Use of New Approach Methodologies for the hazard assessment of nanofibers

Nanocellulose oral exposure: gastrointestinal digestion, nanofibers uptake and local effects

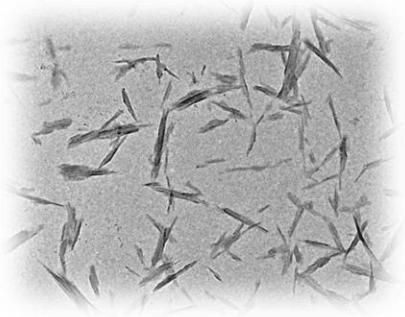
NANOCELLUP

Francesco Cubadda

Dept. Food Safety, Nutrition and Veterinary Public Health

Istituto Superiore di Sanità - *National Institute of Health*

Rome, Italy



Stakeholder workshop on small particles and nanoparticles in food | 31 March – 1 April 2022

Background

Nanocellulose is an emerging material in the food sector:

- Food packaging
- Incorporation into food as thickener, binder or texture modifier
- Novel Foods (e.g. foods for weight control)

Main sources of NC include NC fibres produced by bacterial species (**bacterial NC**, BNC) and other NCs obtained by technological modification of cellulose from plants or other origins, leading to cellulose nanofibers (**nanofibrillated cellulose**, NFC) or nanocrystals (**cellulose nanocrystals**, CNC)

The **biological sources** and **preparation conditions** has been shown to affect several **physicochemical parameters of NC** (size, aspect ratio, morphology, polydispersity, surface charge, surface chemistry and crystallinity index)

The need for a nano-specific assessment

The potential hazards of ingested NC are insufficiently characterized

- **NC nanoscale features require a nano-specific assessment (EFSA SC, 2021) focusing on**
  - **(i) local adverse effect in the gut**
  - **(ii) crossing of the intestinal barrier**
  - **(iii) possible degradation of NC by the human microbiome, potentially delivering smaller fibres**

- **Laboratory animals are not appropriate models because the digestive physiology, microbiome and rate of fibre degradation differ from humans**

EFSA Scientific Committee, et al. 2021. Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. EFSA Journal 2021;19(8):6768, 111 pp. https://doi.org/10.2903/j.efsa.2021.6768

General description of the project

The present project aims to:

- ❑ design and conduct a set of NAM-based studies for addressing the current data gaps on nanocellulose (NC) hazards
- ❑ offer a proposal for including the results in the regulatory hazard assessment of NC for consumers exposed via food

A battery of *in vitro* tests will provide insight into NC hazard and mode of action and will assess if any relationship between toxicity and physicochemical characteristics can be driven

