Meeting

# Annex A to TOX/2023/16 - Review of Efsa Opinion on the Reproductive Toxicity of Titanium Dioxide as a Food Additive

**This is a paper for discussion.**

**This does not represent the views of the Committee and should not be cited.**

## 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 EOGRT study requested from industry by EFSA to support the opinion.

## EOGRT Study Data

### Dosage

2. The study was designed to provide information on the effects of a TiO2 on the integrity and performance of the adult male and female reproductive system.

3. TiO2 was administered in graduated doses to several groups of males and females prior to, during and after mating until weaning of the F1 and F2 Generation.

The F1 Generation was dosed in the same way as the F0 Generation after weaning.

Until weaning, the exposure of the F1 Pups to the TiO2 was indirectly through the breast milk. The pups additionally received the TiO2 directly when commencing feeding by themselves during the last week of the lactation period.

Duration of dosing depended on the requested endpoints for the different cohorts of the F1 Generation. Cohort 1B animals were maintained on treatment beyond PND 90 and bred to obtain an F2 Generation.

4. Key developmental endpoints were examined to identify specific target organs in the offspring; offspring viability, neonatal health, developmental status at birth, and physical and functional development until adulthood.

5. Possible endocrine disruptor effects of the TiO2 were be examined by using laboratory methods T4, T3 and TSH hormone ELISA.

**Animals - F0 Generation**

6. Parental (F0) generation: Mature rats supplied by Charles River Laboratories.

7. The healthy virgin adult animals were randomised and assigned to the treatment groups and cages and held for 6 days of adaptation. Health checks were performed on the day of delivery and at first administration. The body weight range did not exceed 20 % of the mean weight for each sex at the time of selection.

Table 1: Rodent Types for Study.

| Gender | Generation | Species | Body weight at start of dosing | Age at start of dosing |
|---|---|---|---|---|
| Female | F0 | CD® (Sprague Dawley) IGS Rat (Crl:CD(SD)) | 198.8 g – 285.4 g | 71 days |
| Male | F0 | Crl:CD(SD) | 353.5 g – 446.9 g | 71 days |

8. Evaluation of parental animals:

200 female animals were evaluated 14 days pre-exposure for estrous cyclicity to yield 96 females (24 per group) with a regular estrous cycle (4 to 5 days per

cycle) for the main study and 80 for the satellite study. This provided at least 20 pregnant females per group for evaluation of the F0 Generation.

**F0 Satellite Study:**

9. F0 Satellite animals were used for blood sampling for circadian hormone level determination, evaluation of ACF and establishing an F1 Generation of satellite animals that were used as the positive control group in the KLH-assay.

Table 2: Satellite Study Summary.

| **Study** | **Main Study** | **F0 Satellite Study** | **F1 1A** | **F1 1B** | **F0, F1 1A, F1 1B** |
|---|---|---|---|---|---|
| **Males** | 96 | 80 | n/a | n/a | n/a |
| **Females** | 96 | 80 | n/a | n/a | n/a |
| **Monitoring Schedule** | During 10 weeks of pre-mating until evidence of mating. | n/a | Start after onset of vaginal patency until first appearance of cornified cells-<br>- Two weeks starting around PND 75. | Starting from the time of pairing until mating evidence was detected. | On the day of sacrifice. |

| | | | | | |
|---|---|---|---|---|---|
| **Route of administration of TiO2 Test Material** | Oral, via the diet (according to international guidelines) Diet mixtures were freshly prepared once a week. The appropriate amount was weighed into a glass container. A portion and diet was mixed with an impact mill (Grindomix GM300, Retsch GmbH, Haan, Germany) to produce a premix. Then the premix was added to the diet, mixed with a mixer (Röhnradmischer; Typ ELTE 650; J. Engelsmann AG, Ludwigshafen, Germany) for 15 minutes and then transferred to a closable bucket. Each bucket was labelled with group number and dose. To maintain a constant dose level in relation to the animals' body weight, the concentration of the test item in the diet was adjusted based on the mean group food consumption per sex. The concentration was adjusted weekly using the food consumption values from the previous week.Intake | n/a | n/a | n/a | n/a |

