projections); (iii) areas of apoptosis or necrosis, clusters or dispersed populations of ectopic, disoriented or malformed neurons or alterations in the relative size of various layers of cortical structures (indicative of alterations in proliferation, migration and differentiation); (iv) alterations in patterns of myelination; and (v) enlargement of ventricles, stenosis of cerebral aqueduct and thinning of cerebral hemispheres. Finally, a quantitative evaluation, i.e. linear morphometry, was performed (cohort 2A, control and high-dose group) in cerebral cortex, mid-brain, brainstem and cerebellum.

All animals survived until the scheduled necropsy.

Gross pathology – including organ weights – of F0 (all groups; dams and males) and F1 generations (cohorts 1A, 2A and 2B; all groups; males and females) with special attention to the reproductive system and GI tract, did not identify any test substance-related effects. In one animal from the high-dose group (F0, male), 'white fine granular deposit' was observed at the mucosa of the duodenum but this was considered by the authors to be of no toxicological relevance.

The histopathological examination of F0 and F1 generations and comparison of the high-dose group and the control group did not identify any test substance related changes, either in the F0 generation (dams and males) or the F1 generation cohort 1A (males and females).

No test substance-related histological changes were observed in the testis and epididymis in all animals in the control and high-dose groups (F0 and F1 generation cohort 1A). This included an absence of any effects on any spermatogenic phases (proliferative, meiotic and spermiogenic). Furthermore, in the females, no statistically significant differences were observed for the total mean of primordial and small growing follicles as well as corpora lutea for the high-dose group, when compared to control group.

Qualitative and quantitative neurohistopathological examination was performed on cohorts 2A and 2B.

No test substance-related abnormalities were found in the cohort 2A high-dose group animals during qualitative examination. The only abnormality observed appeared in an animal of the control group (eye/optic nerve: slight unilateral retrobulbar haemorrhage). The Panel noted that evaluation of sections in cohort 2A from brain level 1[19] containing variable amounts of olfactory bulbs indicated no abnormalities.

Subarachnoid haemorrhages were observed in animals of both sexes in both the control and high-dose groups of cohort 2B. This was attributed to the preparation and processing of the brain and therefore artefacts. The Panel agreed with this conclusion.

[18] Selected tissues were preserved in a suitable fixative and further processed for histopathology where appropriate: adrenal gland; bone; bone marrow (femur); brain (cerebrum, cerebellum, pons); colon; duodenum; epididymis; eye with optic nerve; heart (3 levels – right and left ventricle, septum); ileum; jejunum; kidney; liver, lymph node, lungs (with main stem bronchi and bronchioles); lymph node (cervical and mesenteric); mammary gland; muscle (skeletal); nerve (sciatic); oesophagus; Peyer's patches; pituitary; prostate; rectum; seminal vesicles with coagulating glands; spinal cord (3 sections); spleen; stomach; testicle; thymus, thyroid (including parathyroids); trachea (including larynx); ureter and urinary bladder.

[19] Brain level 1 refers to the first coronal section of the brain containing the olfactory bulb following a rostral to caudal trimming.

Quantitative neuropathology assessment of cohort 2A and comparison of total means of all measurements in the brain sections examined (cerebrum, mid-brain, cerebellum and brain stem) did not reveal any pathological changes indicative of toxicity for both sexes.

From the results of gross pathology of all animals and the (quantitative) microscopic assessment of animals of the control and high-dose groups of the F0 (dams and males) and F1 generations (cohorts 1A, 2A and B; males and females), the Panel concluded that there were no test-substance related effects.

4.2.6. Aberrant crypt foci examination in satellite F0 animals

Although not a requirement in the OECD TG 443, an evaluation of ACF in the colon of satellite F0 animals (10/sex per group) treated with 0, 100, 300 and 1,000 mg E 171/kg bw per day and terminated after weaning was undertaken. The Panel noted that the design of the study did not include a positive control group (e.g. treatment with a known GIT tumour initiator such as DMH) for the development of ACF.

The colon was excised, opened longitudinally and the contents removed by rinsing with a 0.9% NaCl solution. Thereafter, the tissue was divided in parts of a suitable size for fixation by immersion in 5% buffered formalin. A blind examination of these samples stained with 0.5% (w/v) methylene blue in water was performed under a stereomicroscope at 50x magnification for presence of ACF. The study pathologist followed the definition of ACF given by Shwter et al. (2016), i.e. 'foci containing more than 2 ABCs'.

No ACF were found in the colons of the control and the treated groups that would fulfil the above definition. A mildly increased morphological variability (increased size and intensity of the staining of a small portion) of the crypts in the two caudal parts of colon was observed in seven animals (males: 1/10, 0/10, 1/10, 1/10; females: 1/10, 0/10, 1/10, 2/10 in the control, low-, mid- and high-dose groups, respectively). These changes were assessed by the study pathologist as inconsistent with the appearance and definition of ACF presented in Shwter et al. (2016). Based on the description in the report, the Panel agreed with this conclusion. Furthermore, the incidence of these single crypts observed in the mid and high doses was not significantly different from the control.

The additional submission of data by the interested party (Documentation provided to EFSA No 15) included photomicrographs of mildly increased variability in crypt morphology from all seven animals. In this submission, a re-examination was extended to an additional randomly selected nine control animals (4M and 5F) and eight high-dose group animals (3M and 5F). A transmitted light microscope instead of an incident light microscope was used to improve visualisation. Among these additional animals, a mild increased variability in crypt morphology was observed in eight of the nine (4M and 4F) controls and six of the eight (3M and 3M) high-dose animals.

Based on the evaluation of the data from the first and additional submission, the Panel considered that oral exposure to E 171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon.

4.2.7. Reproductive and developmental toxicity

Evaluation of sexual function and fertility

Male fertility

An overview of results for male fertility parameters is reported in Table 3. No statistically significant or dose-related effects on sperm motility, total spermatids/gram testis, percentage of abnormal spermatozoa and male mating index were observed in the F0. The slight decrease in the number of successful matings at 300 and 1,000 mg/kg bw per day appears unrelated to the male partners, as all males that failed to impregnate their females showed normal sperm motility and sperm counts. Only one of the high-dose males was found to have a lower testicular spermatid content (50% of the group mean), a finding that was also associated with a slightly lower testis weight (85% of the group mean). The number of abnormal sperm was low in all dose groups and remained below 2% in the few males in which abnormal sperm were found. The Panel noted that the epididymal sperm parameters were not evaluated; this is a deviation from the OECD TG 443. The Panel considered that this deviation has no effect on the final conclusion of the study.

There were no effects on any of the sperm endpoints in the cohort 1A.