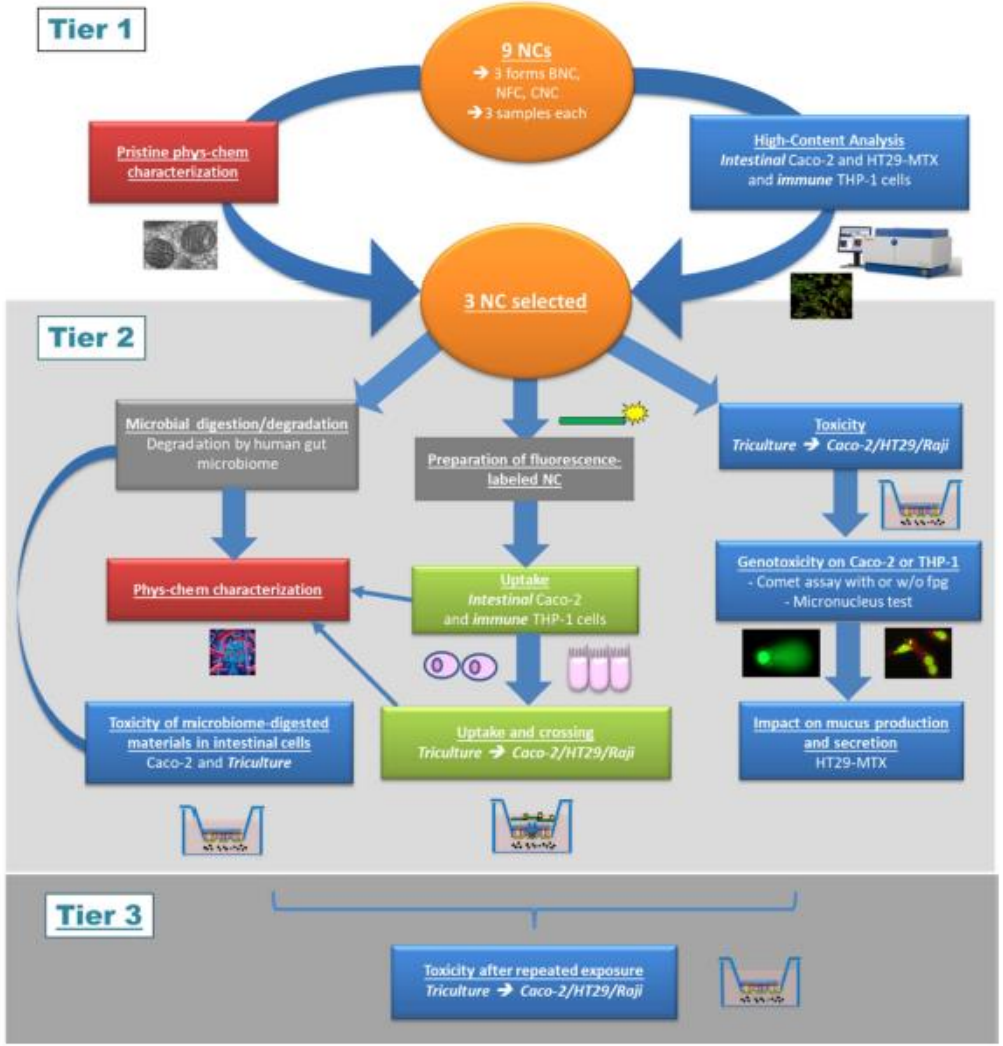
Mono- and co-culture systems will be used and potential effects of NC on integrity of the gastrointestinal barrier, impaired cell viability/cytotoxicity, oxidative stress responses, (pro-)inflammatory responses and genotoxicity will be investigated

The Consortium

Coordinator **ISS**		Italy	Francesco Cubadda, Olimpia Vincentini, Alberto Mantovani, Francesca De Battistis, Francesca Ferraris, Francesca Iacoponi, Andrea Raggi	*Toxicology, Characterisation*
ANSES		France	Valerie Fessard, Kevin Hogeveen, Ludovic Lehegarat	*Toxicology*
SCIENSANO		Belgium	Jan Mast, Eveline Verleysen, Lisa Siciliani	*Characterisation*
INRAE		France	Stephanie Blanquet-Diot, Lucie Etienne-Mesmin, Sylvain Denis, Morgane Brun	*Microbiome*
EC-JRC*		Italy	Susanne Bremer-Hoffmann, Blanka Halamoda-Kenzaoui, Francesco Fumagalli	*Toxicology*

