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EFSA Project on the use of New Approach Methodologies (NAMs) for the hazard assessment of nanofibres. Lot 1, nanocellulose oral exposure: gastrointestinal digestion, nanofibres uptake and local effects

Olimpia Vincentini[1], Anne-Louise Blier[2], Alessia Bogni[3], Morgane Brun[4], Serena Cecchetti[1], Francesca De Battistis[1], Sylvain Denis[4], Lucie Etienne-Mesmin[4], Francesca Ferraris[1], Francesco Sirio Fumagalli[3], Kevin Hogeveen[2], Francesca Iacoponi[1], Andrea Raggi[1], Lisa Siciliani[5], Deborah Stanco[3], Eveline Verleysen[5], Valerie Fessard[2], Jan Mast[5], Stephanie Blanquet-Diot[4], Susanne Bremer-Hoffmann[3], Francesco Cubadda[1]

[1]Istituto Superiore di Sanità - National Institute of Health (ISS), Rome, Italy; [2]French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Fougères, France; [3]European Commission Joint Research Centre (JRC), Ispra, Italy; [4]National Research Institute for Agriculture, Food and the Environment (INRAE) - University of Clermont Auvergne, Clermont-Ferrand, France; [5]Sciensano, Brussels, Belgium

Abstract

Nanocellulose (NC) is an emerging material in the food sector with several prospective application areas. Three main types of NC exist, i.e. bacterial NC (BNC), nanofibrillated cellulose (NFC), and cellulose nanocrystals (CNC). The biological sources and processing conditions affect several physicochemical parameters of NC. In the present project, a NAM-based IATA for addressing data gaps in the assessment of potential hazards associated to NC oral exposure was considered. This IATA focused on three main pillars, i.e. (i) assessment of the uptake and potential crossing of the intestinal barrier by NC, (ii) assessment of local effects, including inflammation and genotoxicity, on the gastrointestinal epithelia, and (iii) assessment of any digestion or degradation of NC by the human microbiome. Eight NC samples belonging to the three NC types, plus a comparator in the micro-range, were selected as study materials and

submitted to a thorough physicochemical characterisation. A battery of *in vitro* tests was used to provide insight into NC hazard and mode of action according to a tiered approach, which lead to selection of three materials belonging to the three main NC types for in depth-testing. Cell uptake of these materials was demonstrated, and such uptake was greater in a triculture model, which better simulates the barrier properties of the human intestinal epithelium, as compared to Caco-2 monolayers. Uptake was the greatest in repeated exposure conditions, in which intestinal barrier crossing was demonstrated for CNC. Pro-inflammatory responses accompanied by massive NC uptake in macrophages, indicative for potential immunotoxicological effects, and barrier function impairment were observed, whereas no indications for genotoxicity were obtained. Finally, no formation of smaller particles following colonic fermentation of NC was observed. For the integration of these results in regulatory hazard assessment of NC after oral exposure, prospective use of NC as novel food or as food additive was considered.

Key words: nanocellulose, NAMs, IATAs, nanospecific assessment, hazard identification, cell uptake, barrier crossing

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Correspondence: MESE@efsa.europa.eu

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Summary

Nanocellulose (NC) is an emerging material in the food sector with several prospective application areas. 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 nanofibres (nanofibrillated cellulose, NFC) or nanocrystals (cellulose nanocrystals, CNC). The biological sources and processing conditions have been shown to affect the size, morphology, and several other physicochemical parameters of NC.

The potential hazards of ingested NC are insufficiently characterised and NC nanoscale features require a nano-specific assessment. The EFSA Scientific Committee Guidance on Nano - Risk Assessment recommends the development of Integrated Approaches to Testing and Assessment (IATAs) based on New Approach Methodologies (NAMs) for covering the nano-specific considerations based on mechanistic understanding of processes at the nanoscale. Based on the available information, current knowledge gaps, and expectations from the nanoscale properties of the different NC forms, hypotheses were formulated regarding nano-specific toxicokinetic and toxicodynamic considerations and a hypothesis-driven problem formulation developed. The NAM-based IATA for addressing data gaps in the assessment of hazards associated to NC oral exposure and for getting mechanistic understanding focused on three main pillars[1]:

1. Assessment of the uptake and potential crossing of the intestinal barrier by NC;
2. Assessment of local effects, including inflammation and genotoxicity, of NC on the gastrointestinal epithelia;
3. Assessment of any digestion or degradation of NC by the human microbiome.

The two main objectives of NANOCELLUP were:

(i) to design and conduct a set of NAM-based studies for addressing the current data gaps on NC hazards according to the developed IATA;

(ii) to offer a proposal for including the results in the regulatory hazard assessment of NC for consumers exposed via food.

Eight NC samples belonging to the three NC types, plus a comparator in the micro-range, were selected as study materials and submitted to a thorough physicochemical characterization. A battery of *in vitro* tests was used to provide insight into NC hazard and mode of action. Mono- and co-culture systems were used and specific endpoints considered to investigate potential effects of NC such as impaired cell viability/cytotoxicity, oxidative stress responses, (pro-)inflammatory responses, integrity of the gastrointestinal barrier, impact on mucus production and secretion, and genotoxicity. A tiered approach was followed (see **Figure 1**). In Tier 1 a maximum amount of information on the cellular responses following exposure to the whole panel of NC materials was obtained. Results from Tier 1 studies were combined with the outcome of the physicochemical characterisation, including the dispersion behaviour of the NCs and

[1] These data gaps and the design of new NAM-based experimental studies for addressing them were discussed in a workshop organised by EFSA in June 2020. The call for proposals GP/EFSA/SCER/2020/03 lists the data gaps and provides the minutes of the workshop (Annex 09).

associated handling issues, to select three NC materials (one CNC and one NFC for complete testing, one BNC for partial testing) for further investigation in Tier 2. Based on the outcome of Tier 2 testing, the CNC sample was prioritized based on the degree of uptake and its association to increased hazard. This material was submitted to Tier 3 testing, where functional impairment of the intestinal barrier, uptake and crossing after repeated exposure were investigated.

Experimental studies were performed in a way to ensure that the results are relevant and reliable in the perspective of their use for regulatory risk assessment. This included: (i) ensuring a high level of standardisation, i.e. establishment of Standard Operating Procedures (SOPs) or internal validation protocols for all experimental procedures; (ii) obtaining results for key NCs under different experimental conditions (single *vs* repeated exposure); (iii) achieving reproducibility of results in terms of adequate number of experimental replicates and replication of key experiments in different laboratories.

With regard to the first question addressed by the IATA, cell uptake was demonstrated with intestinal models for all the three materials investigated, belonging to the three main NC types (NFC, CNC and BNC). Cell uptake was greater in the triculture model, which better simulates the barrier properties of the human intestinal epithelium as compared to Caco-2 monolayers, and was the greatest in repeated exposure conditions. Under these conditions, intestinal barrier crossing was demonstrated. Based on the detected cell uptake for all the NC materials investigated, NC translocation across the intestinal barrier can generally be expected to occur.

As far as the second question is concerned, increased cytokine production was shown, accompanied by massive NC uptake in macrophages. This highlights the immunotoxic potential of NCs and especially CNC. In addition, indications of barrier function impairment for intestinal epithelia exposed to NC consistently emerged. On the contrary, no indications for genotoxicity were obtained.

With regard to the third question, although with a considerable degree of uncertainty, no indications of digestion or degradation of NC by the human microbiome with formation of smaller NC particles were obtained. Toxicity tests did not deliver results supporting higher toxicity of NC undergone colonic fermentation.

For the integration of these results in regulatory hazard assessment of NC after oral exposure, prospective use of NC as novel food or as food additive were discussed, as these are the most likely applications leading to direct exposure of consumers.

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1. Background

1.1. Terms of reference as provided by the requestor

This grant was awarded by EFSA to Istituto Superiore di Sanità (ISS), the Italian National Institute of Health, as the coordinator. Co-beneficiaries were (i) the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), (ii) Sciensano, and (iii) the National Research Institute for Agriculture, Food and the Environment (INRAE).

The grant title was 'Use of New Approach Methodologies (NAMs) for the hazard assessment of nanofibers. Lot 1, nanocellulose oral exposure: gastrointestinal digestion, nanofibers uptake and local effects'. The grant number was GP/EFSA/SCER/2020/04.

The European Commission Joint Research Centre (JRC) contributed to the project as external partner. In particular, the JRC independently replicated the studies carried out at ISS with the triple culture model.

1.2. Additional information

The EFSA Pilot Project on NAMs for the hazard assessment of nanofibers (GP/EFSA/SCER/2020/04) was subdivided into two Lots:

- Lot 1: 'Nanocellulose oral exposure: gastrointestinal digestion, nanofibers uptake and local effects';
- Lot 2: 'Exploring the use of gut-on-a-chip models for risk assessments of nanofibers'.

With the aim to discuss aspects of mutual interest and maximise the results from this activity, cooperation was maintained between the two Lots for all the duration of the project.

1.3. Introduction

Cellulose is a polysaccharide consisting of a linear chain of several hundreds to many thousands of D-glucose units linked by β(1→4) glycosidic bonds, having the molecular formula $(C_6H_{10}O_5)_n$. Cellulose is the most abundant organic polymer on Earth and is an important structural component of the primary cell wall of green plants, several algae and oomycetes; in addition, some species of bacteria secrete it to form biofilms.