Table 3: Male fertility parameters of F0 and F1 (cohort 1A)

Dietary dose (mg/kg bw per day)	F0				F1 (cohort 1A)			
	Control	100	300	1,000	Control	100	300	1,000
No. of males in study	24	24	24	24	20	20	20	20
No. of fertile males[(a)]	24	23	22	22	–	–	–	–
No. of males evaluated	20	20	20	20	20	20	20	20
Testis weight (right), g	1.85	1.88	1.88	1.74	1.74	1.72	1.62	1.65
Spermatids/g testis ($\times 10^6$)	106.2	106.4	102.5	103.2	106.1	107.9	107.5	112.0
% motile spermatozoa	72.0	71.6	70.7	70.7	73.5	74.4	73.8	72.0
No. of males with some abnormal spermatozoa (< 2%)[(b)]	6	4	0	3	0	0	0	0

bw: body weight.
(a): Number of males inducing pregnancy in female partners.
(b): Calculated by the Panel based on individual data.

Female fertility

An overview of results for female fertility parameters is reported in Table 4. No effects on mean oestrus cycle duration were noted in F0 and F1 (cohort 1B) parental generations and all F0 females in the control, 100, 300 and 1,000 mg/kg bw per day groups mated. In the F1 generation 2 and 3 animals from the mid- and the high-dose groups, respectively, were erroneously removed from the study, before mating had been unequivocally confirmed. All other females mated, except one F1 female in the 100 mg/kg bw per day group. With few exceptions, mating occurred at the first oestrus after the females were housed with males. No effects of treatment were observed.

The pregnancy rate was slightly lower in the F0 generation at 300 and 1,000 mg/kg bw per day (100, 96, 92 and 92%). As this finding was not confirmed in the F1 generation (100, 95, 94 and 100%) the Panel considered it as incidental and not treatment-related. No effects were noted on pregnancy duration, number of implantation sites and post-implantation loss. Although they occurred in the mid-and high-dose groups, three single total litter losses, either from total resorption of all embryos or from death of the litter during or shortly before birth, were not considered to be due to treatment. This is because the two F0 dams had unusually small litters of two pups each, which were stillborn, and the F1 dam showed total resorptions of eight implants at necropsy after failing to litter. Live litter sizes and litter weights were comparable to control values in all dose groups in the F0 and the F1 generation.

Table 4: Female fertility and litter data of F0 and F1 (cohort 1B)

Dietary dose (mg/kg bw per day)	F0				F1 (cohort 1B)			
	Control	100	300	1,000	Control	100	300	1,000
No. of females paired	24	24	24	24	20	20	20	20
No. of females mated	24	24	24	24	20	19	18	17
No. of females pregnant	24	23	22	22	20	19	17	17
Live litters	24	23	21	21	20	19	17	16
Total litter loss	0	0	1	1	0	0	0	1
Mean oestrous cycle duration (days)	4.6	4.5	4.5	4.4	4.4	4.4	4.2	4.5
Precoital interval > 4 days[(a)]	0	1	1	1	2	0	3	0
Duration of pregnancy > 23 days[(a)]	0	1	1	1	0	0	0	0
Mean duration of pregnancy (days)	22.6	22.7	22.8	22.9	22.6[(b)]	22.6[(b)]	22.3[(b)]	22.5[(b)]
Implantation sites/dam	14.7	15.5	15.6	15.3	15.6	16.0	16.2	15.3
Total number of implantation sites	294	309	311	306	311	304	275	260
Post-implantation loss	38	24	48	30	20	29	16	28
Post-implantation loss (mean per dam, %)	17.5	8.1	17.0	11.8	6.7	10.1	6.0	12.8

Dietary dose (mg/kg bw per day)	F0				F1 (cohort 1B)			
	Control	100	300	1,000	Control	100	300	1,000
Litters with > 2 post-implantation losses[(a)]	4	3	9	4	2	3	1	3
Live litters with > 1 stillborn[(a)]	0	1	0	1	0	0	0	0
Live litter size	12.7	14.3	13.5	13.7	14.6[(b)]	14.4[(b)]	15.3[(b)]	13.7[(b)]
Litter weight at birth	87.3	97.5	95.3	94.6	97.7[(b)]	97.2[(b)]	97.2[(b)]	96.4[(b)]

(a): Calculated by the Panel based on individual data.
(b): Calculated by the Panel based on individual data and including all 21 pregnant animals with live litters in the study.

Conclusions on sexual function and fertility

No effects of E 171 on sexual function and fertility were observed in either males or females.

Evaluation of developmental toxicity

Pre- and postnatal lethality, structural abnormalities

No treatment-related pre- or postnatal loss was observed in the F0 and F1 generations. The average litter size at birth in all dose groups was comparable, or even higher than in the control group. The sex ratio was unaffected (see Table 5).

No external or internal abnormalities were detected in F1 and F2 pups at termination.

Table 5: Offspring data for F1 and F2 generations

Dietary dose (mg/kg bw per day)	F1				F2			
	Control	100	300	1,000	Control	100	300	1,000
No of pups delivered	309	332	286	294	292	276	261	233
Live-born pups	304[(a)]	317[(a)]	283[(a)]	287[(a)]	291	274	259	233
Stillborn/dead pups on PND 1	5[(a)]	15[(a)]	3[(a)]	7[(a)]	1	2	2	0
% males	52.0[(a)]	52.4[(a)]	49.1[(a)]	51.6[(a)]	54.6	54.7	48.8	49.4
Litter size PND 1	12.7[(a)]	13.8[(a)]	13.5[(a)]	13.7[(a)]	14.6	14.4	15.2	13.7
Litter size PND 4 (pre-cull)	12.3[(a)]	13.2[(a)]	13.1[(a)]	13.2[(a)]	12.9	14.1	14.9	13.4
Litter size PND 4 (post-cull)	8.8[(a)]	9.5[(a)]	9.9[(a)]	9.8[(a)]	–	–	–	–
Litter size PND 21	8.3[(a)]	9.1[(a)]	9.6[(a)]	9.5[(a)]	–	–	–	–
Total litter loss PND 2–4	0[(a)]	0[(a)]	0[(a)]	0[(a)]	1	0	0	0
Litters with pup loss > 1 PND 2–4	1[(a)]	1[(a)]	2[(a)]	2[(a)]	3	1	0	1
Litters with pup loss > 0 PND 5–21	4[(a)]	4[(a)]	3[(a)]	3[(a)]	–	–	–	–

(a): Calculated by the Panel based on individual data for F1.

Growth and sexual development

An overview of the results related to growth and sexual development for the F1 and F2 generations is reported in Table 6. No treatment-related effects were observed in birth weights and growth of the pups. There were no indications for any androgenic and/or oestrogenic effects on the male and female anogenital distance (AGD) and the retention of nipples in males. The Panel noted that instead of examining balanopreputial separation as required by the OECD TG 443 the laboratory examined balanopreputial gland cleavage which does not comply with the OECD TG 443 and cannot be considered a measure of puberty in males.