| | | | | | |
|---|---|---|---|---|---|
| **Dose Rate** | Administration of KLH and cyclophosphamide KLH (Keyhole Limpet Haemocyanin). Route of administration Intravenous bolus injection into a tail vein. Injection speed Dose: 15 sec.  All Cohort 3 animals (groups 1 to 4) KLH positive control animals 941 to 960. Frequency of administration Once Time point of administration 5 days before sacrifice - Sacrifice of cohort 3 animals: PND 53 to 61 - Sacrifice of satellite animals: PND 55 Reconstitution media Aqua ad injectabilia6 Vehicle PBS7 Dosage 1500 µg/animal Administration volume 0.5 mL/animal CPA (Cycloposphamide)8 - KLH positive control animals (nos. 941 to 960) Route of administration Oral, by gavage Animals Satellite animal nos. 941 to 960 Frequency of administration Once Time point of administration On the same day as KLH injection Vehicle 0.5 % Methyl Cellulose9 | n/a | n/a | n/a | n/a |

**Reproductive performance F0 females and Cohort 1B females**

10. Reproductive parameters:

- Number of pregnant females.
- Pre-coital time.
- Gestation length calculated from day 0 of pregnancy Implantation sites.
- Number per dam.
- Distribution in the uterine horns (implantation sites left or right.
- Absolute number per grou.p.
- Mean per group.
- Number of pups per group and per dam - at birth (alive and dead), on post-natal day 4 - on post-natal days 7, 14 and 21 (only F0 females) Number of male and female pups per group and per dam.
- Number of stillbirths - per group - per dam.
- Number of pups with malformations - per group and per dam.
- Values per Dam - Individual Data Relative food consumption Total food given (g) - Total food left (g) (g/kg b.w./day) = Number of animal days# x Body weight (kg) #.

N.B. The term 'animal days' counts one animal day for each animal alive for a whole day; it is assumed that on the day of death an animal does not eat.

**Reproductive indices**

11. For each group of the F0 females and the females of Cohort 1B:

- **Fertility index:** Female Fertility Index [%] = Number of pregnant rats x 100 Number of rats paired with a male.

The female fertility index reflects the total number of dams that had achieved pregnancy, including dams which delivered at term, aborted (not noted in this study) or had fully resorbed litters (Cohort 1B female no. 669 of group 4).

- **Gestation Index [%]** = Number of dams with live pups x 100 Number of pregnant rats.

For each litter and group the following indices were determined for the F0 females and the females of Cohort 1B: Birth Index [%] = Total number of pups born (alive + dead) x 100 Number of implantation scars Live Birth Index [%] = Number of pups alive on day 0/1 of lactation x 100 Total number of pups (alive + dead).

- Viability Index [%] pre-cull = Number of pups alive on day 4 (pre cull) x 100 Number of pups alive on day 0/1.
- Viability Index [%] post cull = Number of pups alive on day 21 x 100 Number of pups alive on day 4 (post cull).
- Post-implantation loss [%] = Implantations - number of pups born alive x 100 Implantations The post-cull viability index could only be calculated for the F0 females.

**Examinations of F1 Pups**

12. As soon as possible after delivery, each litter was examined to establish the number and sex of pups, stillbirths, live births, runts (pups were considered as runts if their weight was less than 70% of the mean litter weight) and the presence of gross abnormalities. Any abnormal behaviour of the offspring would have been recorded. However, no abnormal behaviour was noted for the pups.

13. The following examinations/ observations were done for the offspring: Counting, sexing, weighing.

14. Live pups were counted, sexed and weighed on postnatal days 1, 4, 7, 14 and 21. After weaning they were weighed weekly until sacrifice.