The degree of polymerisation (DP) of cellulose depends on the origin of the cellulolytic material. Cellulose molecular weight has been calculated to be approximately in the range 50,000–2,500,000. In modified celluloses, the chemical and physical characteristics of the native substances are modified in order to confer different technological properties for particular food applications. Microcrystalline cellulose is a purified, partially depolymerised cellulose prepared by treating α-cellulose, obtained as a pulp from strains of fibrous plant material. Celluloses have different uses under EFSA's remit and those used as food additives, including microcrystalline cellulose, have been evaluated by EFSA as of no safety concerns (EFSA ANS Panel, 2018).

Recently, the production and use of cellulose at the nanoscale has attracted increasing interest. Nanocellulose (NC) is an emerging material with potential industrial applications in various domains including medicine, energy, remediation, and the food sector (Portela da Gama and

Dourado, 2018; Mu et al., 2019; Trache et al., 2020; Li et al., 2021). In the food domain, NCs have been identified as emerging in food packaging (Vilarinho et al., 2018), e.g. as coatings and fillers in composites, but they have potential applications in several other areas, e.g. as novel foods (one application already received and under evaluation by EFSA)[2] and food additives (Brand et al., 2022). In addition, the possible presence of NC as a nanosized fraction, e.g. in other types of modified celluloses, cannot be excluded.

Two main sources of NC have been identified, namely 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 nanofibres (nanofibrillated cellulose, NFC) or nanocrystals (cellulose nanocrystals, CNC). The morphological features of the different NC types are quite different. Whereas all NC materials typically have a high aspect ratio, CNC usually consists in rod-shaped crystals, which are generally 50–350 nm long (Brand et al., 2022). On the contrary, NFC consists in fibrils composed of fibres with a length up to 2-3 μm; nanofibers are even longer in BNC and organized in networks. For all the NC types, the diameter can be very small (as low as 5-10 nm) (Brand et al., 2022). In addition to size, aspect ratio, and morphology, the biological sources and preparation conditions has been shown to affect several physicochemical parameters of NC (e.g. polydispersity, surface charge, surface chemistry and crystallinity index). In fact, the variability in NC types and the wide variation in physicochemical properties of individual NCs belonging to each type are key in the development of an ever-increasing number of applications (Sharma and Bhardwaj, 2019; Sheikhi et al., 2019; Trache et al., 2020). In addition to the attractive characteristics of NC, the rising demand for 'green' materials and products may contribute to consumers' acceptance.

The increasing use of NC in food applications requires an assessment according to the EFSA Scientific Committee (SC) Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021). One main concern is that the nanoscale characteristics of the material may influence its toxicokinetic behaviour. In this regard, two main questions to be answered are as follows: i) are the nanosized fibres and crystals able to cross the intestinal epithelia? ii) is the uptake driven by the physicochemical properties of the nanofibres/crystals, leading to selective uptake of nanofibres/crystals with specific characteristics? Another concern is represented by the unknown hazardous properties of cellulose nanofibers/crystals and the possible relationship with characteristics of the material (e.g. length/shape of the fibres/crystals).

The fate of NC in the human gastrointestinal tract is another knowledge gap that impacts the safety assessment of NC (Liu and Kong, 2021). In human nutrition cellulose is a non-digestible constituent of insoluble dietary fibre, which is excreted intact in the faeces. There is some evidence that during the oral, gastric and small intestinal phases of human digestion NC interacts with gastrointestinal tract (GIT) components (e.g. bile salts, digestive enzymes, mucin), influences food digestion and nutrient absorption, and undergoes agglomeration to an extent depending on its physicochemical properties. However, owing to its nature of insoluble and non-digestible fibre, NC reaches the small intestine maintaining its nanoscale features essentially intact.

[2] EFSA-Q-2018-00294. Information retrieved via Open EFSA (https://open.efsa.europa.eu/questions).

In the large intestine, the possible digestion or degradation of NC by the human microbiome, potentially leading to smaller fibres, is an open question. Some animals, e.g. ruminants, can digest cellulose with the help of symbiotic bacteria that live in their GIT. The degradation products are absorbed and used as nutrients. Strategies for maximising this source of energy have been developed by several species and significant degradation has been reported for laboratory animals. For microcrystalline cellulose and powdered cellulose, evidence exists that fermentation during their passage through the large intestine by strains of bacteria found in the human colon might happen, whereas modified celluloses are not fermented and are excreted intact via the faeces (EFSA ANS Panel, 2018). No information is available on the potential degradation of NC by the human microbiome (Liu and Kong, 2021). A single study shows that CNC had higher fermentability than microcrystalline cellulose based on *in vitro* and *in vivo* studies on rats (Nsor-Atindana et al., 2019). However, the NC behaviour in the human colon is essentially unknown.

Studies on the uptake and crossing of the human intestinal epithelium by NC are complicated by the lack of analytical methods for quantitatively measuring NC uptake and fate. Whereas no such studies are available, different systems for tagging NCs have been proposed to improve their detection, i.e. use of fluorescently tagged materials (Mahmoud et al., 2010; Bitounis et al., 2019; Salari et al., 2019; Shazali et al., 2019) or coupling with a biotinylated module (Knudsen et al., 2015).

In addition to the analytical limitations, studies on intestinal uptake and crossing are complicated by the lack of an entirely appropriate cell model. *In vitro* models based on a single cell line (e.g. Caco-2 cells) do not properly represent the complex gut environment. Incorporation of microfold (M) cells and mucus secreting cells into Caco-2 cell cultures can enhance the physiological relevance of intestinal *in vitro* models. A triculture model composed of three different human-derived cell lines (Caco-2 cells, HT29-MTX cells, and Raji B lymphocytes) holds promise in this respect (García-Rodríguez et al., 2018; Kämpfer et al., 2020) and is being investigated in the OECD project 'Integrated in vitro approach for intestinal fate of orally ingested nanomaterials' (OECD, 2020; OECD, 2023). However, in this project the model is being tested with inorganic nanoparticles whereas improvements/adaptations might be required to meet the complexity of carbon-based nanofibres as NC. In addition, a generally accepted SOP is not available yet.

Interaction with mucus appears to be important and capable of affecting the behaviour of different types of NCs. In a study by Lin et al. (2019), NFC showed mucoadhesion by entanglement with mucus in both the gastric and intestinal conditions, whereas CNC showed adhesion by attraction with mucin particles and this phenomenon was especially strong in gastric conditions. As suggested by Lin et al. (2019), NC can induce long-term effects on the mucus structure along the GIT as well as on the turn-over of mucus. Therefore, the production and secretion of mucins should be investigated, which can be done using gene expression or staining of mucins in intestinal cell models (Reale et al., 2020).

The uptake into cells can be affected by the surface charge of the nanofibres. Shazali et al. (2019) documented that cellular accumulation was inhibited with negatively charged CNC due to the electrostatic repulsive force between the CNC surface and the negative charge of the cell membrane. In the presence of BSA, no adsorption was observed onto negatively charged NC

and cellulose fibres as well as on pure cellulose (Ha et al., 1993; Lavenson et al. 2011). Single amino acids do not bind to cellulose as well, independently of the charge of both the amino acid and the cellulose nanocrystals (Lombardo et al., 2017).

As far as toxicity of NC is concerned, effects on intestinal Caco-2 cells have been sparingly reported. Recently, no signs of cytotoxicity (by evaluation of metabolic activity and cell membrane integrity) were detected in Caco-2 cells exposed to NC with various coatings, although the study was carried out with non-differentiated cells that are not fully relevant for mimicking enterocytes (Lopes et al., 2020). Using a triculture model based on Caco-2, HT29-MTX, and Raji B cells, DeLoid et al. (2019) found that 24-hour incubation with digesta of NFC and CNC did not induce significant changes in cytotoxicity, ROS, or monolayer integrity other than a 10% increase over controls in reactive oxygen species (ROS) production with CNC; however extremely high concentrations in the absence of any dispersion protocol were used in this study. Similar results were obtained in a study on CNC having the same methodological limitations (i.e. use of high concentrations in the absence of proper deagglomeration of the test item) (Ede et al., 2020). On the other hand, cytotoxic effects have been shown in other cell types, such as lung cells (Catalan et al., 2015; Menas et al., 2017).

NFCs were reported not to be taken up and induce ROS production in THP-1 macrophages; however, an inflammatory response (increase in TNF-α and IL1-β levels) was detected with unmodified NFC (i.e. the absence of charged groups on the surface) (Lopes et al., 2017). Also Colic et al. (2020) observed that NCs, especially CNC, can induce inflammatory responses upon internalization by macrophages and that the reaction may be significantly modulated by introducing different functional groups on the surface. Differently, Catalan et al. (2015) found that CNC did not trigger an inflammatory response in human monocyte-derived macrophages, whereas microcrystalline cellulose (MCC) was able to induce secretion of both IL-1β and TNF-α. Overall, several studies highlight the relevance of investigating NC inflammogenic potential and immune response towards NC materials (Bhattacharya et al., 2017; Wang et al., 2019; Weiss et al., 2021).