The mean age at vaginal opening was comparable between control and treated groups. The statistically significant lower body weight on the day of vaginal opening in cohort 1A at 300 mg/kg bw per day was not considered to be biologically relevant due to the slightly higher litter sizes in all treated groups, which would have given a growth advantage to the control group.

Table 6: Pup body weight development and attainment of puberty

Dietary dose (mg/kg bw per day)	F1				F2			
	Control	100	300	1,000	Control	100	300	1,000
Birth weight males (g)	7.19[(a)]	7.26[(a)]	7.25[(a)]	7.22[(a)]	7.01	6.94	6.59	6.87
PND 4	9.96[(a)]	9.86[(a)]	9.83[(a)]	9.77[(a)]	9.68	9.58	8.94	9.34
PND 14	32.34[(a)]	30.76[(a)]	30.61[(a)]	31.41[(a)]	–	–	–	–
PND 21	53.49[(a)]	51.92[(a)]	49.56[(a)]	51.02[(a)]	–	–	–	–
AGD/body weight cubed PND 4	17.0[(a)]	16.5[(a)]	17.1[(a)]	17.5[(a)]	15.92	15.38	15.87	15.53
Nipple retention PND 13[(b)]	0	2	3	0	–	–	–	–
Age (PND) at balanopreputial gland cleavage C1A[(c)]	22.3	22.6	22.1	22.2	–	–	–	–
Body weight (g) on day of balanopreputial gland cleavage C1A	59.1	60.4	55.4	57.3				
Age (PND) at balanopreputial gland cleavage C1B[(c)]	22.3	22.4	22.2	22.4				
Body weight (g) on day of balanopreputial gland cleavage C1B	59.2	58.4	56.4	58.4				
Birth weight females (g)[(a)]	6.80[(a)]	6.88[(a)]	6.96[(a)]	6.82[(a)]	6.55	6.55	6.22	6.59
PND 4	9.30[(a)]	9.41[(a)]	9.62[(a)]	9.34[(a)]	9.17	9.15	8.66	9.02
PND 14	30.99[(a)]	29.43[(a)]	29.87[(a)]	29.98[(a)]	–	–	–	–
PND 21	50.67[(a)]	49.52[(a)]	48.48[(a)]	49.18[(a)]	–	–	–	–
AGD/body weight PND 4	8.2[(a)]	8.4[(a)]	8.5[(a)]	8.5[(a)]	7.70	7.64	7.52	7.70
Age (PND) at vaginal opening C1A	33.6	32.8	32.8	32.8	–	–	–	–
Body weight (g) on day of vaginal opening C1A	123.0	117.8	112.4[(d)]	117.5				
Age (PND) at vaginal opening C1B	33.2	32.7	32.9	32.6				
Body weight (g) on day of vaginal opening C1B	120.6	120.2	114.2	117.1				

bw: body weight; PND: postnatal day; AGD: anogenital distance.
(a): Calculated by the Panel for the pups of F0 dams based on individual data.
(b): Calculated by the Panel based on individual data; Litters with > 0 male pups with more than 2 retained nipples.
(c): The Panel noted that this test as conducted by the laboratory does not comply with the OECD TG 443 and cannot be considered a measure of puberty in male.
(d): $p \leq 0.05$ (ANOVA and Dunnett).

Conclusions on developmental toxicity

No effects of E 171 on pre- and postnatal development were observed. Data on the attainment of puberty in males (i.e. an appropriate assessment of the timing of the balanopreputial separation) were missing; however, given the lack of any other treatment-related effects on other parameters, the Panel does not consider this to be critical in this case.

4.2.8. Neurofunctional screening

Male and female F1 cohort 2A offspring were tested for auditory startle response between PND 23 and 25, and for a functional observation battery including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64.

No differences in the response to an auditory startle stimulus were observed between the control and all the tested doses.

Compared to controls, an increase in hindlimb splay was observed in females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

To check whether the significant differences in grip strength and hindlimb splay could be due to systematic bias in group testing order, the testing order was checked (Documentation provided to EFSA No 11). The Panel considered that there was no systematic bias in group testing order and that this was therefore not a plausible explanation for the observed group differences.

Grip strength and hindlimb splay belong to the same domain of neurological function, i.e. motor function and/or sensory–motor coordination. However, the effects observed (i.e. increase in hindlimb splay and increase in mean forelimb grip strength) seem to point in opposite directions when it comes to muscle strength. In particular, an increase in hindlimb splay can be interpreted as muscular weakness whereas an increase in mean forelimb grip strength could be indicative of myotonia. The Panel noted that the effects observed were not correlated to any other changes (e.g. alterations in muscle tone, righting reflex, gait, wire manoeuvre, posture). No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in the functional observation battery measurements or locomotor activity were noted. Furthermore, there were no notable histopathological findings in brain or in peripheral nerve (sciatic). Based on all the above considerations, the Panel considered that the effects on grip strength and hindlimb splay were not treatment-related. However, the Panel noted that quantitative information on peripheral nerves was not available.

Overall, the Panel considered that E 171 had no adverse effects on neurofunctional endpoints in F1 cohort 2A offspring at the doses used.

4.2.9. Developmental Immunotoxicity

Effects on developmental immunotoxicity were determined in the F1 cohort 3 animals through an examination of their ability to raise an antibody response to a foreign antigen. Animals are sensitised and the primary IgM antibody response to the sensitising antigen, in this case to keyhole limpet haemocyanin (KLH) antigen, is measured. The ability of the test compound to modulate serum anti-KLH antibody titre is taken as indicative of a developmental immunotoxic effect. A KLH-immunised control group also exposed to a known immunosuppressant (i.e. cyclophosphamide (CY)), resulting in at least 50% inhibition in serum IgM anti-KLH titre, is crucial for the verification of assay performance.

These data can be considered in combination with additional data related to potential immunotoxic effects. For example, in the F1 cohort 1A animals, the following parameters – even if generally not regarded as having the predictive power of functional tests such as the KLH assay – may contribute to the general assessment for immunotoxicity: weight and histopathology of the spleen, thymus and lymph nodes, as well as bone marrow histopathology, total and differential peripheral WBC count and splenic lymphocyte subpopulation distribution.

