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OPEN Food-grade TiO₂ impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon

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Research conducted in the frame of:

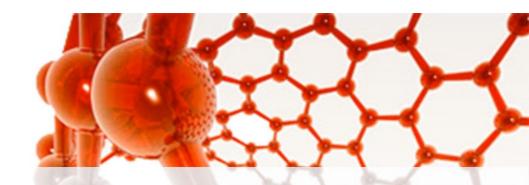
NanoGut

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Physico-chemical characterization what was our strategy in vivo/in vitro ? (E171 vs. P25 NM105)

E171 vs. P25 NM105: a strategy trying to « link » size-to-effects

- STUDY 1: we compared the tissue distribution and immuno-modulatory effects of food-grade TiO₂ (E171 from the French market) versus P25 nanomodel (OECD NM105 series: 22 nm of diameter / 100% nanosized).
 - **How ?** Ultrasonicated E171 or TiO₂-NP model orally administered to rats for **7 days** at a relevant human dietary level (i.e., 10 mg/kg of BW per day gastric gavage)
 - ➤ In case of similar distribution and effects between TiO₂ products, one may suggested the nanoscale fraction of the food grade TiO₂ (E171) responsible for immune effects.
 - Plus, a 100-day treatment with E171 only to investigate chronic effect of food-grade TiO₂ (drinking water).
- **STUDY 2**: we assessed whether E171 may represent a **risk factor** in the **initiation** and/or **promotion of colonic preneoplastic lesions** (i.e., the first stages of colorectal cancer development, **not** tumor formation).

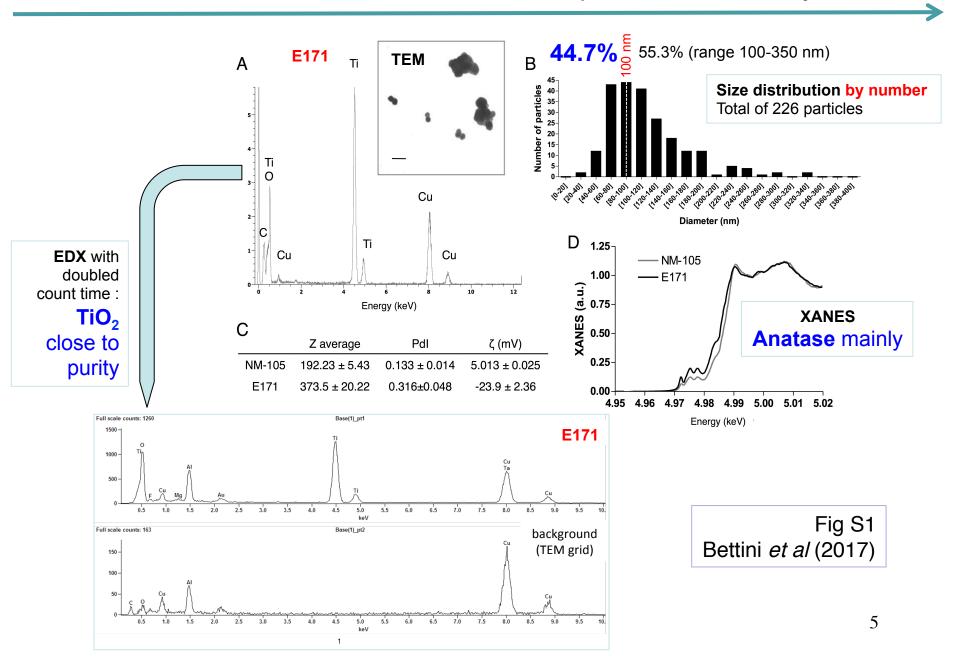
More details later...

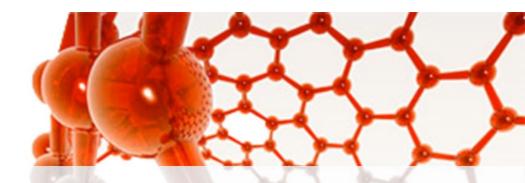
E171 vs. P25 NM105: a strategy trying to « link » size-to-effects

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More details later...