An *in vivo* study (DeLoid et al., 2019) investigating oral administration of 1% w/w NFC suspensions by gavage to rats twice weekly for five weeks did not detect significant effects on hematology, serum markers or histology but detected a biologically relevant decrease in body weight gain (Brand et al. 2022). Khare et al. (2020) found altered microbiome diversity as well as perturbations possibly representing initiation of inflammation. Exposure of C57BL/6 mice to CNC as powder following pharyngeal aspiration induced a more prominent increase in biomarkers of tissue damage than treatment with CNC as gel/suspension (10 wt %), which however caused higher oxidative stress; differences were attributed to the morphology and size distribution of the materials (Yanamala et al., 2014). On the other hand, notable shortcomings - i.e. use of high concentrations (up to 4% w/w) in the absence of any dispersion protocol ensuring appropriate deagglomeration of the test materials (in contrast to the recommendation made by the relevant EFSA guidance, EFSA Scientific Committee, 2021) - affected two subchronic oral toxicity studies carried out on CNC by Ede et al. (2020) and on NFC by Ong et al. (2020), which did not identify adverse effects.

As far as genotoxicity is concerned, data are still scarce (Stoudmann et al., 2020). The brown cotton and curaua nanofibres were genotoxic in plant (*Allium cepa*) and animal cells (human lymphocytes and mouse fibroblasts) (de Lima et al., 2012). *In vitro* studies on pulmonary models showed conflicting results. Whereas Catalan et al. (2015) did not observe induction of micronucleated cells on BEAS2B cells, Ventura et al. (2018) demonstrated a genotoxic effect of NFC in the micronucleus assay on A549 cells at low concentrations (1.5 and 3.12 µg/cm^2); it was unclear whether this effect resulted from a clastogenic or aneugenic mechanism. Some DNA damage was reported in the bronchoalveolar fluid and in the lung of rats 28 days after a single intratracheal instillation (Lindberg et al., 2017). Overall, genotoxicity of some NC fibres cannot be excluded, based on the limited available data set.

Considered in the light of the EFSA SC Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021), the vast majority of the existing *in vitro* toxicological studies are unsuitable for the hazard identification of NC oral exposure owing to the following methodological shortcomings:

- Insufficient physicochemical characterisation of the pristine materials;
- Absence of suitable dispersion protocols;
- Use of extremely high concentrations (well above 100 µg/mL);
- No confirmation of cellular exposure.

The insufficient physicochemical characterisation of the pristine materials makes it impossible to exactly define the NC form studied and assess any relationship between toxicity and physicochemical characteristics. The absence of suitable dispersion protocols violates the basic principle that the test conditions must ensure worst case scenarios, which means exposure to the most dispersed form. This principle is embedded in regulatory data requirements for proper and fresh dispersion of the material before testing, as well as confirmation thereof (EFSA Scientific Committee, 2021; Schoonjans et al., 2023). The use of unrealistically high concentrations with no confirmation of cellular exposure, especially in case of negative results (i.e. no adverse effects detected), is a shortcoming due to the fact that the degree of NC agglomeration increases with increasing concentrations. A proper dispersion is difficult to attain and to keep stable at higher doses and extensive agglomeration generally translates into a decrease in cell uptake, for which size threshold exists (EFSA Scientific Committee, 2021; Schoonjans et al., 2023). This is why *in vitro* studies with exposure concentrations >100 µg/mL are considered inappropriate for hazard characterisation of inorganic nanomaterials, whereas for light (carbonaceous) materials such as NC even a lower threshold appears to be appropriate in order to expose cells to comparable particle number concentrations.

The studies commented above are just some examples from a rapidly increasing body of evidence (Stoudmann et al., 2020; Ventura et al., 2020). Altogether, the existing studies show that the potential hazards of ingested NC are insufficiently characterised. A review of the current knowledge on (possible) adverse health effects of NC upon oral exposure indicated that toxicity data, especially from *in vivo* studies, are limited and outcomes are not unambiguous (Brand et al., 2022). The hazard assessment is further complicated by the diversity in morphologies and surface modifications, the lack of standard reference materials, the limited knowledge about intestinal fate and absorption, the analytical difficulties in detecting NC in biological matrices,

the dispersion issues, and the possible presence of impurities and interferences within biological assays. Two sub-chronic *in vivo* toxicity studies showed no indications of toxicity for two specific NC materials, even at high doses. However, these studies may have missed certain early or nanospecific toxic effects, such as inflammation potential, for which other subacute *in vivo* studies provide some indications. Most *in vitro* studies show no cytotoxicity; however, several indicate that effects on oxidative stress and inflammatory responses depend on differences in size or surface treatments. Further, too few studies assessed genotoxicity of NCs. This recent review concludes that immunotoxicity, oxidative stress and genotoxicity require further attention, as do absorption and effects on nutrient uptake (Brand et al., 2022).

The EFSA SC Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021) recommends the development of IATAs for covering the nano-specific considerations based on mechanistic understanding of processes at the nanoscale. According to the Guidance, NAMs should be first considered to generate such de novo nano-specific information and the resulting evidence should be integrated with animal (and, when existing, human) data according to the relevant IATA. The use of human-relevant NAMs appears as the best option to generate the needed information for the hazard assessment of NC oral exposure for several reasons. First, laboratory animals do not appear to be appropriate models because the digestive physiology, microbiome and rate of fibre degradation differ from humans. Second, the variability in the physicochemical properties of NC make NAMs ideal for efficient testing. Third, the needed studies are technically easier to implement using *in vitro* methods than *in vivo* studies. Whenever hazard assessment requires new animal studies to be performed, NAM-based data are essential for optimizing their design and such *in vivo* studies should be conducted with careful consideration of the relevant animal model, taking into account differences in digestive physiology, microbiome and rate of fibre degradation.

Based on the available information, current knowledge gaps, and expectations from the nanoscale properties of the different NC forms, hypotheses can be formulated regarding nano-specific toxicokinetic and toxicodynamic considerations and a hypothesis-driven problem formulation developed. The NAM-based IATA for addressing data gaps in the assessment of hazards associated to NC oral exposure and for getting mechanistic understanding has to address the main knowledge gaps highlighted above, i.e. (i) the assessment of the uptake and potential crossing of the intestinal barrier by NC; (ii) the assessment of local effects, including inflammation and genotoxicity, of NC on the gastrointestinal epithelia; (iii) the assessment of any digestion or degradation of NC by the human microbiome.

2. Methodologies

The methodologies employed are summarised in Section 2.1, where the IATA and the NAM-based studies for assessing potential hazards from NC oral exposure are presented. A brief description of the *in vitro* models and the analytical techniques used for the physicochemical characterisation is also given. For *in vitro* testing, harmonised conditions were used in the different laboratories with regard to the materials and reagents for cell cultures, the reagents for the assays, the cell lines and the cell culture parameters (Annex A). When the same studies were conducted in different laboratories, specific SOPs detailing study protocols were developed.

Unless otherwise specified, data are reported as means and standard uncertainty or expanded uncertainty (u), obtained by multiplying the combined standard uncertainty by a coverage factor equal to 2 (95% confidence interval). Differences between experimental and control groups were evaluated as effect size (%) and statistical comparisons (Kruskal-Wallis pairwise comparisons test, where a p value <0.05 was considered significant). All analyses were performed by SPSS v28.

2.1. IATA and NAM-based studies for assessing nanocellulose oral exposure hazards

The NAM-based IATA for addressing data gaps in the assessment of hazards associated to NC oral exposure and for getting mechanistic understanding of toxicokinetic and toxicodynamic processes at the nanoscale focused on three main pillars:

1. Assessment of any digestion or degradation of NC by the human microbiome;
2. Assessment of the uptake and potential crossing of the intestinal barrier by NC;
3. Assessment of local effects, including inflammation and genotoxicity, of NC on the gastrointestinal epithelia.

A battery of *in vitro* tests was designed to address the data gaps for each of the three main areas of concern listed above and provide insight into NC hazard and mode of action. Mono- and co-culture systems were used, and specific endpoints considered to investigate potential effects of NC such as impaired cell viability/cytotoxicity, oxidative stress responses, (pro-)inflammatory responses, integrity of the gastrointestinal barrier, impact on mucus production and secretion, and genotoxicity.

A tiered approach was followed (**Figure 1**). The objective of the Tier 1 studies was to obtain a maximum amount of information on cellular responses following exposure to a panel of NC materials and concurrently characterise these materials in terms of their physicochemical properties. Results from Tier 1 studies were used for the selection of NC materials to be submitted to further investigation in Tier 2. In particular, on the basis of the physicochemical and toxicological data obtained in Tier 1, three materials were selected for Tier 2 testing. This second tier entailed in depth toxicological testing, detailed characterization of uptake and crossing in human intestinal epithelia and full physicochemical characterisation. The material showing the most remarkable effect(s) in Tier 2 testing (including the degree of uptake/translocation) or with physicochemical properties likely associated to increased hazard was selected for Tier 3 testing. In Tier 3 testing, effects following repeated dose exposure were investigated.