**Ano-genital distance**

15. On postnatal day 4 before litter adjustment the ano-genital distance (AGD) of all pups was determined using a scale.

**Litter adjustment of F1 Generation on PND 4**

16. After counting on PND 4, the litters were adjusted to 10 pups per litter (5 pups per sex and litter) by eliminating (culling) surplus pups using a randomization scheme generated by Provantis®. Selective elimination of pups e.g. based upon body weight is not appropriate and was not performed. In case of unequal gender distribution, a partial litter size adjustment was performed (e.g. 6 male and 4 female pups). 3.21.4 Blood sampling for thyroid hormone determination On PND 4 (determination of T4) and on PND 22 (determination of T4, T3 and TSH) blood samples for thyroid hormone level determination were taken from 2 selected pups per litter, if possible, from one male and one female pup. On PND 4 the culled surplus pups were used for blood collection and on PND 22 those pups were used which were not selected for the cohorts of the F1 Generation.

**Male nipples / Areolae counting**

17. Nipples were counted in all male pups on PND 13.

**Sexual maturation**

18. All F1 Pups that were selected for the Cohorts of the F1 Generation were evaluated daily for balano-preputial separation or vaginal opening. The body weight of the animals at the time point of balano-preputial separation or vaginal opening was recorded.

**Sex Hormone Levels**

**Males:**

19. No test item-related differences for the examined sexual hormone levels (estradiol, estrone and testosterone) were noted between the control group and the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day). Increased estrone levels in comparison to the control group were noted at the intermediate and the high dose level, statistically significant only at the intermediate dose level (see Text Table 7-10). However, though the mean values were increased in comparison to the control group, the individual values from 10 of 10 animals of the intermediate dose group (range between 8.7 and 14.0 pg estrone/mL) and from 9 of 10 animals of the high dose group (range between 7.4 and 14.4 pg estrone/mL) were in or only marginally above the range of the control group (between 5.8 and 13.5 pg estrone/mL).

20. One increased value was only noted for the high dosed animal no. 168 (36.4 pg estrone/mL). Hence, as this increased value was only noted for 1 animal of 20 animals of the high dose group, the observation was considered to be spontaneous. For the purpose of an assessment of the relevance of any minor alterations in hormone levels, a 24-hour circadian cycle was monitored in male and female animals at two different time points in satellite animals (see section 7.14 ‘Circadian cycles of hormone levels - Satellite animals’). As expected, these measurements document that during a 24-h day, all investigated hormones are subject to substantial variation.

21. Statistically significant changes of the sexual hormone levels that were not considered to be TiO2-related.

**Sexual Hormone Level Summary**

22. Males: The moderate increase is considered to be spontaneous, as the mean values were inside the range of the circadian cycle.

23. Females: No test item-related differences for the examined sexual hormone levels (estradiol, estrone and testosterone) were noted between the control group and the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day).

**Thyroid hormone levels**

24. Male: No TiO2-related differences for the examined thyroid hormone levels (T3, T4 and TSH) were noted between the control group and the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day).

25. Females: No test item-related differences for the examined thyroid hormone levels were noted between the control group and the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day). A slight but statistically significantly increased T3 level in comparison to the control group was noted at the high dose level. However, all mean and all individual values of the control group and the treatment groups were inside the range of the mean values from the examined time points that were measured during the examination of the 24 hour cycle of T3 concentration. Further on, this increase in T3 concentration was observed in females only. Hence, the slight increase was considered to be spontaneous. Statistically significant changes of the thyroid hormone that were not considered to be TiO2-related.

**Circadian Hormone Levels**

26. Satellite animals: It is known that several hormones are subject to variations during a normal 24-hour cycle. In order to be able to put any potential hormone alterations in any of the treatment groups into perspective, an analysis of the 24-hour circadian variation of estradiol, testosterone, T3, T4, and TSH was performed both in male and female satellite animals.

27. Method: For the determination of the daily circadian cycles of estradiol, testosterone, TSH, T4 and T3 satellite animals of the control group and the high dose group were used. Blood withdrawal occurred in a 2-hour interval, starting at 10 a.m. and ending at 8 a.m. on the following day (five animals were used for each time point). The examinations were performed pre-dose (test days 14 / 15) and again after 10 weeks of dosing (test days 84 / 85).

**Sperm**

28. Sperm number: No TiO2-related difference was noted between the rats of the control group and the rats treated with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day for the number of ultrasound-resistant sperm heads (sperm count) per gram testicular tissue.

