Dosing-Related Challenges in Toxicity Studies and Risk Assessment of Titanium Dioxide in Food

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Conflict of Interest Statement

- *The presenter declares that there exists no real or perceived conflict of interest*

- National Institute for Public Health and the Environment (RIVM) is an agency of the Netherlands Ministry of Health, Welfare, and Sport
- Centre for Safety of Substances and Products
  - Main clients are national ministries and inspectorates
  - Also: European Commission and related bodies such as European Food Safety Authority (EFSA) and European Chemical Agency (ECHA)
  - Also: WHO, OECD, etc.

Objectives

- Finding potential causes for differences between diverging outcomes of toxicity studies with titanium dioxide related to dosing
  - Titanium dioxide as example
    - Investigate differences based on overview of studies
    - Discuss potential causes

- Making some recommendations regarding the challenges related to the dosing in *in vivo* studies with nanomaterials

Titanium Dioxide–Introduction 1/2

Titanium dioxide (TiO_2) is used as white pigment

- Oral exposure via
  - Food additive (E171 in EU) in food and food supplements
  - Toothpaste (CI77891)
  - Medicinal products
- Estimated mean lifelong daily intake: 0.19 mg/kg bw/d *(Rompelberg et al., 2016)*
- White color caused by particles of 200-300 nm
- 10-49% of E171 reported < 100 nm (number based)
- E171 no nanomaterial according to the EU definition

Titanium Dioxide–Introduction 2/2

- Titanium dioxide (E171) differs in
  - Particle size distribution
  - Crystal structure: anatase and/or rutile
  - Coating: Aluminum or Silica *(proposal to ban this from E171 specifications)*

- There is a lot of discussion about the safety of TiO_2/E171
- Many contradictory toxicological studies demonstrating no, early or adverse effects upon oral exposure
- E.g., liver edema or fibrosis and induction of intestinal tumor formation

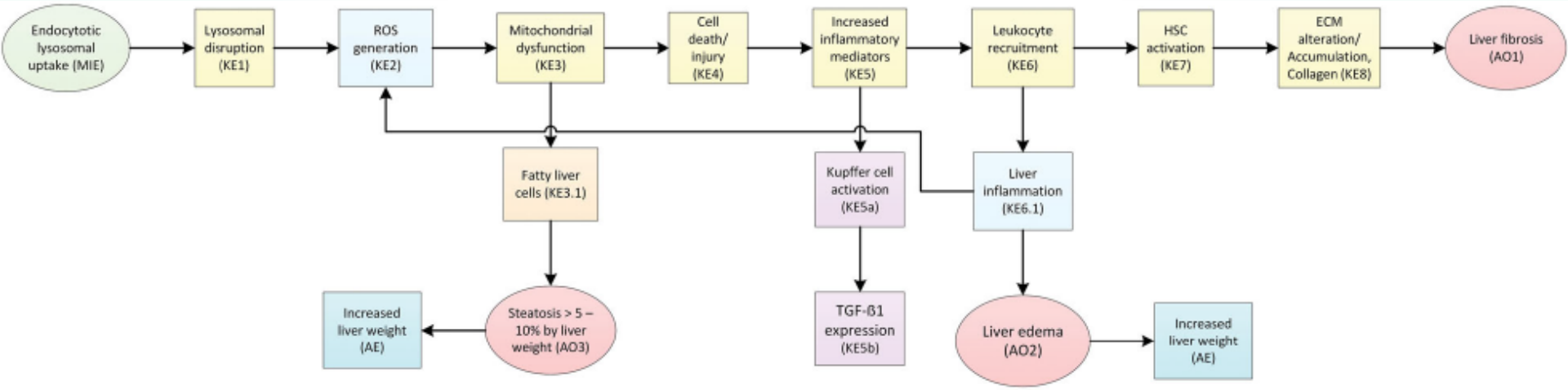
Literature Review

A review of oral *in vivo* studies with TiO_2 (E171 as well as TiO_2 NPs) focusing on liver and intestinal as critical target organs

Structural approach

- According to Adverse Outcome Pathways (AOPs)
  - Differentiate between clinical/histopathological effects (Adverse Outcomes) and early effects (Key Events)
- Including information on internal concentrations
  - Compare to information on Ti-levels in human liver and intestine
- Taking into account study specificities
  - Info on particle characteristics, study design incl those related to dosing