Assessment of NC uptake and crossing in human intestinal epithelia was addressed in Tier 2 and Tier 3. For the intestinal barrier, the human intestinal Caco-2 cell line features among the most well-established models (Frohlich, 2018). However, since nanoparticles cross the intestinal barrier of the small intestine mainly via Microfold (M) cells, Caco-2 monocultures may underestimate permeation. M cells perform transcytosis of antigens across the gut epithelium and play a major role in the induction of efficient immune responses. In this project, Caco-2 cells were cultured alone or in co-culture with mucus-secreting cells (HT29-MTX) and immune cells

(Raji B) to mimic the concurrent presence of key cell types in the intestinal epithelium. In fact, *in vitro* co-culture of Raji B cells and Caco-2 cells induce the M cell-phenotype. By combination with mucus-producing HT29-MTX cells, Caco-2 cells and Raji B cells in direct co-culture form a physiologically relevant model of the human intestine (Frohlich, 2018). This triculture model holds promise for investigating intestinal absorption of nanomaterials (Schimpel et al., 2014; Beloqui et al., 2017; García-Rodríguez et al., 2018; Vincentini et al., 2022).

In Tier 2, the uptake of the three selected NCs at the intestinal level using single cell lines of enterocytes (differentiated Caco-2 cells) and macrophages (differentiated THP-1 cells) was studied a first step. In a second step, the uptake and crossing of the intestinal barrier using a triculture of Caco-2, HT29-MTX and Raji B cells were assessed. In these studies, fluorescence detection was employed (see Section 2.1.6). Uptake and crossing were assessed by using Confocal Laser Scanning Microscopy (CLSM). Co-culture results were compared with Caco-2 monocultures.

Barrier integrity was monitored by Transepithelial electrical resistance (TEER), which also served to evaluate the health of the culture at maturation and before NC translocation/uptake experiments. Permeability was assessed by Lucifer Yellow (LY) and expressed as Apparent Permeability (P_{app}). Protocols were harmonised among the partners by the use of the same cells clones and same culture materials.

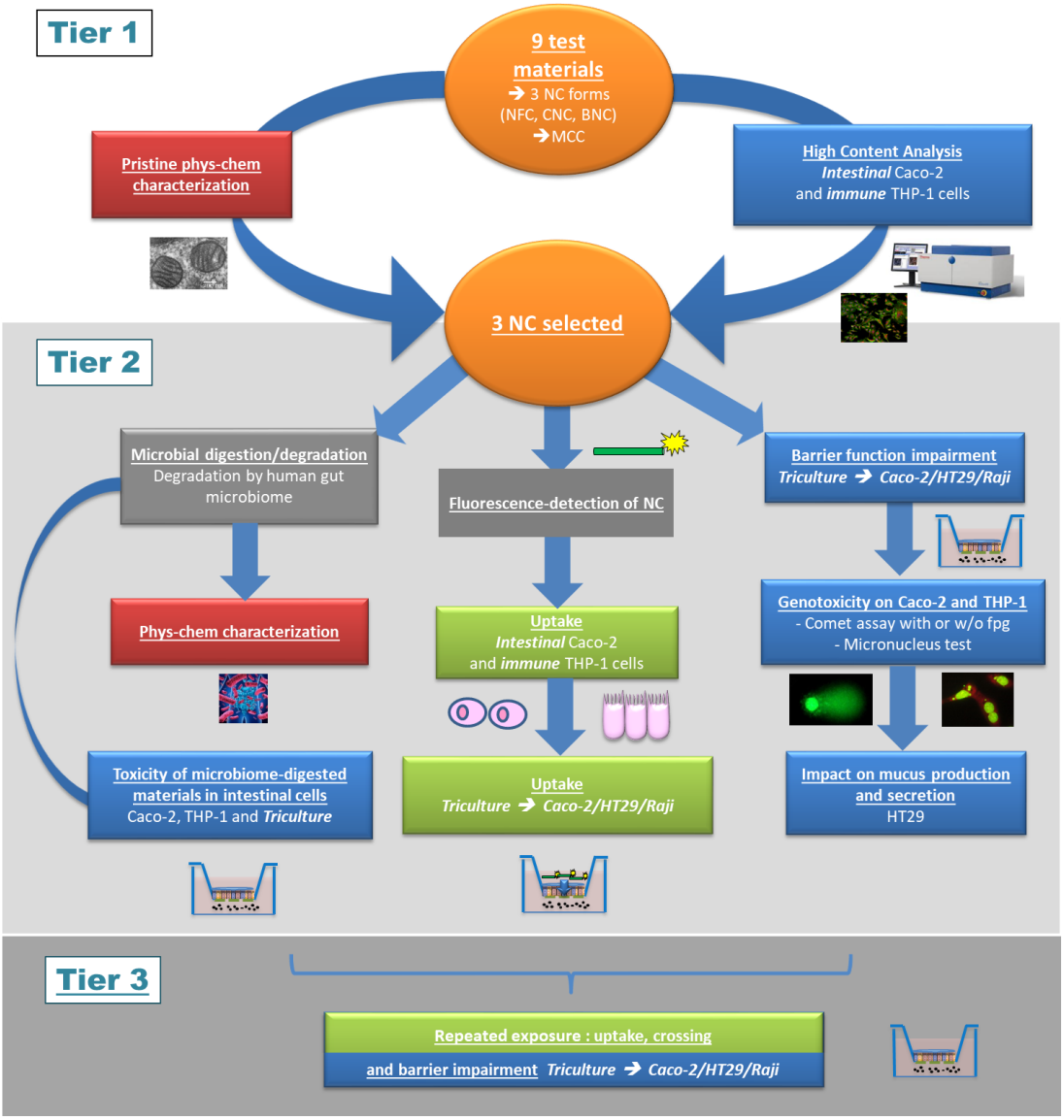

Figure 1: **Tiered approach for NC testing**. Colour code: red = physicochemical characterization, blue = toxicology, green = uptake and crossing, grey = methodological milestones.

All experimental studies were performed to ensure relevant and reliable results in the perspective of their use for regulatory risk assessment. As the first prerequisite, this entailed a detailed physicochemical characterisation and the development of proper dispersion protocols, in line with the EFSA SC Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021). In fact, one key element when testing nanomaterials *in vitro* is the potential for agglomeration as well as stability in different media. This element is important also in relation to the selection of the concentrations/doses to be used. On the one hand, the tested concentrations/doses should be high enough to enable the detection of relevant effects. On the other hand, agglomeration is

expected to increase with the concentration/dose, leading to a reduction in actual exposure levels to NC at high doses. Therefore, in the present project a specific SOP detailing the protocol for NC dispersion was developed to ensure that a similar level of dispersion was achieved through the full dose/concentration range. This SOP made it sure that the dispersion was stable until the exposure of the cells was completed and covered the diverse testing media used in the different cell systems (see Annex B).

For *in vitro* testing, in absence of 'validated' *in vitro* methods, 'valid' methods[3] covering the different endpoints were used, taking into account the recommendations from international bodies, e.g. OECD (OECD, 2108a; OECD, 2018b), from the literature (e.g. Drasler et al., 2017), the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) and in the EFSA SC Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021). In the design of the tests as well as in the reporting, a number of requirements were complied with, including:

i. detailed cell characterisation (i.e. cell source, passage number, cell growth, morphology and differentiation before and during the test performance);
ii. precise description of cell culture method;
iii. exposure and post-exposure times defined and justified with respect to the individual tested parameters;
iv. for mono- or co-culture systems grown on membrane inserts: confluence and viability checked for the appropriate level of resistance by TEER measurements before cytotoxicity assays;
v. careful selection and justification of the concentration/dose levels used;
vi. dosimetry aspects, i.e. assessment of uptake to demonstrate actual cell exposure;
vii. check for the absence of interference, e.g. unwanted reactions of assay components (reagents, proteins, nutrients) or the optical read-out systems of the test with NC;
viii. quality controls including (where available) negative and positive controls and assay reagent controls;
ix. use of two independent in vitro assays per individual endpoint when feasible.

2.1.1. Selection of the test materials

The three main types of NC – i.e. BNC, NFC, and CNC – were investigated in Tier 1. BNC can derive from different bacterial strains whereas NFC and CNC come from processing of cellulose with plant or other origins. The biological sources and preparation conditions have been shown to affect several physicochemical parameters of NC (size, aspect ratio, morphology, polydispersity, surface charge, surface chemistry and crystallinity index). As these physicochemical properties may affect the fate and hazard of NC materials, investigating a broad range of materials is relevant. Inclusion of a micro-sized comparator is also relevant and MCC appeared appropriate for this purpose.

[3] A 'valid' method is, in toxicological testing, a method that has not necessarily gone through the complete validation process, but for which sufficient scientific data exist demonstrating its relevance and reliability (EFSA Scientific Committee, 2021).

In this light, three samples for each of the three types of NC (two for BNC) were studied, for a total of eight test materials (**Table 1**). They were sourced from NC manufactures and, as far as possible, materials that span the range of physicochemical characteristics of each of the three NC types were selected. The NFC and CNC materials have been sourced by commercial providers (including academic-associated centres[4]), whereas the BNC materials have been donated by researchers involved in industry-related R&D activities. The ninth material submitted to Tier 1 testing was a sample of MCC, included in the toxicity testing as comparator in the micro-size range.