* External partner, in kind participation

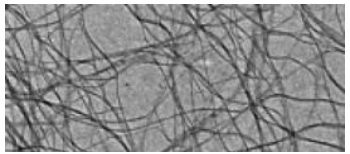
Tiered-approach for testing the NCs

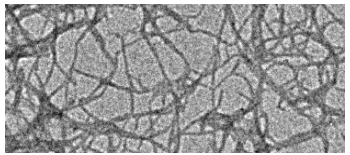


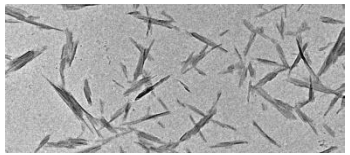
Selection of the study materials

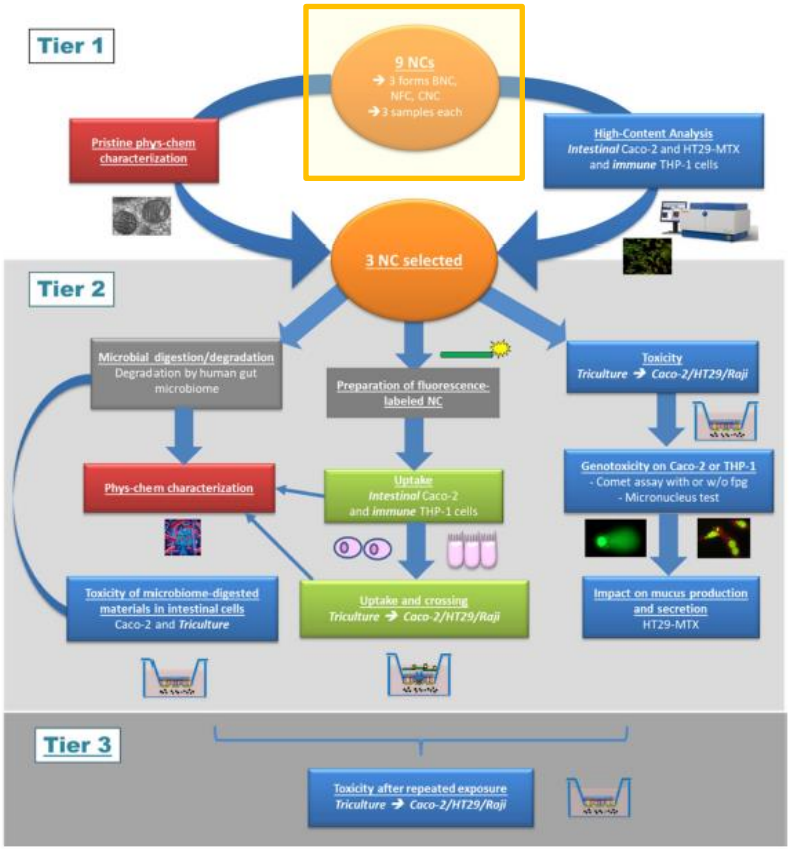
A panel of NC materials (up to 3 for each of the 3 main NC types) will be submitted to Tier 1 (initial physicochemical characterisation and a first tier of *in vitro* testing)

- Bacterial NC (BNC)
- Nanofibrillated cellulose (NFC)
- Cellulose nanocrystals (CNC)









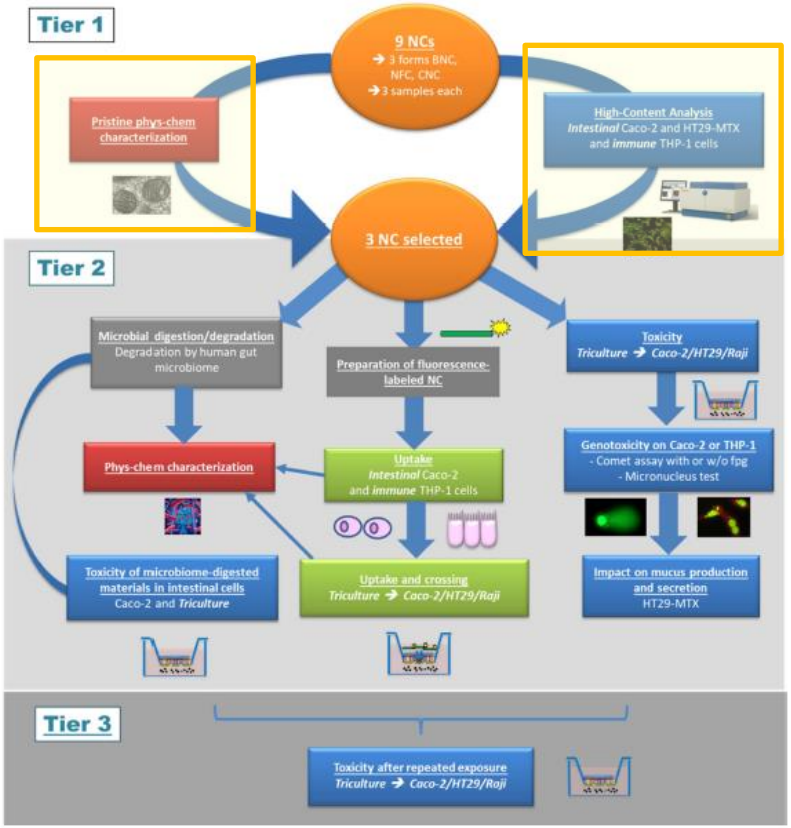
Tier 1 - Physicochemical characterisation and high content analysis