Adverse Outcome Pathway (AOP)–Liver



- Compilation of two adapted AOPs leading to effects on the liver as a result of oral TiO_2 exposure
- Key Events (KEs)
- Adverse Outcome (AOs): Steatosis, edema, fibrosis
- Associated Event (AE): increased liver weight

Studies with TiO_2 on the KEs, AOs, and AEs

- *In vivo* studies
- Positive studies: ROS generation, mitochondrial dysfunction, fatty liver cell formation, cell death, increased inflammatory mediators, leukocyte recruitment, liver inflammation, liver fibrosis, liver edema, and increased liver weight
- Negative studies: no such effects
- Sometimes different studies reporting positive or negative effects for same dose

Overview of Oral *In Vivo* Studies with TiO_2 Investigating AOP Leading to Liver Effects

(different focus, TiO_2, dosing, species, exposure time, etc.)

Dose *(mg/kg bw)*	MIE	Lysosomal disruption (KE1)	ROS generation (KE2)	Mitochondrial dysfunction (KE3)	Fatty liver cells (KE3.1)	Cell injury/ death (KE4)	Increased inflammatory mediators (KE5)	Leukocyte recruitment (KE6)	Liver inflammation (KE6.1)	HSC activation (KE7)	ECM alteration/ accumu-lation, collagen (KE8)	**Liver fibrosis (AO1)**	**Liver edema (AO2)**	**Liver steatosis (AO3)**	**Increased liver weight(AE)**
2															
5															
10															
20															
50															
64															
100															
150															
200															
312.5															
625															
938															
1,000															
1,250															
1875															
2,500															
3,750															
5,000															
7,500															
15,000															
24,000															

no data
Negative (=No) effect
Mixed negative / positive effect
Positive effect

Simplified from Brand et al. (2020)

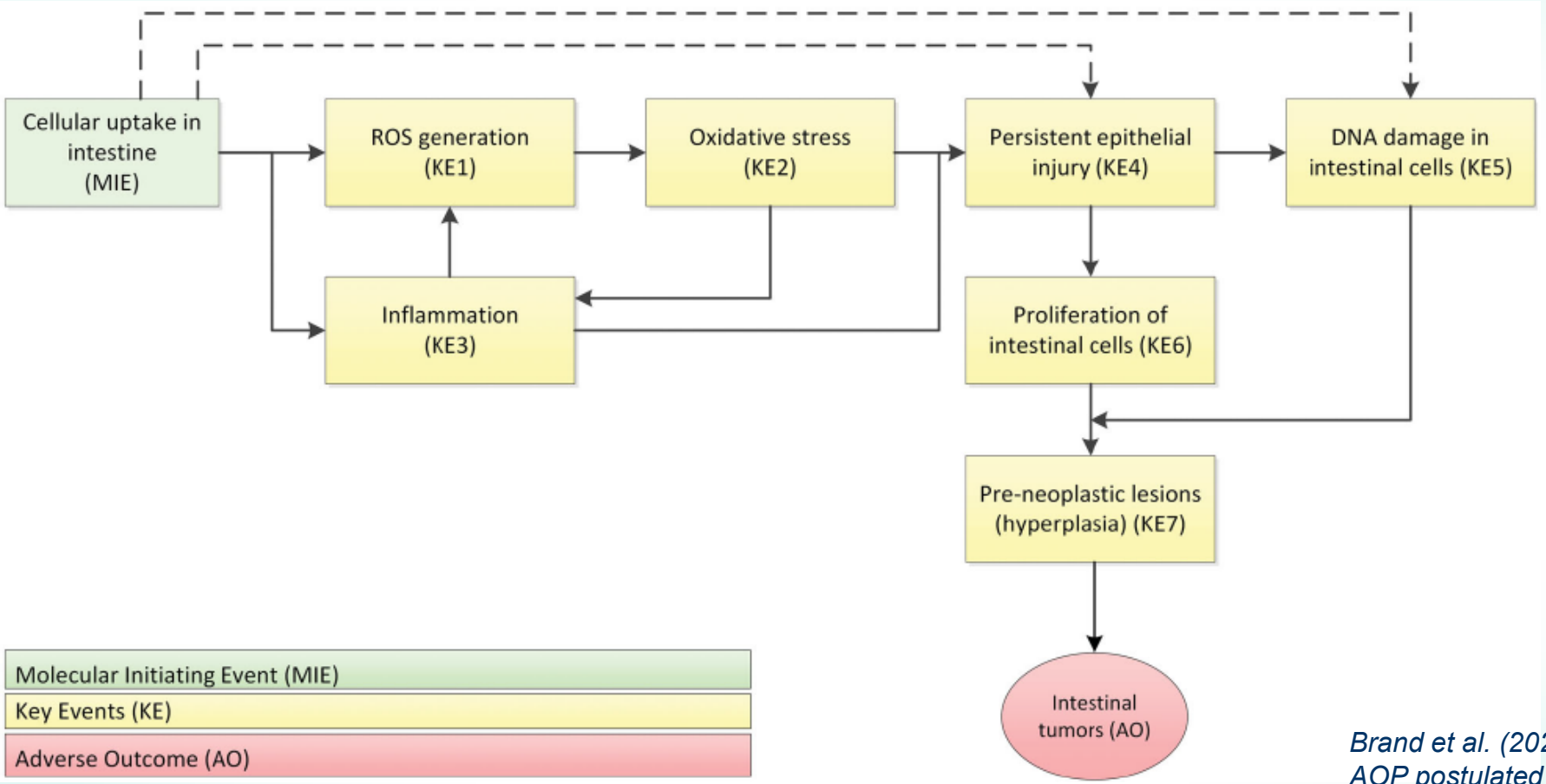
LivercConclusions Regarding Toxicity Studies