Table 1: List of the NC test materials and the comparator in the micro-size range included in Tier 1 testing

Material	Provider
NFC (nanofibrillated cellulose)	
Valida S231	Sappi[1]
CNF High fines slurry	University of Maine-PDC[2]
TOCN[3]	University of Maine-PDC[2]
CNC (nanocrystalline cellulose)	
NG01NC0102	Nanografi[4]
NCV100-NAL90	CelluForce[5]
CNC-PDC	University of Maine-PDC[2]
BNC (bacterial nanocellulose)	
BNC-01-AU (Nata de coco)	Cass Materials Pty Ltd, Perth, Australia
BNC-02-PT	Donation[6]
MCC (microcrystalline cellulose)	
MCC	Merck (Sigma-Aldrich)[7]

[1] https://www.sappi.com/nanocellulose
[2] https://umaine.edu/pdc/
[3] TEMPO-oxidized FPL Cellulose Nanofibrils
[4] https://nanografi.com/
[5] https://www.celluforce.com/
[6] The material was donated by a Portuguese research centre (the supplier requested to remain anonymous)
[7] EP Reference Standard (https://www.sigmaaldrich.com/IT/it/product/sial/y0002021)

All the study materials were obtained as homogenous stock water dispersions. This was considered an important feature in order to limit the variability in the degree of dispersion of particles associated to suspensions obtained from pristine materials in powder form. In addition, all study materials received by the different laboratories originated from the same production batch.

2.1.2. Physicochemical characterisation of the pristine materials and sample handling protocols

[4] Such as the Process Development Center (PDC) of the University of Maine.

Investigating the physicochemical properties of NC materials and how they behave under the conditions applied for *in vitro* testing is a prerequisite for proper hazard identification and for understanding the underlying biochemical mechanisms occurring during NC interactions with living cells and tissues. Therefore, in Tier 1 an in-depth multi-method physicochemical characterisation of the pristine materials as well as a thorough study to establish a proper sample handling before *in vitro* testing were performed as described hereunder.

The physicochemical characterization was carried out in line with the EFSA Guidance on Nano-RA (EFSA Scientific Committee, 2021) by Sciensano and JRC. A descriptive Transmission Electron Microscopy (TEM) analysis of all Tier 1 materials was conducted, and the results summarised in datasheets in order to support the selection of Tier 2 materials. For each examined sample, a set of calibrated, selected and representative TEM micrographs was recorded. The particle diameter and length were estimated based on manual measurements and the properties of the samples were described according to the above-mentioned guidance. The agglomeration state (at low magnification) and the particle integrity (at high magnification) were evaluated under the applied sample preparation conditions.

Key properties were measured for the pristine materials in water suspensions. Material composition and purity, elemental composition, particle size, agglomeration/aggregation state, particle shape, crystal form and phase, and structure were investigated. For the characterisation of the crystalline structure and of the macromolecular chemistry, X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared spectroscopy (FTIR) and Time-of-Flight Secondary Ions Mass Spectrometry (ToF-SIMS) were used.

To perform the above detailed physicochemical characterisation, Sciensano optimised the dispersion conditions of the stock suspensions to avoid any interference on the measurement of the key intrinsic physicochemical properties of the NC particles in the samples investigated in Tier 1. For proper dispersion, sonication using a calibrated probe ultrasound device to deliver an energy of approximately 7 kJ was needed. Furthermore, the dispersion degree and stability of the stock suspension and the influence of different exposure media applied in the *in vitro* experiments on the dispersion of NC particles were assessed. The behaviour of NCs in the cell culture media used for *in vitro* experiments was investigated, focusing on size distribution and dispersion stability. Surface charge under specific conditions and pH was assessed as zeta potential by electrophoretic mobility testing. In order to maintain the degree of agglomeration as low as possible, suitable protocols for handling the stock dispersions and performing the dilutions needed for *in vitro* studies were provided in the form of a SOP for NC samples handling (see Annex B).

2.1.3. High throughput toxicity testing on Tier 1 NCs

This sub-task was performed by ANSES on all Tier 1 samples, with selected confirmatory analyses performed at ISS. The responses of both intestinal (undifferentiated Caco-2 cells) and macrophage (differentiated THP-1 cells) models were investigated using High Content Analysis (HCA) as a preliminary screening tool to generate a maximum amount of information on the cytotoxic responses of cells to the investigated panel of NC materials. HCA is based on automated image analysis and fluorescence quantification in discrete cellular compartments and generates a large quantity of multiparametric cellular data at the single cell level. The use of the HCA

screening approach in Tier 1 facilitated informed selection of NC materials to be further investigated in Tier 2 studies. A wide range of concentrations was tested for each NC (0.4 to 120 µg/mL, 24-h exposure). Images acquired through automated imaging were analysed by specialized algorithms aimed at quantifying fluorescence for each marker in various cellular compartments at the single cell level. Several endpoints (i.e. cytotoxicity, markers of apoptosis, pro-inflammatory effects, DNA damage, and oxidative stress) were assessed with measurements performed using fluorescent probes or fluorescent immunostaining. The experiments were performed twice and, in case of discrepancy, a third experiment was performed. NC toxicity testing included a systematic checking of all possible interferences on the test systems and read-out methods (EFSA Scientific Committee, 2021) as described in several publications (Comfort et al., 2011; Andraos et al., 2020) (see Annex F).

Positive results (i.e. when effects were detected) were confirmed by ISS using classical approaches that, contrary to HCA, provide a response for the whole cell culture, such as release of cytokines in cell medium for inflammation (by ELISA).

2.1.4. Digestion or degradation of nanocellulose by the human microbiome

In Tier 2, a well-characterized dynamic *in vitro* model of the human colon, ARCOL (ARtificial COLon), developed at INRAE by MEDIS, was used to assess potential NC degradation by human microbiome, under physiologically relevant human colonic conditions (Deschamps et al, 2020; Verdier et al 2021; Fournier et al 2022) (Annex J). The main biotic and abiotic parameters of the human colon are reproduced in ARCOL, such as temperature, pH, transit time, nutrient availability from ileal effluents, presence of complex and metabolically active microbiota of human origin and anaerobiosis maintained by the sole activity of resident microbes (no flushing by N_2 or CO_2).

The model was set-up to reproduce the colon of a healthy human adult and inoculated with fresh faecal samples from adult volunteers. Following a 1-day *in vitro* amplification period of the gut microbiota, a physiologically relevant dose of NC was inoculated into the bioreactors for a total exposure time of 10 days. Experiments were performed in triplicate with the faecal samples from three different healthy donors (two females, one male), to take into account inter-individual variability in gut microbiota. Samples were collected daily throughout the fermentation process from the fermentation medium.

Samples were taken also from the fermentation medium and the atmospheric phase to measure fermentation gases and other major end-products of gut microbiota fermentation activities, such as short chain fatty acids. This was necessary for the quality control of the fermentation process, but also to investigate whether NC had any effect on gut microbiome activities. Total DNA from initial faecal samples, at the end of the stabilisation phase and after NC treatment in the colonic luminal compartment, i.e. inside the bioreactor) were extracted, quality controlled and analysed by Metabarcoding (Illumina 16S rRNA sequencing). This analysis allowed to assess whether NC exposure affected microbiota composition, i.e., whether it induced microbial perturbations.

In order to assess possible modifications of NC by gut microbiota, colonic luminal samples collected at the end of stabilisation phase (negative control) and at the end of the treatment with NCs were submitted to physicochemical characterisation (see Section 2.1.5). In addition,

to test whether NC microbiome-induced modifications are associated to any change in toxicity, the same samples were investigated as detailed in Section 2.1.12. Before resorting to the ARCOL model, preliminary experiments were conducted in simpler batch fermentation models in order to optimise the physicochemical characterisation of the NC fibres and crystals in complex samples.

2.1.5. Tier 2 physicochemical characterisation

The two materials selected for full Tier 2 testing were characterised in terms of number-based size distributions of constituent particles by quantitative TEM analysis at Sciensano (ANNEX Q). The distribution of aspect ratios (shape descriptor) was also characterised.

Colonic luminal samples collected as described in Section 2.1.4 were examined at Sciensano to investigate the extent of digestion or degradation of NC (if any) by the human microbiome. The number-based size (length and width) and shape distributions of each pristine and treated (i.e. undergone colonic fermentation) material were assessed using state-of-the-art TEM-based methodologies.

Internalisation of NC in the human intestinal epithelia as well as NC translocation across the same was studied by CLSM (see Section 2.1.6). To visualize the localisation of individual NC fibres/crystals at the ultrastructural level (within lysosomes) CLSM and TEM imaging in ultrathin sections were used.

2.1.6. Fluorescence detection

Fluorescence staining/labelling to facilitate detection of NC (Bitounis et al., 2019) is essential to assess uptake by intestinal cells and crossing of the intestinal barrier via CLSM. Fluorescent labelling is prone to alter the properties of NC fibres and crystals, affecting the cellular uptake (EFSA Scientific Committee, 2021[5]; Snipstad et al., 2017). Thus, fluorescent staining was used for all the NC materials submitted to the uptake and crossing studies (Tier-2 materials) at ISS. Two alternative methods – namely based on (i) Calcofluor white (CFW) or (ii) Protein with a Carbohydrate Binding Module and N-terminal Green Fluorescent Protein (GFP-CBM) – were used and compared in terms of selectivity and sensitivity (Annex N).

2.1.7. Uptake in Caco-2 and THP-1 cell monocultures

Tier 2 NC materials were submitted at ISS to experiments on uptake in differentiated Caco-2 cells and macrophage differentiated THP-1 cells, used as first screening models to assess cell uptake. Cells were exposed for 24 h and 72 h at a concentration of 30 µg/mL on the 20th day of culture. Fluorescence detection and CLSM imaging were used, as detailed in Section 2.1.6.