Handling protocol and physicochemical characterisation

- SOPs for the preparation of NC suspensions in the most disperse state (maximal deagglomeration) are being developed
- Physicochemical characterisation, including particle size, agglomeration/aggregation state, particle shape (all by TEM), and crystallinity (by XRD)

High throughput toxicity testing

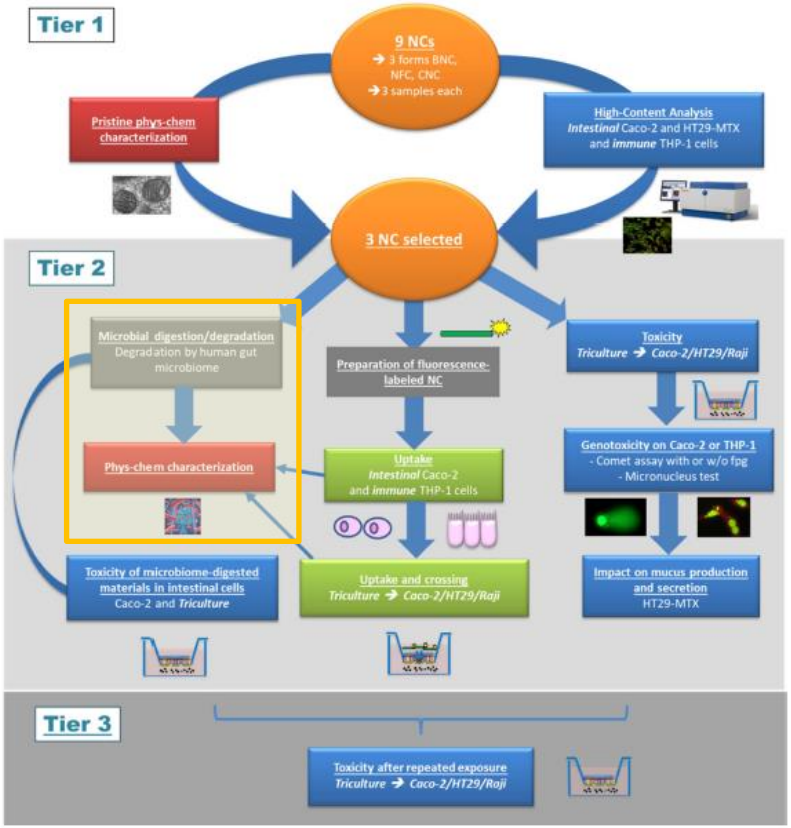
- The responses of both intestinal and macrophage cell models (Caco-2 and THP-1 cell lines) will be investigated using high content analysis (HCA) as a preliminary screening tool to generate a maximum amount of information on cytotoxicity (multi parametric cellular data at the single cell level)



Tier 2 - Digestion or degradation of NC by the human microbiome

ARCOL: a validated, dynamic *in vitro* model of the human colon

- ARCOL will be used to follow NC degradation by human microbiome, under physiologically relevant human colonic conditions. Three bioreactors will be set in parallel to test the three Tier 2 NC forms
- Furthermore, it will be assessed if NC exposure has an effect on microbiota composition, i.e. if it induces microbial perturbations or not
- Colonic luminal and mucosal samples will be examined by TEM to investigate the extent of digestion or degradation, including surface modifications, of NC by the human microbiome