E171 vs. P25 NM105: characterization of particle size and crystal form





STUDY 1.1

Fate of TiO₂ particles along the gut-liver axis

tissue distribution after one week oral treatment in rats

(E171 vs. P25 NM105)



E171 vs. P25 NM105: methods used for dispersion and oral treatment



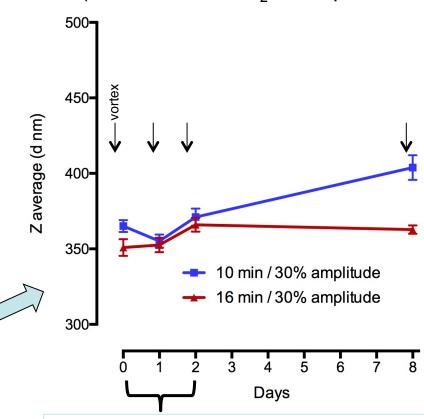


2.56 mg/ml NM Stock Suspension in EtOH / BSA (instilled, diluted or dosed into specific test mediums)

Ultrasonication with probe preliminary tests 10 vs.16 min at 30% amplitude

EFSA query 1: YES, the dispersion state was checked by DLS before treatment to rats (no change upon 48h)

Hydrodynamic diameter (DLS) with time (ultrasonicated TiO₂ re-suspended in water)

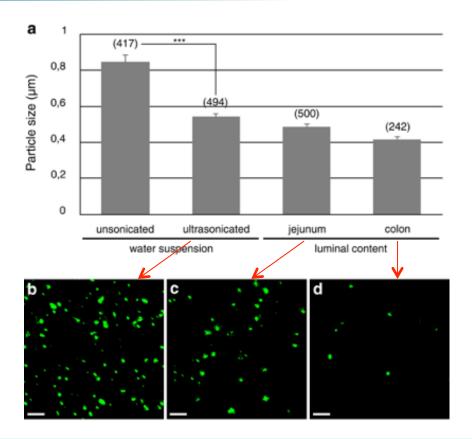


- **stable dispersion for 1 week** using a 16 min ultrasonication with 30% of amplitude
- **for chronic oral study**: renewal of the E171 suspension in drinking water **every 2 days**



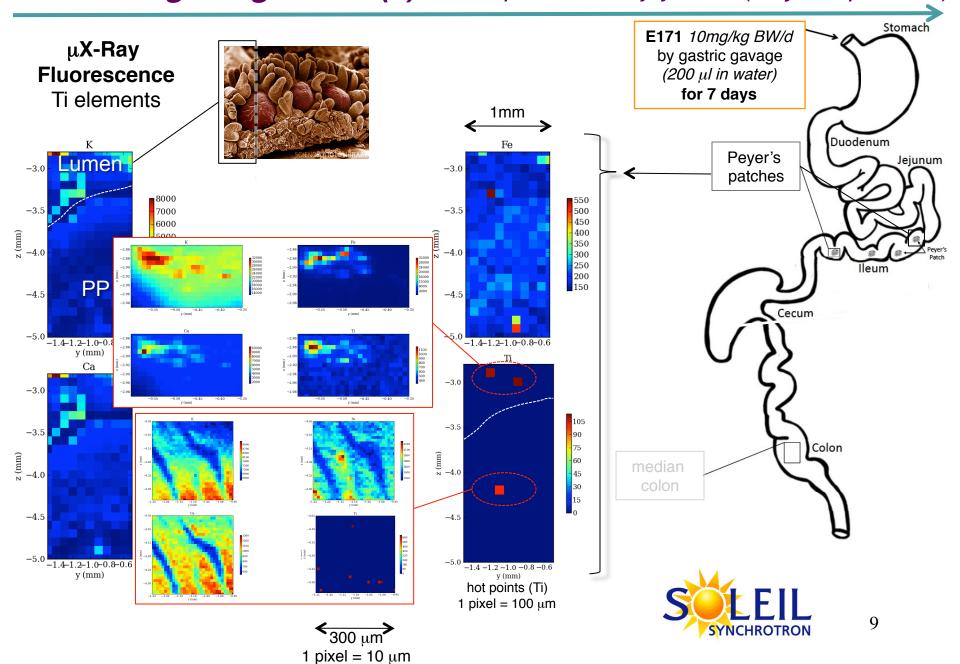
Fate of E171 in the gut lumen : no re-agglomeration in vivo – figure S2