- Early Key Events seem to be triggered by TiO_2 exposure
  - E.g., ROS generation, increased inflammatory mediators
  - Already at low (external) exposure of ~ 5/10 mg/kg bw/d

    (note: the estimated human mean lifelong daily intake = 0.19 mg/kg bw/d)
  - KEs can be reversable or unable to trigger next KE or AO
- Adverse Outcomes (fibrosis, steatosis, edema) are reported in some studies but not in others

1 Talamini et al. 2019 (3 weeks 3 doses/week, E171)
2 Wang et al. 2007 (single oral dose 5000 mg/kg bw, 25 nm, 80 nm and 155 nm)

Adverse Outcome Pathway–Intestine



Brand et al. (2020), AOP postulated by Braakhuis et al. (2021)

Overview of Oral *In Vivo* Rat Studies with TiO_2 Investigating AOP Leading to Effects Intestine

Dose *(mg/kg bw)*	Cellular uptake (MIE)	ROS generation (KE1)	Oxidative stress (KE2)	Inflammation (KE3)			Persistent epithelial injury (KE4)	DNA damage (KE5)	Proliferation of intestinal cells (KE6)	Pre-neoplastic lesions (hyperplasia) (KE7)	Intestinal tumors (AO)
				Tissue resident cell activation (KE3.1)	Increased pro-inflamm. mediators (KE3.2)	Leukocyte recruitment / activation (KE3.3)					
0.2											
0.5											
1.30											
1.81											
3.50											
4.76											
10											
22											
31.43											
50											
100											
175											
200											
250											
300											
374											
500											
550											
1,000											
1,250											
1,750											
2,000											
2,500											
5,000											
24,000											

no data
Negative (= No) effect
Mixed negative / positive effect
Positive effect

Simplified from Brand et al. (2020)

Intestine–Conclusions Regarding Toxicity Studies

- Key Events seem to be triggered in some studies at relatively low (external) exposure
  - E.g., oxidative stress, leukocyte recruitment/inflammation, DNA damage
  - Already at low (external) exposure of ~10 mg/kg bw/d

    (note: the estimated human mean lifelong daily intake=0.19 mg/kg bw/d)
  - KEs can be reversable or unable to trigger next KE or AO
- No Adverse Outcome is found (e.g., intestinal tumours)

General Conclusions Regarding Toxicity Studies

- Relatively highly dosed studies just focussing on AO are negative, while relatively low dosed studies report effects at early KEs (but usually do not regard later AOs)
- There are studies with positive AOs and studies with negative AOs
- What causes differences in outcome of these studies?
- Including information on internal concentrations

Significant Increased Ti Levels Liver in Animal Studies (Oral Expos.)