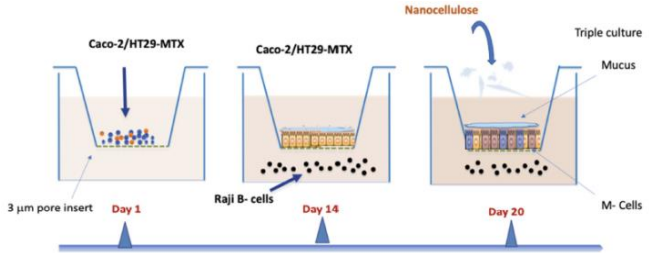
Tier 2 - Uptake and barrier crossing

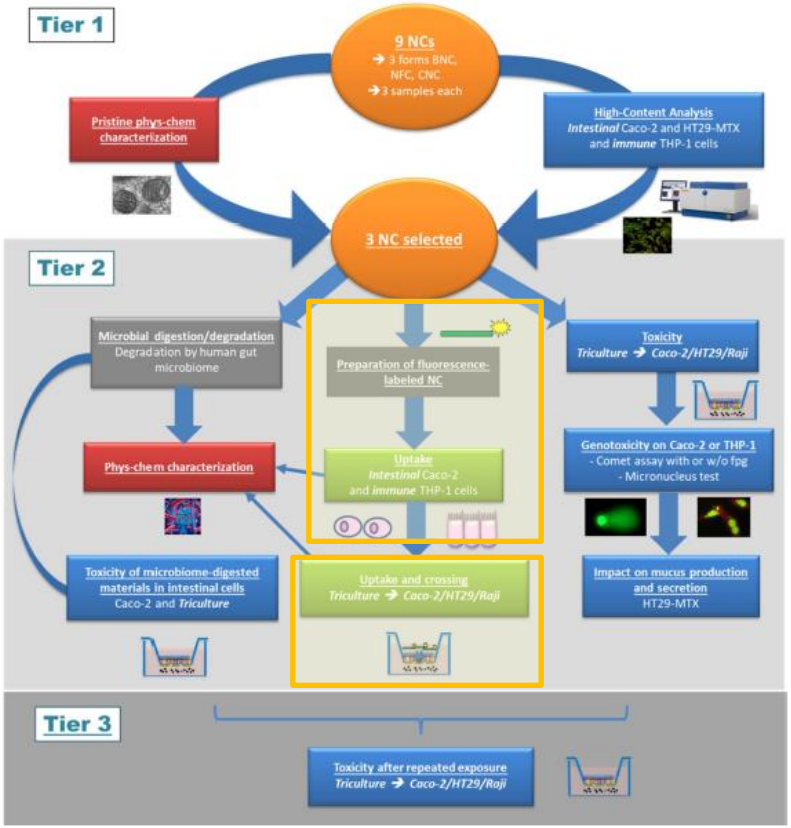
Uptake in Caco-2 and THP-1 cell monocultures

- Uptake of fluorescently labeled materials will be studied both in differentiated Caco-2 and HT29 cells, on the one hand, and THP-1 macrophages, on the other hand (by confocal microscopy).

Uptake and barrier crossing using a tri-culture model

- Fluorescently labeled materials and co-cultures of Caco-2 and HT29-MTX intestinal cells with Raji B cells on inserts will be used:





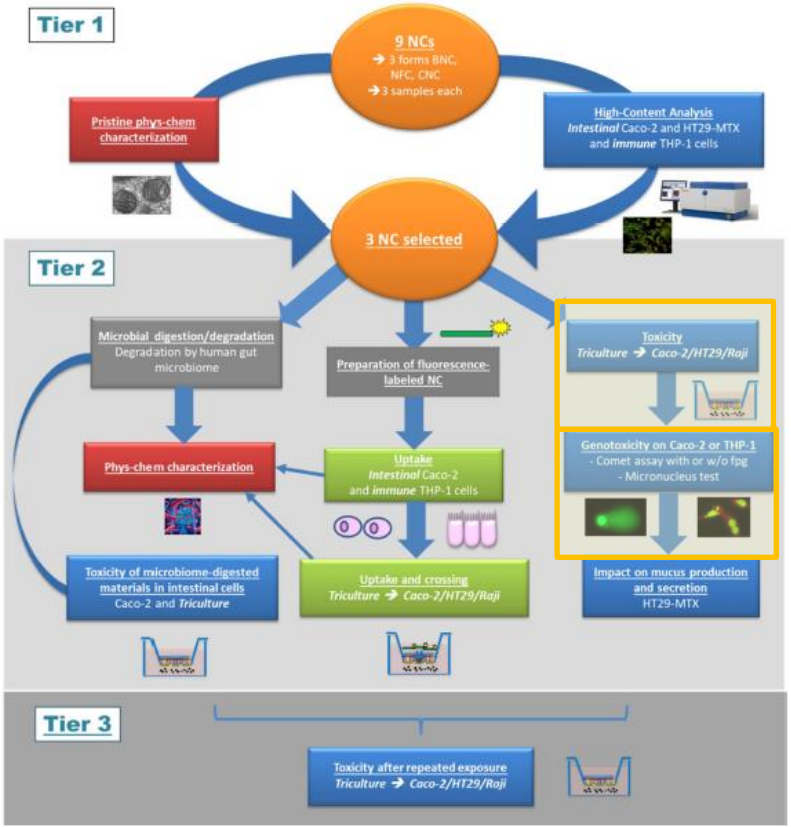
Tier 2 - Toxicity testing

Toxicity in the tri-culture model

- The tri-cultures of Caco-2/HT29-MTX/Raji-B cells will be used for toxicity testing of (non-labeled) materials

Genotoxicity

- The comet assay (alkaline version) detects DNA breaks and alkali-labile sites. Besides the alkaline version, the modified Fpg-comet assay will be also performed as it can favour the detection of oxidative DNA lesions due to oxidative species formation. Depending on the results in Tier 1, this assay will be performed on differentiated Caco-2 or THP-1 cells
- The micronucleus assay that detects chromosome and genome mutations will be also carried out on differentiated Caco-2 cells (OECD TG 487)



Tier 2 - Mucus production/secretion, toxicity of microbiome-digested NC

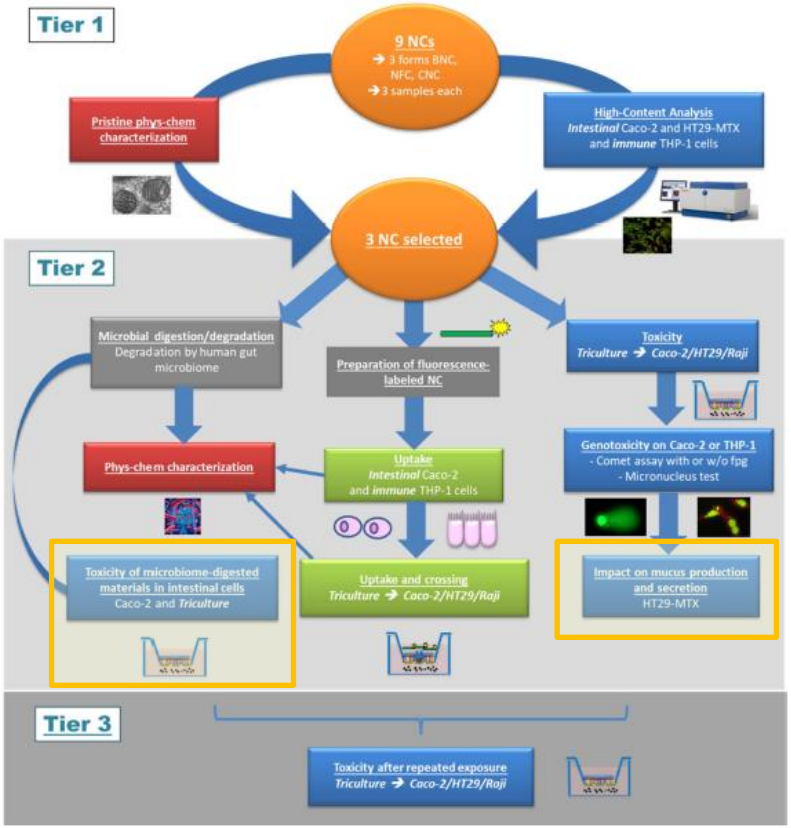
Effects on mucus production and secretion