2.1.8. Uptake in the triculture model

The protocol for the triculture model was developed following the latest advances (including OECD and EU projects-related developments) and led to the establishment of a specific SOP (Annex O). The co-cultures of Caco-2 and HT29-MTX intestinal cells with Raji B cells were carried out on inserts with a pore size of 3 µm (Figure 2). Uptake studies of Tier 2 NC materials with

[5] See section 7.6.2.

this triculture model were performed at ISS and replicated at JRC. Monolayer integrity (TEER, LY) was checked both before and after NC exposure. Uptake was evaluated by CLSM (as detailed in Section 2.1.6). Cells were exposed for 24 h and 72 h at a concentration of 30 µg/mL on the 20th day of culture.

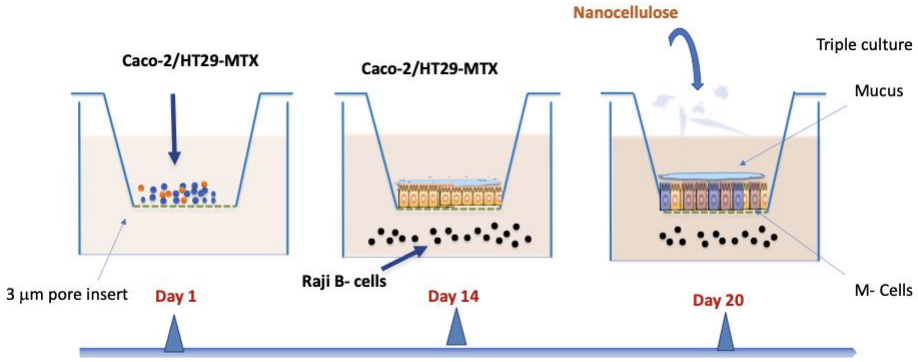

Figure 2: Scheme of the Caco-2/HT29-MTX/Raji-B triculture *in vitro* model.

2.1.9. Barrier function impairment in the triculture model

The triculture model used for the characterization of NC uptake (Section 2.1.8) was used to assess any functional impairment of the intestinal barrier resulting from exposure to Tier-2 materials. Experiments were performed at ISS. Cells were exposed for 24 and 72 h at a concentration of 30 µg/mL on the 21st day. TEER was measured to monitor the cell monolayer integrity and LY translocation to assess paracellular permeability before and after NC exposure.

2.1.10. Genotoxicity of Tier 2 materials

In addition to the data generated by HCA on genotoxicity and oxidative stress (which can be an indirect genotoxicity mechanism), further genotoxicity assays were performed. The comet assay (alkaline version) was used to detect DNA breaks and alkali-labile sites. In addition to the alkaline version, the modified Fpg-comet assay was also performed as it can favour the detection of oxidative DNA lesions due to oxidative species formation. The assay was performed on differentiated THP-1 cells on Tier 2 materials at ANSES. The recommendations provided by the European projects that investigated the genotoxicity of nanomaterials (e.g. RiskGONE) and by the EFSA Guidance on Nano-RA (EFSA Scientific Committee, 2021) were followed.

In addition to the comet assay, the phospho Histone H3 (PH3) test, capable of discerning aneugenic and clastogenic mechanisms of genotoxicity (Kopp et al., 2018), was performed on undifferentiated Caco-2 cells.

In all treatments NC concentrations of 0.3, 3 and 30 µg/mL were used. Each test was performed in duplicate independent experiments and a third one was performed in case of discrepancy.

Interferences with the assays, including the inhibition of the Fpg enzyme in the modified comet assay, were investigated as detailed in the literature (Kain et al., 2012; George et al., 2017).

2.1.11. Impact of Tier 2 materials in mucus production and secretion

As it was shown that mucus may play a role in the response of the intestine exposed to NC, this study investigated the impact of Tier 2 NCs on both production and secretion of mucins. The experiment was performed at ANSES on differentiated HT29-MTX cells with production, followed by gene expression, of key mucins by RT-QPCR and secretion quantified by image analysis on histological section of HT29-MTX cells cultures on inserts. For secretion, acidic and neutral mucins were stained with Alcian blue and periodic acid Schiff respectively. Both mucus area and density were measured as previously done in Reale et al. (2020).

2.1.12. Toxicity of microbiome-digested materials in intestinal models

NC materials from the digestion study with human microbiota (Section 2.1.4) were tested on proliferating Caco-2 cells and differentiated THP-1 cells at ANSES as well as with the triculture model at ISS. The samples (3 replicates) and associated negative controls were diluted in the cell culture medium as appropriate and tested to assess if degradation by gut microbiota influences NC toxicity. The potential toxic effects were investigated by HCA at ANSES as described in Section 2.1.3. In the triple culture model, TEER was measured to monitor the cell monolayer integrity before and after NC exposure.

2.1.13. Tier 3: functional barrier impairment, uptake and crossing in the triculture model using repeated exposure

The NC sample selected for Tier 3 was submitted at ISS to repeated dose testing using the same model and experimental conditions as above (Sections 2.1.8 and 2.1.9), but with cell exposure from the 14th to the 21st day. The culture medium was changed every 48 h, with the NC material freshly dispersed at the selected concentration. At the end of the treatment TEER was measured to monitor the cell monolayer integrity and LY translocation to assess paracellular permeability and compared to pre-exposure values. In addition, uptake and crossing was investigated by fluorescent staining and detection by CLSM (Section 2.1.6). Possible accumulation in the lysosomes was investigated by CLSM and TEM analysis (Section 2.1.5).

2.2. Integrating the results in the regulatory hazard assessments of nanocellulose after oral exposure

The evidence gained in the present project was used to fulfil key data gaps in risk assessment of NC according to the developed IATA.

Use of NC as novel food and as food additive, which are the most likely applications leading to direct exposure of consumers, have been considered in the perspective of translating the results of the present project into regulatory hazard assessment strategies. One request for assessment of a BNC aqueous suspension in view of a Novel Food Authorisation has been already received by EFSA.[6] Regulatory applications for the use of NC as food additive are not yet there (at least

[6] EFSA-Q-2018-00294. Information retrieved via Open EFSA (https://open.efsa.europa.eu/questions).

in the EU), but based on current literature there are ongoing research and development efforts towards such prospective use (Khan et al., 2018; Portela da Gama and Dourado, 2018; DeLoid et al., 2019; Ede et al., 2020; Ong et al., 2020; Brand et al., 2022).

Elements considered for integrating the results in the regulatory hazard assessments of NC after oral exposure were:

- the type of NC used (BNC, NFC, CNC);
- the specific physicochemical properties;
- the NC physical form (solid, liquid suspension);
- the intended use, including the surrounding matrix (e.g. NC used in tablets, i.e. 'pure', *vs* as a bulk in foods containing several other ingredients);
- the use levels and associated exposure scenarios (including likelihood for occasional *vs* daily, long-term exposure).

NC can also be considered as a potential impurity in food products, e.g. as a fraction of the microcellulose or other celluloses used as food additives. Additional considerations regarding this different scenario were elaborated.

Finally, prospective applications of NC as a material to be used in food contact materials (FCMs) and in feed additives were also considered. These uses would only imply a potential for indirect exposure of consumers to NC (e.g. as residue in products of animal origin or following migration from FCMs). In case of use in plastic FCMs, migration of NC into food seems very unlikely. Migration might occur from other types of FCMs, but this scenario is considered too speculative and cannot be meaningfully explored. On the other hand, potential exposure of final consumers following use in feed additives would imply absorption of NC by the producing animals and deposition in tissues or excretion in products (e.g. milk, eggs) subject to human consumption. It is noted that this scenario would be highly dependent on the GIT physiology of the producing animal, also in respect to any ability to possibly digest/degrade cellulosic materials. Since relevant information on this topic are not available, also this scenario was considered too speculative and was not further explored.

3. Results

3.1. Tier 1

3.1.1. Physicochemical characterization of the pristine materials

Dispersion protocols allowing maximal de-agglomeration without altering the size and shape of the constituent particles (i.e., NC fibres and crystals) were developed for each study material (Annex B). The concentration of each study material was optimised in order to obtain a suitable amount of particles on the TEM grid, for the physicochemical characterisation, and relevant exposure conditions for subsequent *in vitro* testing. Because most of the as-supplied NC materials have high viscosity (gel-like appearance), they were sampled using a mass-based approach which allowed to determine the resulting concentration accurately. A concentration of 60 µg/mL, 600 µg/mL and 1.2 mg/mL resulted for CNC, NFC and BNC materials, respectively, in highly concentrated samples suitable for physicochemical characterisation by TEM. An exception was the TOCN material, for which an optimal concentration of 6 µg/mL was determined. Due to the size and weight of the agglomerates of micrometer-sized fibres in the

MCC material, no suitable concentration resulting in a well dispersed material could be determined. Even so, at a concentration of 600 µg/mL, such agglomerates of large fibres and a fraction of nanosized fibres could be observed on the TEM grid.

Visual inspection demonstrated for all materials that sonication reduced agglomeration without damaging the individual fibres, except for the BNC-02-PT material, for which some broken fibres were observed after sonication. When sonication was applied to the MCC material, the agglomerated micrometer-sized cellulose fibres were broken down to single nanometre-sized cellulose fibres, with a shape similar to the CNC materials, and small agglomerates (**Annex C**).