EFSA query 2: NO, rats were not fasted before oral treatments with TiO₂

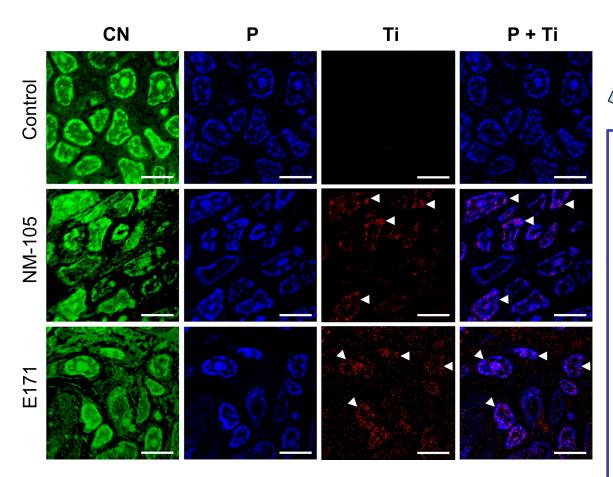


- ➤ E171 particle size (TiO₂ matter distribution) evaluated by confocal microscopy from suspensions spread on microscope slides, and showing similar dispersion state from the initial water suspensions (b) to luminal contents recovered from the small (c) and large intestine (d).
- \rightarrow The laser reflection of TiO₂ was detected with a magnification given 1 pixel = 50 nm.

Fate along the gut wall (1): absorption in the jejunum (Peyer's patches)



E171 or TiO₂-NP model in Peyer's patches : nanoSIMS analyses



Subcellular Ti distribution into immune cells
Mass spectrometry detection of ⁴⁸Ti¹⁶O clusters
(1 pixel ≈ 80-100 nm / Scale bars 5 μm)

Fig 2 Bettini *et al* (2017) E171 = P25 NM-105

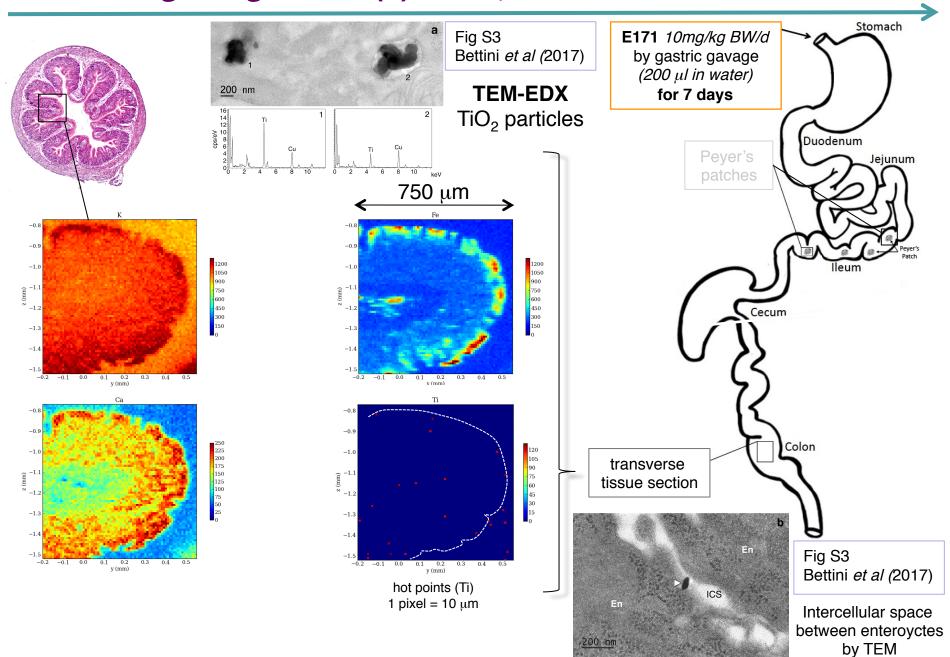




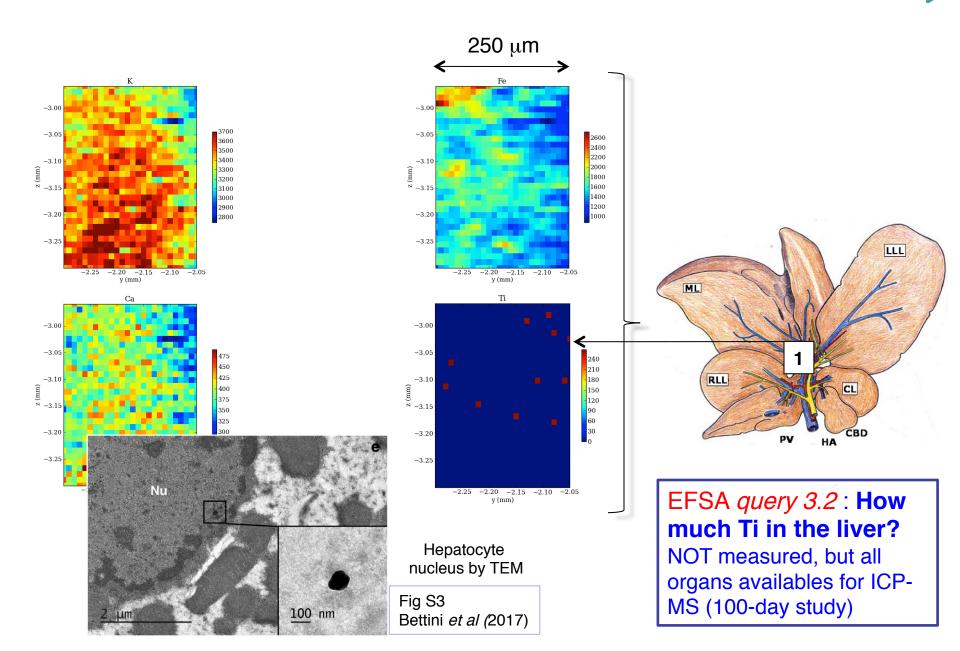
EFSA *query 3.1*: why « no background » in control rats?