- Mucus may play a role in the response of the intestine exposed to NC: the impact of NC on both production and secretion of mucins will be investigated

Experiment will be performed on differentiated HT29-MTX cells, followed by gene expression of key mucins by RT-QPCR and secretion quantified by image analysis on the histological section of HT29-MTX cells cultures on inserts

Toxicity of microbiome-digested NC

- One of the Tier 2 materials from the digestion study with human microbiota will be further tested on differentiated Caco-2 cells (with prospective additional studies on THP-1) as well as with the tri-culture model to assess if degradation by gut microbiota influences NC toxicity

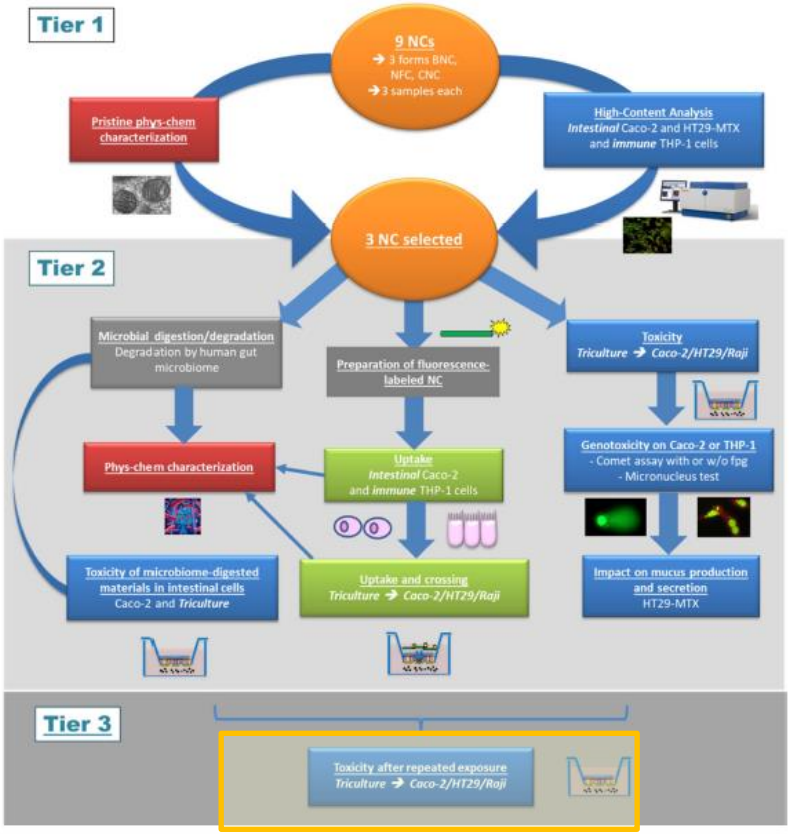


Tier 3 - Toxicity in the tri-culture model using repeated exposure

Toxicity in the tri-culture model using repeated exposure

- The NC sample selected for Tier 3 will be submitted to repeated dose toxicity testing using the tri-culture model model previously described, but with cell exposure from the 14th to the 21st day