- Different studies, contradicting results
- Liver concentration in mice 0.94 mg/kg with inflammation after 3 weeks exposure to 5 mg/kg bw/d E171 *(Talamini et al. 2019)*
- AE (increased liver weight) at 4 mg/kg liver

External dose *(mg/kg bw/d)*	Ti in liver (mg/kg)		
0.5	**~2.1** (Control: ~0) (45 d exposure)		
5	**0.94** (Control: 0.26) (3 w exposure)		
10	**~2.1** (Control: ~1.6) (26 w exposure)		
50	**~2.8** (Cntrl: ~1.6) (26 w exposure)	**~2.2** (Cntrl: ~1.2) (26 w exposure)	**~1.1** (Cntrl: ~0.6) (8 w exposure)
64	**~1.9** (Control: 0.6) (28 w exposure)		
100	**0.94** (Control: 0.46) (28 d exposure)		
1,000	**~0.8** (Control: ~0.2) (2 w exposure)		
5,000	**~4.0** (Control: ~0.1) (single dose)		

no effects studied
no effect observed
effect observed

Significant Increased Ti Levels in Intestinal Tissues in Animal Studies (Oral Exposure)

- Tissue concentrations are hardly ever determined
- One study *(Talamini et al., 2019)*: increased Ti concentration in colon tissue, but not in small intestine, after 3 week exposure of mice to 5 mg/kg bw/d E171
  - At this concentration KE1 and KE3 were triggered

External dose *(mg/kg bw/d)*	**Ti in intestinal tissues (mg/kg)**
2	**0.13** (control: 0.08) in small intestine (5 d exposure) No histomorphological changes
5	**1.07** (control: 0.60) in colon (3 w exposure)
10	**~3.4** (control: ~2.1) in small intestine (26 w exposure)
50	**~5.3** (control: ~2.1) in small intestine (26 w exposure)

no effects studied
no effect observed
effect observed

Ti Concentration in Human Post-Mortem Organs 1/2

TiO_2 (nano)particles are present in human organs, including liver and intestinal tissues *(Heringa et al. 2018, Peters et al. 2020)*

- Post-mortem tissues (from University Medical Centre Utrecht)
  - Individuals (n=30) who donated their bodies to science
  - Age: 56-104 years (average: 86)
  - No medical history or histopathology performed

- Analysis by WFSR (RIKILT), Wageningen, the Netherlands
  - Total Ti measured by ICP-MS, measurement of TiO_2 particles sp-ICP-HRMS, confirmation by SEM-EDX

Ti Concentration in Human Post-Mortem Organs 2/2

- Human liver:
  0.01-0.16 mg Ti/kg
- Human intestinal tissues:
  0.02-2.04 mg Ti/kg

Organ	Subjects		Total Ti *(mg/kg) in n>LOD*				
	n	*n>LOD*	*min*	*max*	*median*	*average*	*SD*
Liver	30	18	**0.01**	**0.16**	**0.03**	0.04	0.04
Jejunum	12	12	**0.02**	**2.04**	**0.14**	0.37	0.59
Ileum	12	12	**0.06**	**1.41**	**0.26**	0.43	0.43
Spleen	30	27	0.02	0.40	0.04	0.07	0.09
Kidney	15	14	0.01	0.37	0.06	0.09	0.09

- Total Ti caused by TiO_2 particles, almost exclusively originating from oral exposure

Brand et al. (2020), Heringa et al. 2018, Peters et al. (2020)

Comparing Ti Levels Liver in Animal Studies with Human Levels

- Liver concentration in mice with inflammation responses at concentration 6 or 30 times higher than median and max liver concentration found in humans, respectively. After 3 weeks exposure to 5 mg/kg bw/d E171 *(Talamini et al. 2019)*

External dose *(mg/kg bw/d)*	Ti in liver (mg/kg) *Human levels: 0.01-0.16*		
0.5	**~2.1** (Control: ~0) (45 d exposure)		
5	**0.94** (Control: 0.26) (3 w exposure)		
10	**~2.1** (Control: ~1.6) (26 w exposure)		
50	**~2.8** (Cntrl: ~1.6) (26 w exposure)	**~2.2** (Cntrl: ~1.2) (26 w exposure)	**~1.1** (Cntrl: ~0.6) (8 w exposure)
64	**~1.9** (Control: 0.6) (28 w exposure)		
100	**0.94** (Control: 0.46) (28 d exposure)		
1,000	**~0.8** (Control: ~0.2) (2 w exposure)		
5,000	**~4.0** (Control: ~0.1) (single dose)		

no effects studied
no effect observed
effect observed

Comparing Ti Levels in Intestinal Tissues in Animal Studies with Human Levels

- Ti concentration in colon tissue after 3 week exposure of mice to 5 mg/kg bw/d E171 (triggering KE1 and KE3) similar to concentration in small intestine in humans *(human median is a factor 4-8 lower)*