29. Sperm motility: No test item-related differences were noted between the rats of the control group and the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day) for the percentage of motile spermatozoa in the epididymal cauda on the total number of motile and non-motile spermatozoa.

30. Sperm morphology: The examination of spermatozoa from the epididymal cauda revealed no increased number of spermatozoa with a malformation in the treatment groups (100, 300 or 1000 mg Titanium dioxide E171/kg b.w./day) in comparison to the control group. In detail, the examination of 4000 spermatozoa (200 per animal) from each test group revealed 11 spermatozoa with a malformation in the control group, 6 in the low dose group and 5 in the high dose group. In all cases, the observed malformation was in the form of a banana-like sperm head.

**Female Fertility**

**Monitoring of estrous cycles**

31. Exposure period: After the allocation of the animals to the test groups and the start of treatment on test day 15, the estrous cycles were further monitored during the pre-mating and mating period until one day before a positive mating sign was noted. No TiO2-related differences were noted for the mean length and the mean number of estrous cycles per dam during the pre-mating period between the female animals of the control group and the female animals of the treatment groups (100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day)

# Summary of Results

## F0, F1 Pups and Satellites Cohort Summary

**Parental male and female animals (F0 Generation):**

32. None of the animals died prematurely. No test item-related changes were noted in behaviour and the external appearance. Pale faeces that were noted in a dose response-related manner in all treatment groups were caused by the white colour of the test item and were considered to be of no toxicological relevance. No test item-related influence was noted for the male and female animals on body weight, food consumption, drinking water consumption, the haematological and biochemical parameters, urinalysis, the levels of the thyroid and sexual hormones and the sperm parameter.

33. No test item-related changes were noted during the macroscopic examination, the examination of bone marrow and for the relative and absolute organ weights. No test item-related observations were noted during the histopathological examination of the male and female animals of the high dose group. No test item-related induction of aberrant crypt foci (ACF scoring) was noted in colons from the male and female animals of the treatment groups.

**N.B.** The mean actual intake of the test item via the diet over the whole study period was 104, 312 and 1050 mg/kg b.w./day for the male and 109, 319 and 1085 mg Titanium dioxide E171-E/kg b.w./day for the female animals. Hence, the actual values were in the range of the nominal values with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day.

**Parental females:**

34. No test item-related influence was noted on the length and numbers of the estrous cycles during the pre-mating period, the fertility index, the gestation index, the duration of the pre-coital time interval and the gestation period.

**Pups:**

35. No test item-related effect was noted on the prenatal development of the pups (birth and live birth index, percentage of post implantation loss). Furthermore, no test item-related effect was noted on the postnatal development of the pups regarding the viability indices (pre- and post-cull), the pup body weight, the ano-genital distance and the nipple retention.

36. The examination of the thyroid hormone levels on lactation days 4 and 22 revealed no test item related differences. Any incidental alterations were minor and well within the circadian variation for each gender. The gross inspection (external and / or internal) of the pups at necropsy on lactation days 4 (culled pups) and 22 revealed no test item-related changes.No test item-related

influence was noted on the organ weights from pups sacrificed on lactation day 22.

## F1 Generation Cohort 1A

37. Necropsy was carried out between postnatal days 91 and 96 (males) or 86 and 92 (females). None of the animals of cohort 1A died prematurely. No changes were noted in behaviour, the external appearance and the consistency of the faeces. Pale faeces that were noted for all male and female animals of the high dose group were caused by the white colour of the test item and, hence, were considered to be of no toxicological relevance. No influence was noted for the male and female animals on body weight, food consumption, the haematological and biochemical parameters, lymphocyte typing in the spleen, urinalysis, the levels of the thyroid and sexual hormones, the sperm parameter and sexual maturation.

38. No test item-related changes were noted during the macroscopic examination, the examination of bone marrow and for the relative and absolute organ weights. The histopathological examination of the organs, the detailed examination of testis and epididimydes and the quantitative examination of primordial and small growing follicles and the corpora lutea did not reveal any test item-related changes or differences.