- Need repeated exposure (e.g., daily for one week and more) to observe particles into the gut mucosa and cells.
- Studies performed with SIMS and µXRF imaging: no Ti level measurements (e.g., ICP-MS).

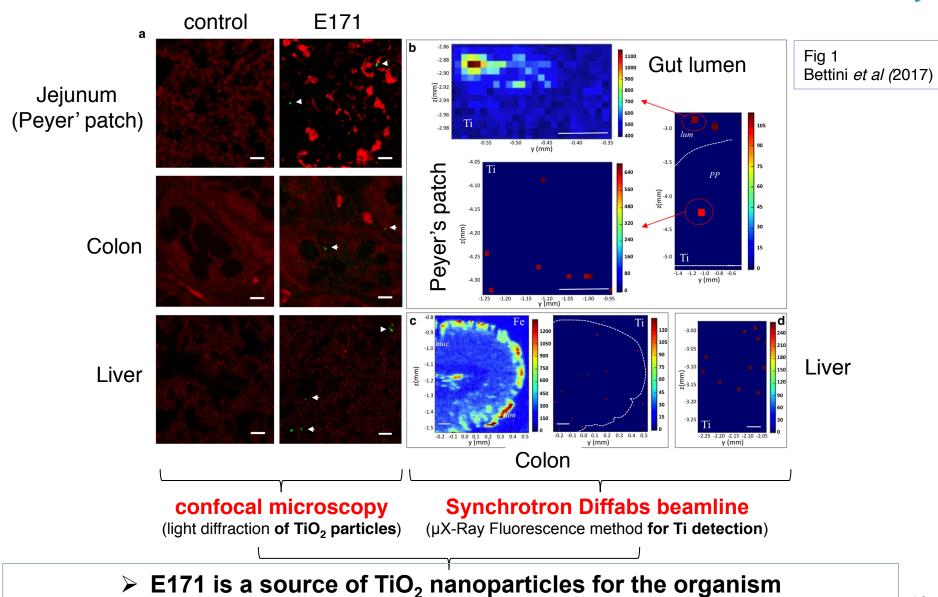
Fate along the gut wall (2): absorption in the colon



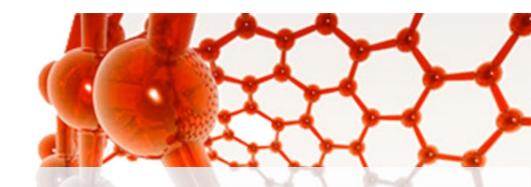
Gut-to-liver passage (3): systemic distribution



Food grade TiO₂: concluding remark from Confocal/SIMS/TEM-EDX studies



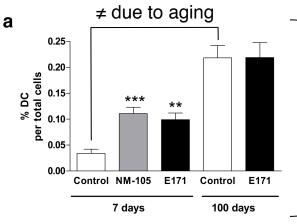
> Ti[O₂] (food grade) reachs immune cells in PP, similarly to a TiO₂-NP model



STUDY 1.2
Effects on local and systemic immune system
one week vs.100-day treatment

Immune cell frequency: figure 3 - Bettini et al 2017

e



Dendritic cells frequency **/**

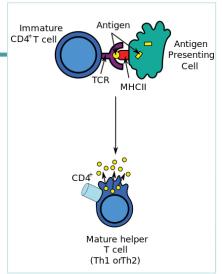
activated antigen-presenting cells

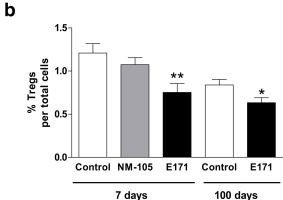
(i.e. CD11b/c+ CD103+ expressing the major histocompatibility complex MHC-II+ on their surfaces for antigen presentation):