External dose *(mg/kg bw/d)*	**Ti in intestinal tissues (mg/kg)** *NB Human levels: 0.02-2.04*
2	**0.13** (control: 0.08) in small intestine (5 d exposure) No histomorphological changes
5	**1.07** (control: 0.60) in colon (3 w exposure)
10	**~3.4** (control: ~2.1) in small intestine (26 w exposure)
50	**~5.3** (control: ~2.1) in small intestine (26 w exposure)

no effects studied
no effect observed
effect observed

Conclusions Regarding Toxicity Studies Taking into Account Internal Organ Concentrations

- Internal organ concentrations (liver): Increased Ti concentrations with or without effects (and effects without increased Ti concentration).
- Relationship internal exposure with external dose not clear
- Sometimes effects at levels not much higher than human levels
- Unclear if TiO_2 can lead to irreversible adverse effects in liver (or intestine) in humans due to oral exposure

- What causes differences in outcome of these studies?

Causes of Differences in Outcome Studies with TiO_2

Properties of TiO_2 used

- Particle size-distribution, crystal-structure, coating

Exposure duration

Animal species?

Administration methodology

- Dose?
- Formulation?
- Method of administration?

Dose and Formulation?

Dose

- Differences in outcome high dose vs. low dose in animal studies
- High doses can reduce intestinal uptake and subsequent effects

Formulation

- Dispersion in water
- Aggregation/agglomeration potentially negatively affecting bioavailability

Method of Administration?

Method of administration

- Oral gavage, dripping water into the mouth, or mixed through diet
- The studies with suspension in drinking water caused KEs to occur
- Studies with dietary exposure (only 2) showed no induction of effects

- How do these difference relate to realistic human exposure?

Study Comparing Different Dosing Regimes

Only one study comparing different dosing regimes TiO_2 *(by Rodriguez-Escamilla et al. (2019) - Universidad Nacional Autónoma de México)*

- Studied toxicological effects on testis in mice (10 wk exposure to E171) with different dosing regimes:
  - Oral gavage: suspension in water (5 mg/kg bw/d)
  - Dietary: through feed pellets (three dose levels equivalent to 102, 682, or 1379 mg/kg bw/d)
  - Effects through oral gavage similar to up to 260 times higher dose through pellets illustrating importance of dosing regime

Summary–Take Home Messages

Administration methodology including dose, formulation, and method of administration likely affects oral absorption, toxicokinetics, and subsequent toxicological effects

- **Recommendations:**

- Pchem properties of material at doses delivered and in the delivery medium must be characterized
- Research need to investigate effects of dose and method of administration (including degree of agglomeration) on absorption
- High doses should be treated carefully
- Formulation should be well dispersed (and measured)
- Internal concentrations in key tissues should be determined

References

- Braakhuis et al. (2021) Mechanism of action of TiO_2: Recommendations to reduce uncertainties related to carcinogenic potential. *Annu Rev Pharmacol Toxicol 61*: 203-223.
- Brand et al. (2020) Possible effects of titanium dioxide particles on human liver, intestinal tissue, spleen and kidney after oral exposure. *Nanotoxicology 14*(7): 985-1007.
- Heringa et al. (2018) Detection of titanium particles in human liver and spleen and possible health implications. *Part Fibre Toxicol 15*(1): 15.
- Peters et al. (2020) Silicon dioxide and titanium dioxide particles found in human tissues. *Nanotoxicology 14*(3): 420-432.
- Rodriguez-Escamilla et al. (2019) Food-grade titanium dioxide (E171) by solid or liquid matrix administration induces inflammation, germ cells sloughing in seminiferous tubules and blood-testis barrier disruption in mice. *J Appl Toxicol 39*(11): 1586-1605.
- Rompelberg et al. (2016) Oral intake of added titanium dioxide and its nanofraction from food products, food supplements and toothpaste by the Dutch population. *Nanotoxicology 10*(10): 1404-1414.
- Talamini et al. (2019) Repeated administration of the food additive E171 to mice results in accumulation in intestine and liver and promotes an inflammatory status. *Nanotoxicology 13*(8): 1097-1101.

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Thank you for your attention!