T-cell-dependent anti-KLH response (KLH assay)

The determination of serum anti KLH-IgM antibodies was performed in F1 cohort 3 (10/sex per group, PND 53–61) using an enzyme-linked immunosorbent assay (ELISA). The animals were sacrificed 5 days after intravenous bolus injection (tail vein) of KLH, blood was withdrawn and the level of anti-KLH IgM was measured in serum. In addition, satellite animals of F1 (10/sex, PND 55) were immunised with KLH and treated with CY (single administration of 40 mg/kg bw by gavage on the same day of KLH treatment) to provide a positive control (for an inhibition of immune response).

A slight, but statistically significant decrease in the antigen specific IgM level was measured at the highest dose tested (1,000 mg/kg bw per day) in males only (–9%) and without an apparent dose response.

In addition, the Panel noted that treatment with CY was not performed at the same time as the rest of F1 cohort 3, without a separate control for the CY response, conducted at the same time (Documentation provided to EFSA No 11). Since the results from the CY positive control were not valid, the sensitivity of the test was not demonstrated.

It was noted that the assay conditions may have not been optimal resulting in an apparent low antibody response to KLH when compared to literature (Gore et al., 2004), as also pointed out by the study authors (Documentation provided to EFSA No 11).

The study authors considered that all tested animals in the study had a weak immunogenic response to KLH that was insufficient to identify a T-cell-dependent immunotoxic effect of E 171. The study authors therefore considered that no conclusion can be drawn on the effect of E 171 on the developing immune system.

The Panel agreed with the conclusion of the study authors.

Assessment of pathology, haematology and splenic lymphocyte subpopulations

At necropsy, pathology of lymphoid organs, haematology and lymphocyte subpopulations in the spleen were investigated. The spleens of all animals were cut in two, and histopathology was performed on one of the spleen halves of the F1 cohort 1A animals only. From the remaining half spleen from animals of F1 cohort 1A (20/sex per group, PND 87–96) and from the spleens of the animals of F1 cohort 3 (10/sex per group, PND 53–61), single cell suspensions were prepared for the analysis of the subpopulation of lymphocytes. The following lymphocyte subpopulations were determined via flow cytometry analysis (FACS): T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells.

The Panel noted that haematology, spleen weight and histopathology of lymphoid organs in animals from F1 cohort 1A did not indicate any dose-related effects. As regards the splenic lymphocyte subpopulation analysis, no statistically significant differences were observed in the percentage of T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells of any of the treated groups compared to control in both sexes. The study authors concluded that no test substance-related effect was observed on the proportion of the examined lymphocyte subtypes.

The Panel agreed with the study author conclusion that the splenic lymphocyte subpopulations in this cohort were not affected. However, the Panel considered that an isolated observation in F1 cohort 1A is not sufficient to conclude on immunotoxicity.

According to the study authors, when compared to animals of F1 cohort 1A, F1 cohort 3 animals showed a shift in the lymphocyte subpopulation that indicated activation of the immune system by injection of KLH, and concluded that increased B-cell proliferation may have led to the production of antigen-specific antibodies. In F1 cohort 3 animals, no differences in the relative size of the lymphocyte subpopulations were observed between the control group and the E 171-treated groups, after immunisation of the animals with KLH. The study authors argued that the B-cell shift in F1 cohort 3 was caused by KLH immunisation, supported by the fact that there was no such shift found for the positive control animals that were sensitised to KLH and treated with CY.

The study authors have acknowledged that the positive control of F1 cohort 3, i.e. the effect of CY on the antibody response to KLH, has not met with expectations. However, the authors considered that KLH induced an immune reaction, and that this response was influenced by CY in the way it would be expected (i.e. a shift in T-/B-cell populations in the spleen). According to the study authors, KLH would increase the percentage of splenic B cells and decrease the percentage of T cells. Therefore, they concluded that the immune response was affected by CY but was not adversely affected by the TiO_2 test substance.

The Panel did not agree with the conclusion of the study authors that a shift to B cells by KLH was substantiated. The Panel considered that it is incorrect to compare the groups of F1 cohort 1A and of F1 cohort 3 because the groups of animals of F1 cohort 3 had a different age than that of the animals in F1 cohort 1A at the time of sacrifice (PND 87–96 vs. PND 53–61, respectively). In addition, the FACS analyses on the splenic cell suspensions were not all performed in the same round of analysis but were performed separately, while it is known that this may have influenced staining and subsequent quantification.

The study authors suggested that even if the positive CY control did not perform as expected, the data still indicate there is no effect of E 171 on sensitisation to KLH. However, the Panel did not agree with this conclusion and overall considered that the data did not allow to conclude on developmental immunotoxicity with respect to E 171.

4.2.10. Uncertainty

The Panel identified the following uncertainties regarding the EOGRT study with respect to its validity to fully identify all potential adverse effects of E 171 when used as a food additive:

i) The extent to which the particle size distribution of the E 171 used in the EOGRT study is reflective of the particle size distributions of E 171 when added to foods.
ii) The extent to which the particle size distribution of E 171 in transit through the GIT in the EOGRT study was affected by the concentration in the diet (i.e. dose).

The selected test material was representative of E 171 containing a large proportion (around 50% by number) of constituent particles below 100 nm (E 171 sample E reported in EFSA FAF Panel, 2019). The particle size distribution of the E 171 in samples of the test diet was also analysed after applying a sample dispersion protocol that aims to extract E 171 particles from the feed matrix ((Documentation provided to EFSA No 16) and the results show that the particle size distribution of the constituent

particles was similar to that of pristine E 171 after dispersion (EFSA FAF Panel, 2019; Verleysen et al., 2020).

However, neither of these procedures were considered by the Panel to reliably determine the particle size distribution of E 171 in the feed. The Panel acknowledges that methods for determining particle size distributions in complex foods and feeds *in situ* are not currently available. Accordingly, the Panel considers that the extent to which the particle size distribution of the E 171 used in the EOGRT study is sufficiently reflective of the particle size distributions of E 171 when added to foods remains uncertain.

The interested business operator considered that mixing of two dry components (feed and E 171) was the best possible option to retain the particle size distribution properties of the original E 171 sample, and that the use of liquid dispersion would add further superfluous unknowns (Documentation provided to EFSA No 11).

The Panel considered that E 171 has a broad size distribution of constituent particles (from about 40 to 250 nm); considered that in dry form, this size distribution of the constituent particles is expected to be stable and further, that homogenous mixing of E 171 with dry diet is a pragmatic approach to adopt in terms of performing an animal study over an extended time frame such as the EOGRT study. The Panel considered this approach to be representative of some uses of E 171 in food (e.g. E 171 in confectionary coatings and fillings and in ready to use sauces ((Documentation provided to EFSA No 11)). However, the Panel also noted that this approach may not be fully representative for all uses of E 171 in food since liquid dispersion of E 171 was reported to be used, potentially along with additional processes, to reduce the formation of agglomerates in suspension in some products (e.g. incorporation of E 171 into a tablet coating or capsule (Documentation provided to EFSA No 17).