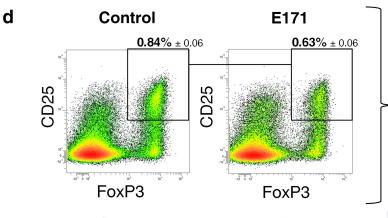
A TRANSIENT effect

E171 = P25 NM-105

i.e., return to basal after 100 days of E171 treatment







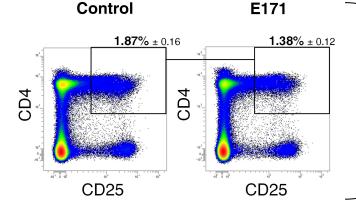


(CD4+ CD25+ FoxP3+)
immunosuppressive
activity (IL-10, TGFβ):
They organize oral
tolerance to food

antigens, self-tolerance, and regulate inflammatory responses

100 days

7 days



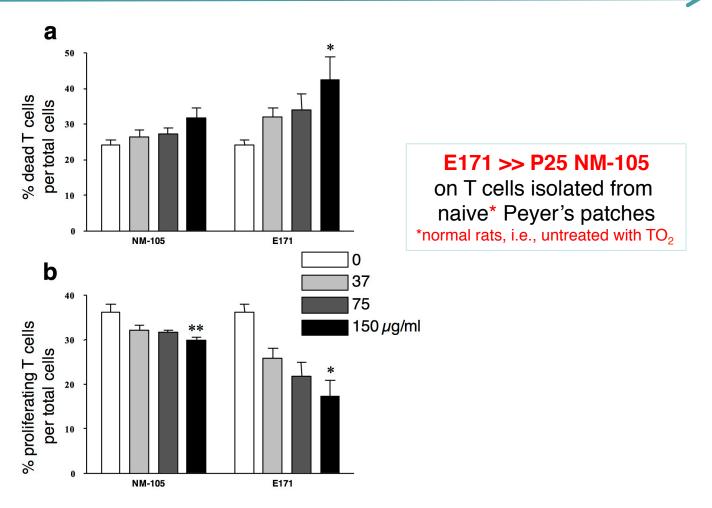
A CHRONIC effect

E171 >> P25 NM-105

Total T cells 🔌

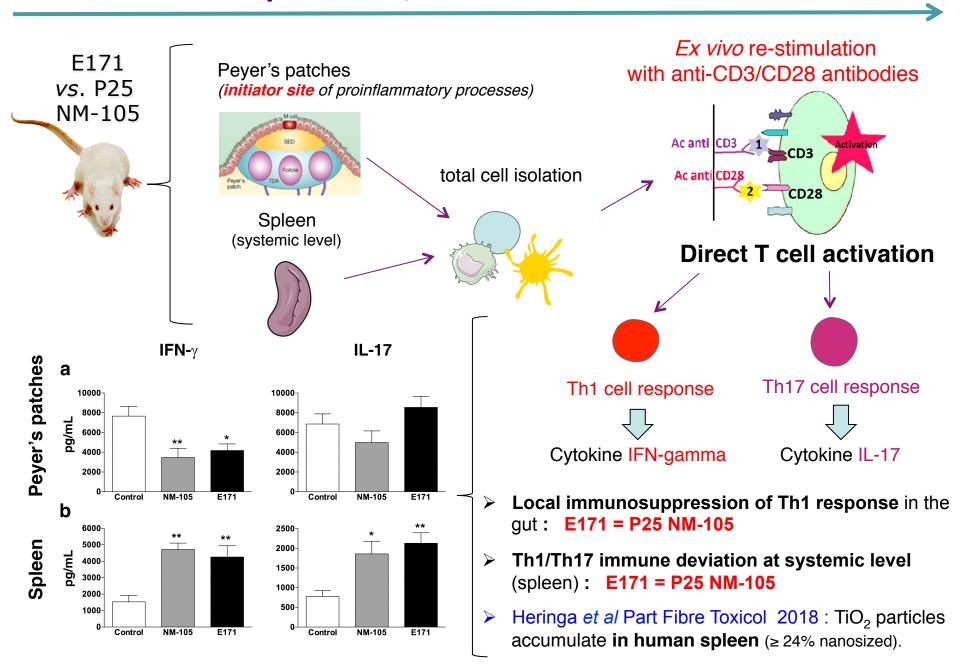
(CD4+ CD25+)
include T-helper (Th)1
+Th2+Th17+Tregs