At the end of the treatment cells will be analysed for TEER, permeability by FITC dextran flux, perturbation of thigh junctions and cytoskeleton proteins by confocal microscopy. Possible accumulation in the lysosomes will be investigated by colocalization with confocal microscopy and TEM analysis



Integrating the results in the regulatory hazard assessments

- Experimental studies will be performed so as to ensure that the results are relevant and reliable in the perspective of their use for regulatory risk assessment. In order to demonstrate this, the level of reporting will be adequate to allow risk assessors to assess reliability and relevance

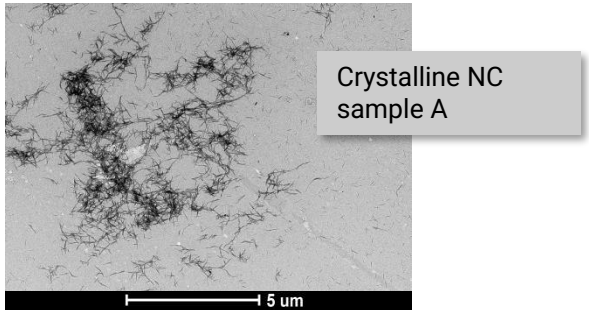
- Elements will be considered for setting the quality criteria for the different datasets in order to be fit-for-purpose for use in regulatory risk assessment

- The evidence gained in the present project will be used to fulfil key data gaps in risk assessment of NC according to the structured approach provided by the EFSA Guidance on Nano-RA and the methodology defined therein

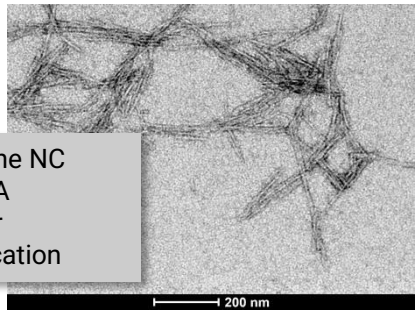
- Use of NC as novel food and as food additive, which are the most likely applications leading to direct exposure of consumers, will be thoroughly considered in the perspective of translating the results of the present project into regulatory hazard assessment strategies

Conclusions

- The increasing use of NC in food applications requires an assessment according to the Guidance for Risk Assessment of Nanomaterials
- A battery of *in vitro* tests providing insight into NC uptake and crossing, hazard and mode of action is being used
- A tri-culture of Caco-2, HT29 and Raji B cells is being applied for the first time to complex nanomaterials of organic nature



Crystalline NC sample A



Crystalline NC sample A at higher magnification

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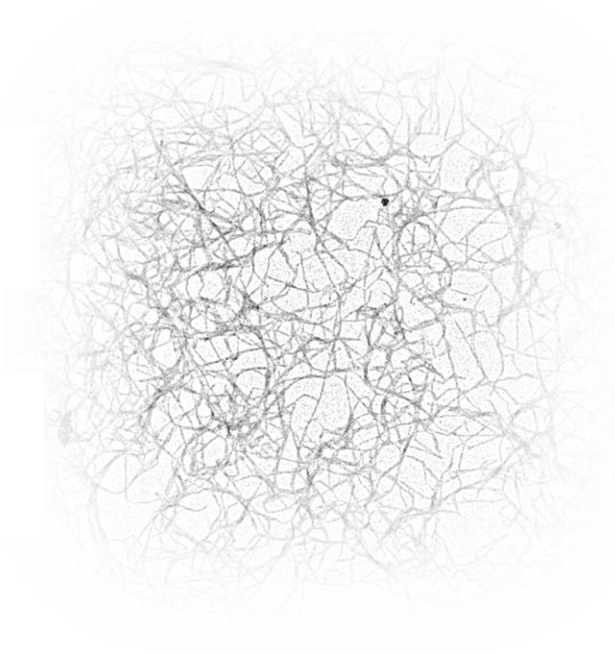

European Food Safety Authority

Acknowledgements

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EFSA contact person: Jose TARAZONA
e-mail: Jose.TARAZONA@efsa.europa.eu