The Panel considered that investigations of Ti levels in tissues would have reduced uncertainty regarding dose dependency of internal exposure. However, the Panel noted that the EOGRT study demonstrated unequivocally low levels of internal exposure to TiO_2 in animals that were fed a diet prepared by addition of E 171 to dry feed.

Dispersed NPs show a greater tendency to agglomerate when suspended in liquid media at higher concentrations. This concentration effect on agglomeration and/or resistance to de-agglomeration may also exist in the GIT at high-dose levels (Appendix E). The Panel therefore considered that there remains an uncertainty regarding the effects of dose levels/concentrations in feed and the extent to which agglomeration occurred in the GIT. However, the Panel considered the propensity for this agglomeration is likely reduced when exposure is via feed rather than through bolus gavage administration of E 171.

4.3. Genotoxicity

The genotoxicity of TiO_2 was reviewed by the ANS Panel in the 2016 opinion on the re-evaluation of the food additive (E 171) (EFSA ANS Panel, 2016). According to the data provided by interested parties and from the literature at that time, TiO_2 as E 171 was not considered to be a nanomaterial according to the EU Recommendation on the definition of a nanomaterial (i.e. 'a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm'). Therefore, the ANS Panel had considered that the data on TiO_2 as a nanomaterial were not directly applicable to the evaluation of the food additive. The EFSA ANS Panel (2016) had noted mixed results in *in vitro* genotoxicity studies, which had provided some evidence of *in vitro* genotoxicity for TiO_2 micro- and nanoparticles. The ANS Panel had considered that most positive results were reported under experimental conditions associated with the induction of oxidative stress (as shown by increased 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), LPO and reactive oxygen species (ROS) generation), and that the genotoxic effects observed mainly concerned indicator assays (comet and H2AX histone phosphorylation), which in some studies were shown not to be associated with permanent chromosome damage such as chromosome breaks visualised as micronuclei (MN).

In vivo, overall negative results were obtained in genotoxicity studies with microsized TiO_2 pigment. Limited evidence of genotoxicity, if any, was provided by studies with orally administered TiO_2 NPs. Limited or no indication of the genotoxicity of TiO_2 NPs was provided by studies using an intravenous route of administration, which allows maximum exposure of target tissues. Overall, the ANS Panel had concluded that the use of E 171 as a food additive did not raise a concern with respect to genotoxicity (EFSA ANS Panel, 2016).

Upon request by the European Commission, in 2018, the ANS Panel had evaluated four new studies on the potential toxicity of E 171. One of these studies (Proquin et al., 2017) investigated the potential genotoxicity of three different TiO_2 test materials in two human colon cancer cells lines. The study authors reported genotoxic activity *in vitro* of E 171 in a comet assay and in a cytokinesis-block MN assay. The ANS Panel had noted that, in view of the composition of the tested E 171 (containing around 40% of nanoparticles by number), and of the available evidence of genotoxicity *in vitro* of both TiO_2 microparticulate (MP) and TiO_2 NPs, a genotoxic activity of E 171 under *in vitro* conditions could already be anticipated. In this respect, the ANS Panel also had noted that overall negative results were obtained in studies *in vivo* with both TiO_2 NPs and TiO_2 MP (EFSA ANS Panel, 2016), indicating that TiO_2 MP and TiO_2 NPs, and consequently E 171, did not raise concern for *in vivo* genotoxicity (EFSA ANS Panel, 2018).

In 2019, EFSA published a statement on the review of the risks related to the exposure to E 171 performed by ANSES (2019). With respect to genotoxicity, the ANSES opinion made reference to a systematic review of *in vitro* genotoxicity studies on nano titanium dioxide (Charles et al., 2018). According to the authors of this review, the majority of the publications analysed had shown that the *in vitro* genotoxic effect of TiO_2 NPs was linked to a secondary mechanism consequent to oxidative stress, although primary genotoxic effects could not be excluded. The only additional *in vivo* study identified (Jensen et al., 2019) was considered by ANSES to have methodological limitations that reduced the reliability of the negative results observed. According to the ANSES opinion, although there were no studies showing direct interaction of E 171 with the DNA and/or the mitotic apparatus, a direct effect of E 171 on genetic material or other molecules interacting with the genetic material could not be excluded. ANSES recommended further investigation of *in vivo* genotoxicity. Overall, EFSA noted that the new genotoxicity studies assessed in the ANSES's opinion did not add new elements to the previous conclusions by the EFSA ANS Panel and did not provide any reason to revise the conclusion on genotoxicity of E 171, previously drawn by the EFSA ANS Panel (2016, 2018). EFSA considered that a review of the overall genotoxicity database may be needed once the characterisation of titanium dioxide used as the food additive E 171 is completed (see EFSA, 2019).

The focus of the current updated assessment was to gather any relevant information from the newly available evidence that could be used to refine the risk assessment and to reduce the uncertainties identified by the ANS Panel in its earlier evaluation. In particular, the FAF Panel examined whether new data from the published literature could provide new evidence on the potential genotoxicity of E 171. To this aim, a literature search was performed as reported in Appendices A and B. Based on new information regarding the constituent particle size distribution of E 171 (EFSA FAF Panel, 2019) and on the updated EFSA Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health (EFSA Scientific Committee, 2018a) also the references from the previous ANS Panel opinion (2016) have been considered in the present assessment. In addition, data submitted in the context of the NANOGENOTOX project, 2013 (Documentation provided to EFSA No 7, 8, 9 and 10), publications reported in the OECD dossier (OECD, 2016) and documentation provided by interested business operators (IBOs) (Documentation provided to EFSA No. 14 and 15) have been included in the current assessment using the same appraisal criteria applied to the newly published data.

Genotoxicity studies considered for this assessment are:

- *in vitro* and *in vivo* studies retrieved from the literature search (Appendices J, K),
- *in vitro* and *in vivo* studies considered in the re-evaluation of E 171 (EFSA ANS Panel, 2016) (Appendices L, M),
- *in vitro* and *in vivo* studies reported in the OECD (2016) ((published papers and results from NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7, 8, 9 and 10)) (Appendices N, O) and
- *in vitro* studies submitted by IBOs (Documentation provided to EFSA No 14 and 15) (Appendix P)
- studies considered relevant for the assessment of E171 in addition to studies considered in the re-evaluation of E171 (EFSA ANS Panel, 2016) or retrieved in the updated literature search (see Section 2.2)

The score for NSC related to specific aspects in the study design (dispersion and/or confirmation of internal exposure) for all studies has been performed according to Appendix E.