Dose-dependent cytotoxicity on T cells: figure S5 – Bettini et al 2017

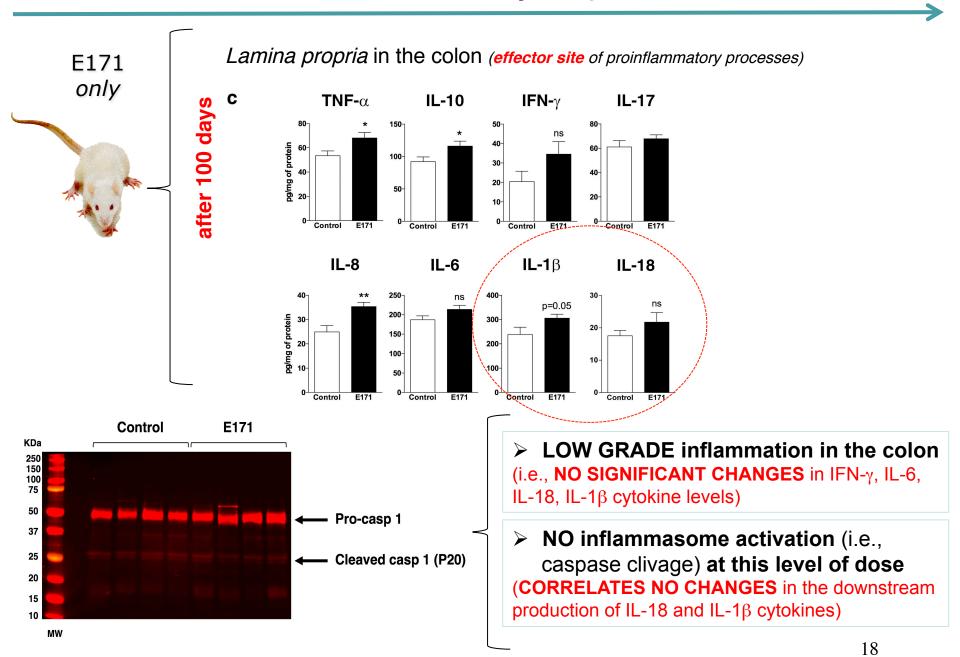


- Food grade TiO₂ (E171) more cytotoxic than TiO₂-NP model (P25 NM-105) on total T cells ex vivo (Th1, Th2, Th17, Tregs)
- Data in support of in vivo observations

Immune cell response : figure 4 – Bettini et al 2017

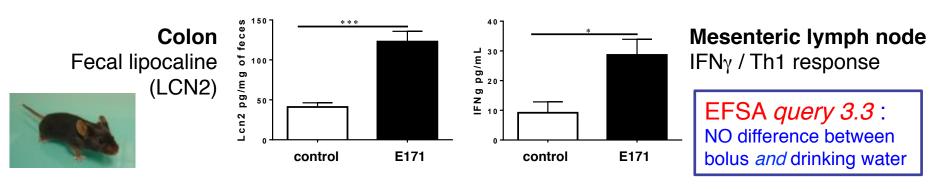


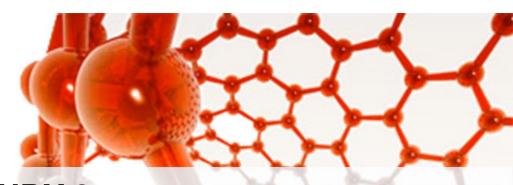
Mucosal inflammation after 100 days: figure 6 - Bettini et al 2017



Conclusions (study 1)

- Food-grade TiO₂, per os in rats at a human relevant dietary level,
 - A source of TiO₂-NPs for the intestinal mucosa (and beyond)
 - Translocate to immune cells in Peyer's patches, similarly to NP model
- E171 absorption is not "neutral" for the organism through,
 - Imbalance of immune homeostasis in the gut (T cell frequency and activity, in vivo + in vitro): E171 effects ≥ TiO₂-NP model
 - Th1/Th17 immune deviation in splenocytes (ex vivo), suggesting susceptibility to proinflammatory responses at the systemic level: E171 effects = TiO₂-NP model
- Low-grade inflammation in the colon (i.e., no sign of IBD-like inflammation) after chronic exposure for 100 days
- ➤ Of note: similar observations in a mouse model (unpublished) orally exposed to E171 (same batch *and* dose) for 60 days by gastric gavage, e.g.:





STUDY 2

Effects on the initiation and promotion of preneoploastic lesions in the colon

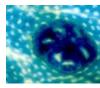
oral treatment for 100 days in rats (E171 only) in vitro studies (E171 vs. P25 NM105)

Models and strategy used: a strategy trying to « link » size-to-effects

STUDY 2: we assessed whether E171 may represent a **risk factor** in the **initiation** and/or **promotion of colonic preneoplastic lesions** (i.e., the first stages of colorectal cancer development, **not** tumor formation).