**N.B.** The mean actual intake of the test item via the diet over the whole study period was 100, 305 and 1066 mg/kg b.w./day for the male and 105, 320 and 1101 mg Titanium dioxide E171-E/kg b.w./day for the female animals. Hence, the actual values were in the range of the nominal values with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day.

## F1 Generation Cohort 1B

### General toxicity

#### Parental male and female animals (F1 Generation):

39. None of the animals died prematurely. No changes were noted in behaviour, the external appearance and the consistency of the faeces. Pale faeces that were noted for all male and female animals of the high dose group were caused by the white colour of the incorporated test item and were not considered to be of toxicological relevance. No influence was noted for the male and female animals on body weight, food consumption, water consumption, the

sexual hormones and sexual maturation.

40. No test item-related changes were noted during the macroscopic examination at necropsy and for the relative and absolute organ weights.

**N.B**. The mean actual intake of the test item via the diet over the whole study period was 104, 314 and 1046 mg/kg b.w./day for the male and 103, 328 and 1089 mg Titanium dioxide E171-E/kg b.w./day for the female animals. Hence, the actual values were in the range of the nominal values with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day.

# Reproductive toxicity

## Parental females:

41. No influence was noted on the fertility index of the females, the gestation index, the duration of the pre-coital time interval and the gestation period.

**Pups:**

42. No test item-related effect was noted on the prenatal development of the pups (birth and live birth index, percentage of post implantation loss).Furthermore, no test item-related effect was noted on the postnatal development ofthe pups until postnatal day 4 regarding the viability index (surviving live born pups until lactation day 4), the pup body weight on lactation days 1 and 4, and the ano-genital distance.The gross inspection of the pups at necropsy revealed no test item-related changes.

## F1 Generation Cohort 2A and 2B

43. The neurological screening was carried out between postnatal days 58 and 64 (males and females) and the preparation of the brain occurred between postnatal days 84 to 90. The animals of cohort 2B were used for the histopathological examination of pup brain development and sacrificed on their postnatal days 21 to 23.

## Cohort 2A

44. None of the animals of cohort 2A died prematurely. No changes were noted in behaviour, the external appearance and the consistency of the faeces.

Pale faeces that were noted for all animals of the high dose group were caused by the white colour of the test item and, hence, were not considered to be of toxicological relevance. No influence was noted for the male and female animals on body weight, food consumption, the levels of the sexual hormones and sexual maturation.

45. The neurological screening and the examinations of grip strength and spontaneous motility revealed no test item-related effects on the neurological function. No observations were noted during the macroscopic examination of the animals at necropsy and for the relative and absolute brain weights. The microscopic examination of different brain regions of the male and female animals of the high dose group revealed no test item-related effects when compared to the control animals.

**N.B.** The mean actual intake of the test item via the diet over the whole study period was 102, 308 and 1028 mg/kg b.w./day for the male and 116, 312 and 993 mg Titanium dioxide E171-E/kg b.w./day for the female animals. Hence, the actual values were in the range of the nominal values with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day.

## Cohort 2B

46. No test item-related influence was noted on the levels of the sexual hormones. No observations were noted during the macroscopic examination of the animals at necropsy and for the relative and absolute brain weights. The microscopic examination of different brain regions of the male and female animals of the high dose group revealed no test item-related effects when compared to the control animal.

## F1 Generation Cohort 3

47. None of the animals of cohort 3 died prematurely. No changes were noted in behaviour, the external appearance and the consistency of the faeces. Pale faeces that were noted for all male and female animals of the high dose group were caused by the white colour of the test item and, hence, were not considered to be of toxicological relevance. No influence was noted for the male and female animals on body weight, food consumption and sexual maturation. The examination of lymphocyte subpopulations in the spleen and the KLH assay did not reveal any immunosuppressive effect of the test item. No test item-related changes were noted during the macroscopic examination at necropsy.

**N.B.** The mean actual intake of the test item via the diet over the whole study period was 88, 270 and 956 mg/kg b.w./day for the male and 97, 287 and 1043 mg Titanium dioxide E171-E/kg b.w./day for the female animals. Hence, the actual values were in the range of the nominal values with 100, 300 or 1000 mg Titanium dioxide E171-E/kg b.w./day.