The genotoxicity studies have been assessed using a scoring system for reliability based on criteria published by Klimisch et al. (1997) as explained in Appendix D. In a second step, the relevance (high, limited, low) of study results was assessed based on reliability of the study, some general aspects e.g. genetic endpoint, route of administration and status of validation of the assay and specific NSC (see Appendix D).

Genotoxicity studies evaluated as of low relevance have not been further considered in the assessment. The assessment of *in vitro* and *in vivo* studies of high and limited relevance is reported in the following sections.

4.3.1. Gene mutation

In vitro gene mutation studies

Fourteen studies investigated the ability of TiO_2 NPs to induce gene mutations in mammalian cultured cells. Seven of these studies were considered of high or limited relevance and consequently were taken into account in the assessment of genotoxicity (Appendices J, L, N). Positive results were reported in two hypoxanthine-guanine phosphoribosyl transferase (HPRT) studies performed in Chinese hamster V79 lung fibroblasts (Chen et al., 2014; Jain et al., 2017) and in a Spi^- gene mutation assay in primary embryo fibroblasts from gpt delta transgenic mouse (Xu et al., 2009). On the contrary, no mutagenic effect was observed in two HPRT assays, one in V79-4 cells (a cell line derived from V79) (Kazimirova et al., 2020) and one in Chinese hamster ovary cells CHO-K1 (Wang et al., 2011). In a mouse lymphoma assay (Demir et al., 2017), no statistically significant increase over the untreated control was reported and the global evaluation factor (GEF) was never exceeded; however, there was a statistically significant concentration–effect relationship in all six experiments performed, therefore the overall result was considered equivocal. Negative results were reported in another mouse lymphoma study ((NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8)).

In addition, the data set included seven bacterial reverse mutation studies, plus one recently submitted by industry. However, all these studies were considered of low relevance, due to limitations in the penetration of particles through the bacterial cell wall and the lack of internalisation in bacteria (EFSA Scientific Committee, 2018a).

In vivo gene mutation studies

The induction of gene mutations was investigated *in vivo* in six studies considered of high or limited relevance (Appendices K, M, Table 7). All studies were performed with TiO_2 NPs (< 30 nm).

Table 7: Summary table of test results of *in vivo* gene mutation studies

Study design	Test material	Results	Reliability/ Relevance	Reference
Oral				
C57BL/6Jp^{un}/p^{un} mice *In vivo* DNA deletion assay in the p^{un} locus Drinking water for 10 days to pregnant mice; 500 mg/kg	NSC: 2, TiO_2 NPs (P25), **15–24 nm**	**Positive**	2/limited	Trouiller et al. (2009)
Intraperitoneal injection				
Male B6C3F1 mice Pig-a gene mutation assay in peripheral blood reticulocytes and in total red blood cells; 0.5, 5.0 and 50 mg/kg bw per day for 3 days	NSC: 1, TiO_2 NPs, anatase, ellipsoidal shape (TEM), minor axes **12.1 ± 3.2 nm**	**Negative**	2/limited	Sadiq et al. (2012)
Intravenous injection				
Gene mutation in *gpt*, Spi^- (liver) and pig-a (erythrocytes) Delta transgenic C57BL/6J mice; 2, 10 and 50 mg/kg bw for 4 weeks	NSC: 1, TiO_2 NPs (P25), **15–24 nm**	**Negative**	1/limited	Suzuki et al. (2016)

Study design	Test material	Results	Reliability/ Relevance	Reference
Gene mutation in *gpt*, Spi⁻ (liver) Delta transgenic C57BL/6J mice; 0, 2, 10 and 50 mg/kg bw for 4 weeks	NSC: 1, TiO_2 NPs (P25), **15–24 nm**	**Negative**	2/limited	Suzuki et al. (2020)
LacZ mutation assay in liver and spleen C57BL/6 transgenic mice; 0, 10 and 15 mg/kg bw, i.v. on 2 days	NSC: 1, TiO_2 NPs (NM-102), **21–22 nm**	**Negative**	1/limited	Louro et al. (2014)
Intratracheal instillation				
Male Sprague–Dawley rats Pig-a gene mutation assay in peripheral blood reticulocytes and in total red blood cells; 3 endotracheal instillation over 8 days; 0.5, 2.5 and 10 mg/kg (a total particle surface area lung deposition of 87, 437 and 1,700 cm^2/lung)	NSC: 1 TiO_2 NPs (P25), **15–24 nm**	**Negative**	2/limited	Relier et al. (2017)

NSC: nanoscale considerations; NP: nanoparticle; i.v.: intravenous.

There are three *in vivo* gene mutation assays that were performed in mice with intravenous application, all with negative results. TiO_2 NPs (< 30 nm) were administered to male gpt Delta transgenic C57BL/6J mice at doses up to 50 mg/kg bw once a week for 4 weeks, then Pig-a mutation was analysed in erythrocytes and gpt and Spi⁻ mutation in liver (Suzuki et al., 2016). In another assay performed in the same laboratory the gpt and Spi⁻ genes were investigated in liver 90 days after the last injection (Suzuki et al., 2020). Louro et al. (2014) treated intravenously C57BL/6 transgenic mice and analysed *LacZ* mutations in liver and spleen.

Pig-a gene mutation was analysed also in two studies in peripheral blood reticulocytes and in total red blood cells: one in male B6C3F1 mice, after intraperitoneal administration (Sadiq et al., 2012); one in male Sprague–Dawley rats after intratracheal administration (Relier et al., 2017). In both these studies, no mutagenic effects were reported.

In contrast, Trouiller et al. (2009) found a significant increase in large DNA deletions in an eyespot assay performed in fetuses after a 10-day administration of TiO_2 (P25) suspended in drinking water to pregnant C57BL/6Jp^{un}/p^{un} mice.