How ? Ultrasonicated E171 was orally administered to rats for 100 days (200 μg and/or 10 mg/kg of BW per day) through drinking water,

ACF



In vivo assessment of spontaneous development and/or promotion of preneoplastic lesions (Aberrant Crypt Foci, ACF) in the rat colon (i.e., with or without dimethylhydrazine (DMH)-induced carcinogenesis, respectively).

Plus cellular models (E171 vs. P25 NM-105),

Apc +/+

➤ Apc +/+ (normal) and Apc Min/+ (preneoplastic, i.e., mutated on the Adenomatous Polyposis Coli gene) mouse epithelial cells,

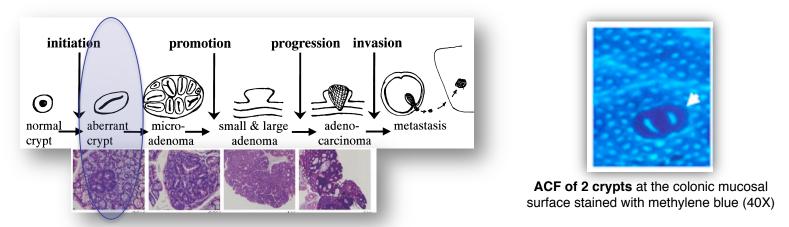


- \triangleright Ultrasonicated TiO₂ suspension in DMEM without serum (2.56 μg/mL or 25.6 μg/mL) for 24h,
- Cytotoxicity (MTT assay) and genotoxicity (γH2AX test).

⇒ consistency of the stages of carcinogenesis between animal and cellular models

Aberrant crypt foci (ACF)

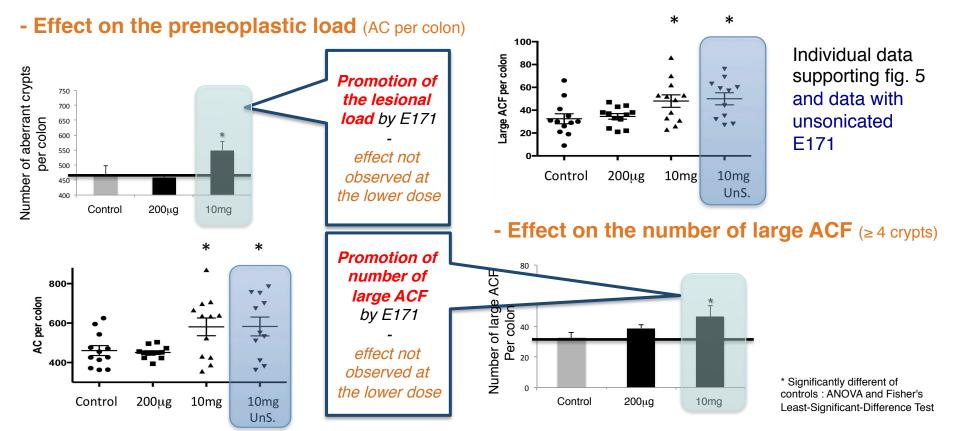
- The earliest histopathologic lesions associated with colorectal cancer
- Commonly used as a surrogate marker for studies on colorectal cancer chemoprevention in rodents (Pierre et al 2003, 2004, 2008, 2013)
- ➤ Also observed in humans (Kim et al 2008). Several genetic and epigenetic changes are common in both ACFs and CRC (Lopez-Ceron et al 2012)



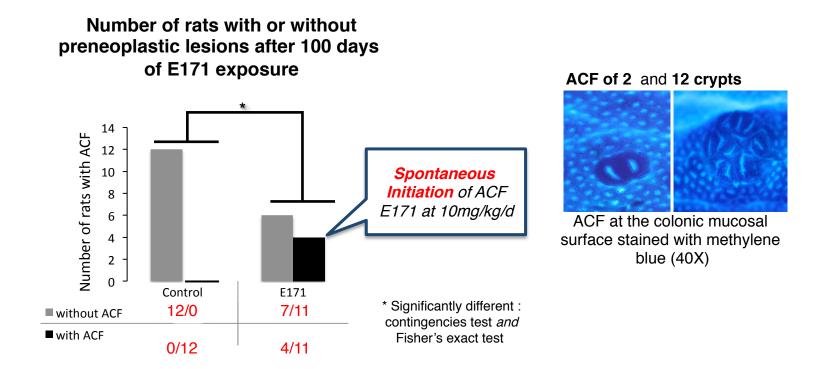
- In the STUDY 2, ACF were counted under light microscope, after fixation with 10% buffered formalin and tissue staining with methylene blue, in duplicate by two independent readers, blinded for the origin of the colon.
 - Number of lesions per colon : ACF / colon
 - Size of lesions : Crypts / Foci
 - Number of aberrant crypts per colon: i.e., preneoplastic load = AC/colon
- Statistics: t-test and contengency test for 2 comparison groups (initiation) and ANOVA for 3 comparison groups (promotion)