At the optimal dispersion conditions, the zeta potential of the NC materials was determined in double-distilled water (DDW) as an indicator of stability of the dispersion[7]. For all materials, the zeta potential measurements were negative (Annex C). The CNC materials showed the greater (most negative) zeta potential, i.e., between -60 and -54 mV, whereas the NFC and BNC materials displayed a lower (less negative) zeta potential, i.e., between -36 and -26 mV. The zeta-potential of the TOCN material was in between that of the CNC and other NFC materials, which could be explained by its straight rigid structure that deviates significantly from the other NFC materials. The zeta potential of the MCC material was the smallest in absolute value (-20 mV).

A descriptive TEM analysis was realized for all NC materials in line with the relevant EFSA Guidance (EFSA Scientific Committee, 2021) (Annex C). All the eight investigated NC materials were confirmed to be nanomaterials[8] on the basis of the minimum external dimension (i.e., fibre diameter for NFC and BNC materials), with 100% of particles having such dimension <100 nm. The estimated median diameter is in the range 5-9 nm for all materials. A difference was noted between the two BNC materials, one (BNC-02-PT) having a diameter almost twice as large as the other one (BNC-01-AU). The estimated median length is in the range 169-220 nm in the three CNC materials, whereas it is >1 µm for the fibres composing the NFC and the BNC materials. The chemically-modified (TEMPO-oxidized) NFC, i.e., the TOCN, has a median length of 188 nm.

To evaluate the presence of inorganic contaminants, all materials were analysed by energy dispersive X-ray spectroscopy. Samples NCV100-NAL90, CNC-PDC, NG01NC0102, TOCN, Valida S231, and BNC-01-AU showed high purity, whereas in CNF High fines slurry, BNC-02-PT and MCC materials, iron containing particles were detected, which were not mentioned to be present in the sample by the suppliers.

At the JRC, a set of orthogonal nano-analytical techniques was used to generate a dataset of physicochemical properties for each NC material and the MCC comparator (Annex D). A CNC standard reference material was used in these studies. The ultimate goal was to obtain a detailed physicochemical characterisation with the potential to offer an interpretative framework for the subsequent toxicity experiments. The study involved X-ray diffraction analyses (XRD) for the characterization of the crystalline structure, X-ray photoelectron spectroscopy (XPS) and Fourier-transform infrared spectroscopy (FTIR) for the characterization of the functional groups' chemistry and Time-of-Flight Secondary Ions Mass Spectroscopy (ToF-SIMS) for the analysis of

[7] Greater absolute values are associated to a higher tendency of the particles to repel each other, i.e., to not agglomerate.

[8] As considered in EFSA Scientific Committee (2021).

macromolecular chemistry. The NC materials were ranked in order of decreasing crystallinity, as measured by XRD. The BNC materials exhibited the highest crystallinity value (93%), followed by the CNCs (84-87%), MCC (82%) and the NFCs (43-52%). The TOCN material showed the lowest crystallinity value (10%).

Each sample was further grouped by chemical structure affinity analysing ToF-SIMS data using a multivariate approach (principal component analysis, PCA) that separates the samples based on a first component describing the glucose monosaccharide structure and a second component describing oxidized groups. The PC1-PC2 scores plot divides the sample set in 4 groups. The first group (positive scores for PC1 and negative for PC2) includes the BNCs and appears to feature the highest affinity with ideal NC chemistry and the higher proportion of surface oxidized groups (from the glucose rings). Another cluster of samples (negative PC1 and PC2) includes MCC and the two unmodified NFCs (Valida S231 and CNF High fines slurry). These samples show a facets arrangement similar to the previous group but with elements not directly attributable to the NC chemistry. A third group (positive scores for both PC) includes the three CNC materials, even though they show a looser inter-sample clustering. These samples appear to exhibit both high purity in terms of NC chemistry and a certain extent of spurious oxidation and hydrocarbon presence. Finally, a single-element group comprises the TOCN material, exhibiting the same (lower) level of NC chemistry as the other NFC samples and MCC, but also showing an increased extent of spurious oxidation and hydrocarbon presence. It is interesting to note that this grouping described by the PC1-PC2 scores plot of ToF-SIMS data qualitatively reflects the samples differences highlighted independently by XRD and Z-potential analysis.

Functional groups chemistry analysis via XPS and FTIR showed the appearance of COO– functionalities in the TOCN (i.e., catalytic oxidised) sample that are absent in the other materials. The appearance of these residual groups can be ascribed to the interaction of NC hydroxyl functionalities and the catalyst oxygen radical. The resulting disruption of the hydrogen bonds network between the NC chains can be further correlated to the loss of crystalline organization observed via XRD.

3.1.2. Sample handling and dilution in testing media

The degree of dispersion of particles in the stock dispersions and in exposure media is known to be a major factor determining variability of results between replicates, experiments, and laboratories. To limit this variability, Sciensano assessed the influence of different exposure media, applied in the *in vitro* experiments, on the dispersion of NC fibres and crystals. The agglomeration state, stability and particle coating by the media were investigated by TEM.

Based on the optimal concentration in water, the NC materials were dispersed in Gibco Dulbecco's Modified Eagle Medium (DMEM) and Gibco Roswell Park Memorial Institute (RPMI) 1640 Medium with and without applying probe sonication. When the CNC and MCC materials were dispersed in DMEM or RPMI 1640, a higher degree of agglomeration was observed compared to dispersions in distilled water. For the NFC and BNC materials, the degree of agglomeration remained comparable to dispersion in distilled water. Sonication reduced the agglomeration significantly and ensured stable dispersions. However, when the CNF High fines slurry material was dispersed in RPMI 1640, a higher degree of agglomeration was observed than when it was dispersed distilled water or DMEM. Moreover, sonication did not improve the dispersion stability in RPMI 1640 for this material. In all cases, the electron micrographs showed

the deposition of compounds from the media on the fibres or crystals, coating them with a corona (Hadjidemetriou and Kostarelos, 2017).

The impact of addition of foetal bovine serum (FBS) to the media at concentrations of 1%, 5% and 10% was also evaluated, along with the stability of these dispersions over 24 h. In the presence of 10% FBS in the test media, the background of the TEM images was high, making visualization of the NC particles impossible. In the presence of 1% or 5% FBS in the culture media, combined with probe sonication, TEM analysis could still visualise the NC fibres, although the proteins corona reduced contrast in negative staining. The dispersions were shown to remain relatively stable for at least 24 h.

All sample were stored at 4°C during the dispersion tests. To allow sample storage over longer time periods, the impact of sample freezing was examined by freezing the materials at -20°C for at least 24 h, unfreezing and dispersing them using the pre-determined optimal conditions in distilled water for TEM analysis. No morphological alterations of the constituent particles for any of the NC materials after freezing at -20°C were observed, expect for the CNF High fines slurry material which started to precipitate as soon as it was dispersed in water. As indicated by the supplier, this material cannot be frozen.

Based on these results, suitable protocols for handling the stock dispersions and performing the dilutions needed for *in vitro* studies were provided in the form of a SOP for NC samples handling (Annex E). The maximum concentration applicable in *in vitro* studies according to this SOP was 30 µg/mL. Accordingly, the typical concentration range investigated in the experiments was 0.3-30 µg/mL. The specific concentrations used in each test are detailed in the relevant sections hereunder.

3.1.3. Assessment of local effects, including inflammation, on the GI epithelia: high throughput toxicity testing and confirmatory experiments

A series of toxicity endpoints was assessed in undifferentiated intestinal Caco-2 cells and in THP-1 macrophages using an image-based HCA approach (Annex F). HCA is based on automated image analysis and fluorescence quantification in discrete cellular compartments and generates a large quantity of multiparametric cellular data at the single cell level. This multiparametric approach was used in order to quantify a variety of cellular effects in response to a 24 h treatment with a wide range of NC concentrations.

Endpoints of cytotoxicity included direct cell counts, nuclear size and nuclear intensity. Furthermore, markers of apoptosis (active Caspase-3), pro-inflammatory effects (NF-κB, IL-8), DNA damage (γH2AX, pATM S1981), and oxidative stress (DCFDA) were also quantified. For HCA testing, pending the finalisation of the handling SOP, the Nanogenotox dispersion protocol was used and the concentration range tested was 0.4-120 µg/mL.

Positive findings were subjected to replication at ISS using classical approaches that, contrary to HCA, provide a response for the whole cell culture.

Treatment of Caco-2 cells with the eight NC materials had negligible effects on the toxicity endpoints evaluated. Only the NG01NC0102 and Valida S231 materials had effects following a 24 h exposure, and these effects were only observed at the highest concentration tested (120 µg/mL). Both NG01NC0102 and Valida S231 showed only slight effects on cell counts. The small

decrease in cell numbers 24 h after exposure with the NG01NC0102 material was accompanied by slight increases in nuclear intensity, apoptotic markers (active Caspase-3), and markers of DNA damage, only at the highest concentration tested (120 µg/mL). No effects were observed for oxidative stress or pro-inflammatory markers for any material. No effects were observed for MCC with or without sonication.