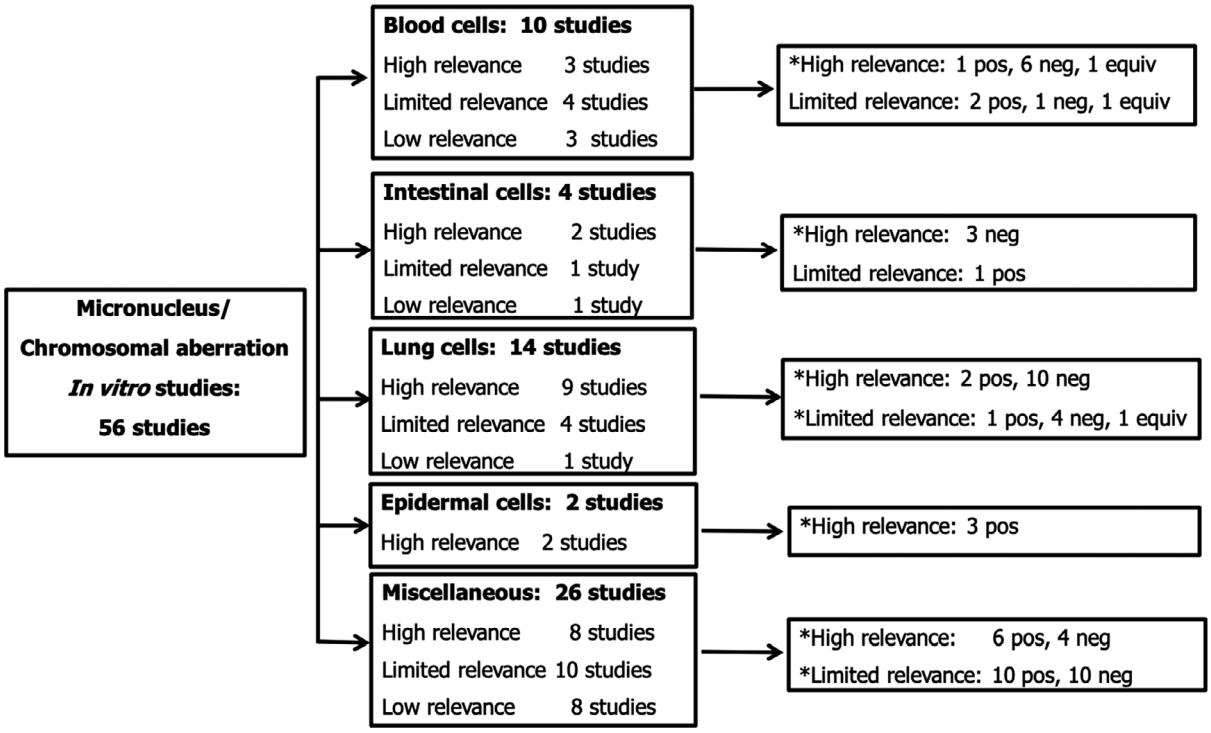
Concluding remarks

Several *in vitro* studies demonstrated the ability of TiO_2 NPs to induce gene mutations in cultured mammalian cells. One *in vivo* study indicated the induction of large DNA deletions, however four other studies, that investigated different molecular targets suitable for identification of point mutations and small deletions, gave consistently negative results. Overall, the available experimental data do not confirm the potential of TiO_2 NPs (< 30 nm) to induce gene mutations *in vivo*.

4.3.2. Induction of micronuclei/chromosomal aberrations *in vitro* and *in vivo*

***In vitro* micronuclei/chromosomal aberrations**

Fifty-six *in vitro* studies on MN frequency and structural chromosomal aberrations (CAs) in different cell lines were available for the evaluation ((44 papers, NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8) and industry (Documentation provided to EFSA No 15)) (Appendices J, L, N, P). Of 56 studies, 43 were classified as of high or limited relevance and further considered in the assessment (Figure 1).



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

Figure 1: *In vitro* micronucleus and chromosomal aberrations studies. Summary of test results from 56 studies reported in 44 papers, NANOGENOTOX Project, 2013 (Documentation provided to EFSA No 7 and 8) and Documentation provided to EFSA No 15

Three out of seven studies performed with primary human lymphocytes to which high or limited relevance was assigned showed positive results. In a study, classified of high relevance, a concentration-dependent increase of MN frequency was observed in peripheral blood lymphocytes from healthy subjects and colon cancer patients (Kurzawa-Zegota et al., 2017). Positive results in cultures of human peripheral lymphocytes were also reported in two studies with limited relevance (Turkez and Geyikoglu, 2007; Kang et al., 2008). Negative or equivocal results were described in four studies classified at high or limited relevance (NANOGENOTOX Project 2013 Documentation provided to EFSA No 7 and 8; Tavares et al., 2014; Andreoli et al., 2018; Osman et al., 2018)).

Three out of four studies performed with intestinal cells were considered relevant. One study, classified at high relevance, showed negative results with MN assays in Caco-2 cells exposed at different concentrations of TiO_2 NPs (Zijno et al., 2015). The outcome of this study is consistent with the results reported in the same cell line by the NANOGENOTOX Project, 2013 (Documentation provided to EFSA No 7 and 8). A single study showed concentration dependent increase of MN frequency in human colon adenocarcinoma (HCT116) cell line (Proquin et al., 2017).

Thirteen studies performed with lung cells were classified as relevant. Four out of five studies available in human lung epithelial cells (BEAS-2B) were negative with MN tests after exposure at different concentrations of TiO_2 NPs and for different times ((NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Vales et al., 2015; Di Bucchianico et al., 2017; Zijno et al., 2020). In Falck et al. (2009), negative (rutile, 5,000 nm) and equivocal (anatase, < 25 nm) results were reported. Positive results with the MN test were reported in BEAS-2B cells only using a treatment medium that minimised the nanoparticle agglomeration (Prasad et al., 2013). Inconsistent results were reported in studies in human lung carcinoma cell line (A549). Two out of five studies were evaluated as positive (Srivastava et al., 2013; Stoccoro et al., 2017). Negative results were reported in two studies ((NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Brandão et al., 2020) classified at high relevance and in a study with limitations (Jugan et al., 2012). Negative results with the CA test were described in a Chinese hamster lung cell line (CHL/IU cells) (Nakagawa et al., 1997).

Two studies in human epidermal cell lines (A431, NHEK) to which high relevance was assigned were positive ((NANOGENOTOX Project, 2013 Documentation provided to EFSA No 7 and 8); Shukla et al., 2011).

Twenty-six studies carried out in various other types of cell lines of different origin, reporting results on MN frequency or on structural CAs were evaluated: eight of them were classified of high relevance and ten of limited relevance. The differences in the results observed in different studies could not be attributed to a certain parameter such as the crystalline form, particle size, degree of aggregation, treatment medium used, concentrations applied and treatment time.

The Panel noted that around 60% of the available results were obtained with TiO_2 NPs < 30 nm. The majority of *in vitro* MN or CA tests gave negative results, regardless of the size of the tested particles (55% for TiO_2 NPs < 30 nm and 67% for TiO_2 NPs > 30). A single study tested E 171 in intestinal cells and reported positive results (Proquin et al., 2017).

In vivo micronuclei/chromosomal aberrations

Twenty-six *in vivo* studies addressing the potential of TiO_2 to induce MN and structural CA through various routes of exposure were available for the evaluation. After a screening for reliability and relevance of the results (Appendices K, M), 15 studies, ranked as of high (one study) and limited (14 studies) relevance, were selected for further consideration in the assessment (Table 8, Figure 2).