Promotion of preneoplastic lesions: figure 5 - Bettini et al 2017

- Effect on the number of ACF per colon : no significant difference between groups

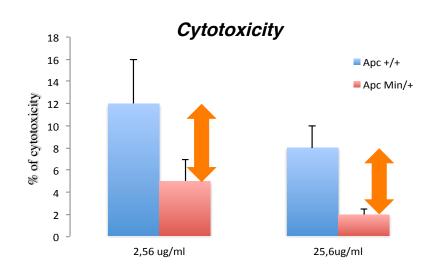


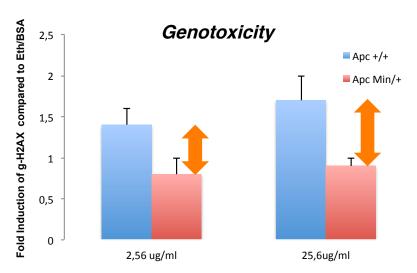
- Promoting effect of E171 on tumor formation reported in a colitis-associated carcinogenesis model in mice (i.e., several 7-day cycles of DSS inflammation into mucosae) Urrutia-Ortega et al. Food Chem Toxicol 2016
- ➤ Bettini et al Sci Rep 2017 provides demonstration on a normal colon mucosa with conclusion limited at the preneoplastic stage + Idem with non sonicated E171 (unpublished)



- ➤ The food grade TiO₂ (ultrasonicated E171) induces preneoplastic lesions in the rat colon, at 10 mg/kg BW/d for 100 days.
- It is however insufficient to conclude on a risk at the tumor stage, that requires the set up of a carcinogenic study (OCDE guidelines #451) with a E171 batch of similar nanosized fraction (i.e., ≥ 40% by number)

Cytotoxic and genotoxic in vitro assays: figure 5 d - Bettini et al 2017





Cytotoxicity more important on normal cells:
Apc mutation makes cells more resistant to cytotoxicity of TiO₂

Risk => positive selection of preneoplastic cells

Genotoxicity more important on normal cells: Apc mutation makes cells more resistant to TiO₂-inducing genotoxicity

25

Risk => mutation in normal cells?

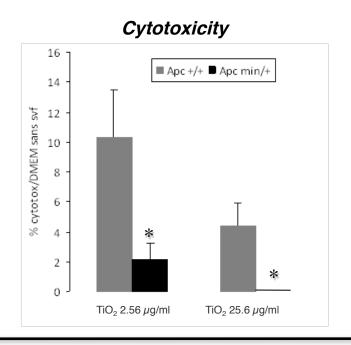


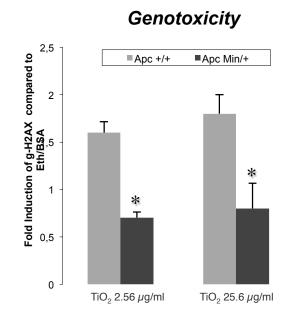
Could participate to

- (1) spontaneous initiation of ACF (genotoxicity on normal cells)
- (2) promotion of ACF (cytotoxicity on normal cells)

Cytotoxic and genotoxic in vitro assays: figure S7 - Bettini et al 2017

Assay with P25 NM-105 as TiO₂-NP model







Similar results with P25 and E171

Suggest that the promotional effect of E171 in our premalignant model could be linked to the nanosized fraction of TiO₂ particles present in the food additive

Conclusion (study 2)

- ➤ Oral exposure to food-grade TiO₂, at a representative dose of human exposure, induces and promotes preneoplastic lesions in colon after chronic exposure (100 days)
- These effects were observed on :
 - the lesional load
 - the number of large preneoplastic lesions
- Effect correlated with (no causal demonstration):
 - a developing "low-grade inflammation" into the colonic mucosa (see study 1)
 - a positive selection of pre-neoplastic cells and genotoxicity on normal cells (in vitro)

THANKS to all partners,

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Christel Cartier





cea







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Sylviane Taché
Nathalie Naud



Elisa Boutet





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