Table 8: Summary table of test results of micronucleus and chromosomal aberrations *in vivo* studies. 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				
MN in bone marrow rats 10–200 mg/kg per bw 30 days	NSC: 2, TiO_2 NPs, Anatase **75 ± 15 nm**	**Negative**	2/limited	Chen et al. (2014)
MN and CA in bone marrow mice 200 and 500 mg/kg per bw 90 days	NSC: 4, TiO_2 NPs, crystalline form unknown, mean **58 nm**	**Positive**	2/limited	Chakrabarti et al. (2019)
MN in bone marrow mice 10, 50, 100 mg/kg per bw 14 days	NSC: 1, TiO_2 NPs Anatase, **20–50 nm**	**Positive**	1/high	Shukla et al. (2014)
CA in bone marrow mice 0.2–0.8 mg/kg per bw 28 days	NSC: 2, TiO_2 NPs, Rutile, **21–31 nm**	**Positive**	2/limited	Manivannan et al. (2020)
MN in bone marrow mice 50, 100 and 200 mg/kg bw 60 days	NSC: 2, TiO_2 NPs Anatase, **5–12 nm**	**Positive**	2/limited	Grissa et al. (2015)
Intraperitoneal injection				
MN assay in peripheral blood and in bone marrow mice 250, 500 and 1,000 mg/kg (1st exp.), 500, 1,000 and 1,500 mg/kg bw (2nd exp.)	NSC: 3, TiO_2 Anatase **> 100 nm**	**Equivocal**	2/limited	Shelby et al. (1993)
Chromosomal aberration in bone marrow mice 625–2,500 mg/kg bw Single administration	NSC: 3, TiO_2, Anatase, **> 100 nm**	**Negative**	2/limited	Shelby and Witt (1995)
MN in bone marrow mice 500–2,000 mg/kg bw 5 days	NSC: 1 TiO_2 NPs, anatase/rutile, **44 nm**	**Positive**	1/limited	El-Ghor et al. (2014)
MN in bone marrow mice 0.1–3 g/kg bw Single administration	NSC: 2, TiO_2 NPs, Rutile, 28.88 nm (XRD) and **5–45 nm** (TEM)	**Positive**	2/limited	Lotfi et al. (2016)

Study design	Test material	Results	Reliability/ Relevance	Reference
MN in bone marrow mice 9.38–150 mg/kg bw 5 days	NSC: 2, TiO_2 NPs Anatase, **< 30 nm**	**Positive**	1/limited	Fadoju et al. (2019)
MN in bone marrow mice 10, 100 and 500 mg/kg bw Single administration	NSC: 2, TiO_2 NPs Anatase, 20.17 nm (XRD) and **1–25 nm** (TEM)	**Equivocal**	2/limited	Zirak et al. (2016)
MN assay in peripheral blood reticulocytes Mice 0.5, 5.0 and 50 mg/kg bw/day, for 3 days	NSC: 1, TiO_2 NPs, Anatase, ellipsoidal shape (TEM), minor axes 12.1 ± 3.2 nm	**Negative**	2/limited	Sadiq et al. (2012)
Intravenous injection				
MN test in bone marrow PCE and reticulocytes Rats 5 mg/kg bw Single administration	NSC: 2, TiO_2 NPs (NM-105), **15–24 nm**	**Positive**	2/limited	Dobrzynska et al. (2014)
MN in peripheral blood reticulocytes Transgenic mice 2–50 mg/kg bw per week for 4 consecutive weeks	NSC: 1, TiO_2 NPs (P25) **15–24 nm**	**Negative**	2/limited	Suzuki et al. (2016)
MN test in peripheral blood reticulocytes Transgenic mice 10 and 15 mg/kg bw 2 days	NSC: 1, TiO_2 NPs (NM-102), **21–22 nm**	**Negative**	2/limited	Louro et al. (2014)

NSC: nanoscale considerations, MN: micronucleus assay, CA: chromosomal aberration assay, XRD: X-ray diffraction; TEM: transmission electron microscopy, PCE: polychromatic erythrocytes; bw: body weight.

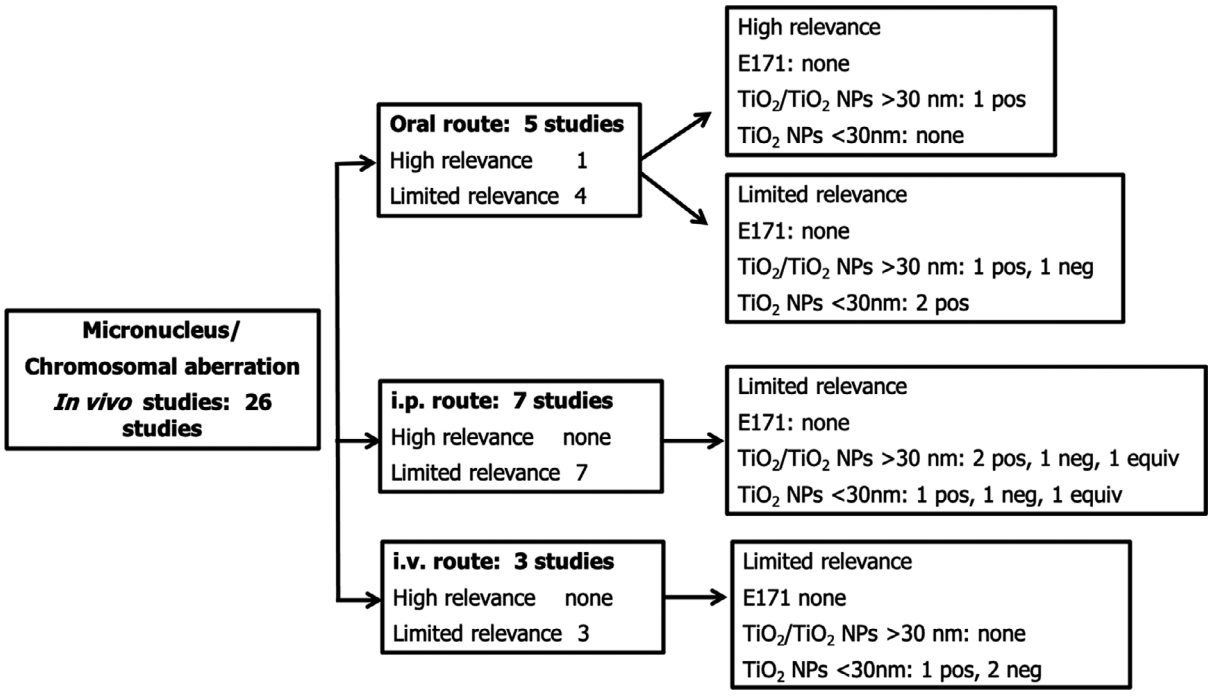


Figure 2: *In vivo* micronucleus and chromosomal aberration studies – Summary of test results