In contrast to Caco-2 cells, pronounced effects on some toxicity endpoints were observed in PMA (phorbol 12-myristate 13-acetate) differentiated THP-1 cells. TOCN and the nanofibrillated Valida S231 induced cytotoxic effects in THP-1 cells, with significant reductions in cell counts, at the highest concentrations tested. CNC-PDC induced increases in the apoptotic marker active Caspase-3 while interestingly, Valida S231 resulted in decreases in this marker. A slight increase in DNA damage marker γH2AX was observed for the nanofibrillated Valida S231 material at the highest concentration tested. CNC-PDC induced increases in pro-inflammatory markers (NFkB) at low concentrations (≥15 µg/L).

The most remarkable effects observed were associated with IL-8 secretion. Except for NCV100-NAL90 and BNC-01-AU, all NC materials induced significant increases in IL-8 secretion (CNC-PDC>TOCN>BNC-02-PT>CNF>Valida S231>NG01NC0102), in many cases at very low concentrations (i.e. in the range 0.475-3.75 µg/L).

Interestingly, a significant effect of sonication was observed for MCC. A considerable decrease in cell count at high concentrations was observed in THP-1 cells treated with sonicated MCC, whereas no effect was observed for the non-sonicated material. In addition, sonicated MCC induced significant increases in IL-8 secretion at much lower concentrations compared to the non-sonicated material.

Confirmatory experiments were conducted at ISS for the most prominent findings with differentiated THP-1 cells by HCA. IL-8 release in macrophage differentiated THP-1 cell cultures was analysed by ELISA after 24 h treatment with five NC materials dispersed in the culture medium according to the handling SOP. Trends in IL-8 release were in general agreement with those observed by ANSES and the CNC-PDC sample was confirmed to be the one inducing the highest secretion of the cytokine.

In order to rule out any possible interference on IL-8 release, endotoxins were quantified in all the eight NC samples (Annex G). No correlation was found between background endotoxin concentration and IL-8 release measured by ELISA at ANSES on the whole set of test materials.

The Neutral Red Uptake (NRU) and Trypan Blue assays were conducted to confirm the cytotoxicity and viability data obtained by HCA, respectively. The results were again in broad agreement with those obtained at ANSES (Annex H).

3.2. Tier 2

3.2.1. Selection and number-based size characterisation of Tier 2 test materials

CNC-PDC exhibited a marked pro-inflammatory effect (IL-8 release) in PMA differentiated THP-1 cells. Results were independently confirmed at ANSES and ISS, notwithstanding a different protocol for macrophage differentiation of THP-1 cells used in the two laboratories. The two differentiation protocols, compared by TEM evaluation of cell ultrastructure (cytoplasm/nucleus ratio, pseudopodia, vacuoles, polymorphous nuclei), were both found to successfully induce

macrophage differentiation (Annex I). Overall, the evidence on the pro-inflammatory potential of this material was considered robust. From the physicochemical point of view, CNC-PDC is a typical nanocrystalline material (see especially particle size data by TEM and crystallinity index), with a remarkable potential for cell uptake. Taken all together, these features led to the selection of CNC-PDC as a Tier 2 test material.

NFC (Valida S231) induced cytotoxic effects in THP-1 cells, independently confirmed at ANSES and ISS using different methodologies. From the physicochemical point of view, it presents the features of a typical nanofibrillated material and, as CNC-PDC, could be effectively dispersed in testing media and thus properly handled during *in vitro* testing. For all these features, Valida S231 was selected as the second Tier 2 test material.

The two materials were characterised in terms of number-based size distributions of constituent particles by quantitative TEM analysis at Sciensano. CNC-PDC crystals had a median diameter of 7 nm (mean diameter 7 nm) and median length of 173 nm (mean length 176 nm). In terms of shape, the median aspect ratio was 24 (mean 25). The NFC Valida S231 fibres had a median diameter of 5 nm (mean diameter 5 nm) and median length of 2.5 µm (mean length 2.7 µm). The median aspect ratio was 500 (mean 540).

In addition to these two materials to be submitted to full testing, it was then considered that a BNC material was worth investigating in Tier 2. Based on Tier 1 toxicity data, BNC-02-PT was selected. Since complete testing was not possible due to time and resource constraints, this material was submitted to partial testing in Tier 2.

3.2.2. NC digestion/degradation in an *in vitro* model of the human colon

To investigate NC degradation by human microbiome, two sets of experiments have been conducted at INRAE with (i) simple batch assays and (ii) the ARCOL model, respectively (Annex J). In both assays, fresh faecal samples from 3 adult volunteers were used to inoculate either penicillin flasks (for batch assays) or bioreactors (for ARCOL).

The ARCOL model was set up to reproduce the colon of a healthy human adult and was inoculated with fresh faecal samples from the adult volunteers. Two bioreactors were set to test in parallel two NC materials. CNC or NFC were inoculated daily into the bioreactors for a total exposure time of 10 days. Experiments were performed in triplicate with the faecal samples from three different healthy donors, to take into account inter-individual variability in gut microbiota.

The test samples were the NFC material Valida S231 (Sappi) and the CNC material NCV100-NAL90 (CelluForce). Due to time constraints, the experiments were performed before the selection of Tier 2 test samples was finalised and the CNC material used in the colonic fermentation studies differed from the one selected for general testing in Tier 2 (i.e., CNC-PDC). However, considering the similarity of the two CNC materials and the purpose of the colonic fermentation studies, the deviation is considered unimportant.

The tested concentration was 2 g/L. Such concentration was considered relevant in the light of potential human exposure from realistic use levels according to prospective NC food-related applications (DeLoid et al., 2019).

Gut microbiota composition was studied by qPCR and 16S sequencing whereas gut microbiota activity was assessed by gas production and composition and by SCFA composition as indicators

of fermentation activities. Gut microbiota activity was not markedly affected by CNC and NFC treatments, whereas both NC materials did impact gut microbiota β-diversity.[9]

3.2.3. Characterization of NC microbiota modifications via TEM

A descriptive TEM analysis of the physicochemical properties of NC fibres or crystals after fermentation in the ARCOL system to investigate any possible degradation and potential formation of smaller particles was performed (Annex K).

A first set of test samples was produced for the optimisation of the extraction of the NC fibres or crystals from the matrix and the preparation of specimens fit for descriptive TEM analysis. These samples originated from the preliminary experiments conducted in batch fermentation mode. The NC samples were incubated under anaerobiosis with human faecal matter of three different donors during 24 h at a concentration of 2 g/L. Samples were collected immediately after adding the NC fibres (T1) and after 24 h (T24). In addition, control microbial experiments without NC fibres were sampled (T0). The T1 and T24 samples, containing NC at a concentration of 2 g/L, were dispersed in water to obtain a concentration of ~60 µg/mL for the CNC and ~600 µg/mL for NFC, the previously determined optimal concentrations for TEM analysis (handling SOP, Annex E).

The main findings of the descriptive TEM analysis can be summarised as follows:

- No NC fibres were observed in the negative control samples at T0 (initial time point). Other types of fibres, originating from the donor, were observed in the control samples. These could be distinguished from the NCV100-NAL90 crystals and the Valida S231 NFC fibres based on their morphology.
- For all donors, the T1 and T24 samples contained NC fibres. Simple dilution up to concentrations of ~60 µg/mL for the CNC and ~600 µg/mL for NFC worked well to obtain a distribution of NC fibres on the TEM grids, suitable for descriptive TEM analysis.
- No differences were observed between the T1 and T24 samples. The fibres observed in the T24 samples did not show any signs of degradation or digestion. The size and morphology of the NC fibres looked similar to those of the pristine material.

As far as the ARCOL samples are concerned, in order to assess possible modifications of NC by gut microbiota, samples were collected at the end of stabilisation phase before adding NC fibres (day 0) and at the end of the treatment with NCs, at days 9 and 10 (containing NC at a final concentration of 2 g/L). In addition, negative control samples were provided. The samples were dispersed in water to obtain a concentration of ~60 µg/mL for the CNC and ~600 µg/mL for NFC.

The main findings of the descriptive TEM analysis of the ARCOL samples can be summarized as follows:

- No NC fibres were observed in the negative controls for the three donors. Other types of fibres, probably originating from the donor (most likely of dietary origin), were observed in

[9] Bacterial β-diversity based on distance-based redundancy analysis (RDA) excluding the donor effect showed that both CNC and NFC treatments significantly impact gut microbiota structure. Some bacterial populations changed in a similar manner following treatment with CNC or NFC. In particular, the relative abundance of the Acutalibacteraceae and Bacteroidaceae increased in presence of CNC and NFC, whereas the relative abundance of Lachnospiraceae, Oscillospiraceae and Ruminococcaceae increased following CNC treatment.

the control samples. These could be distinguished from the test NC materials based on their morphology.

- No NC fibres were observed in the D1 samples (sampled after stabilisation phase, before NC was added) of the three donors. Other types of fibres, originating from the donors, were observed in the D1 samples. These can be distinguished from the test NC materials based on their morphology.
- For donors 1 and 2, the D9 and D10 samples contained NC fibres. Simple dilution up to concentrations of ~60 µg/mL for the CNC and ~600 µg/mL for NFC worked well to obtain a distribution of NC fibres on the TEM grids, suitable for descriptive TEM analysis. The fibres observed in the D9 and D10 samples did not show any signs of degradation or digestion. When comparing them to the pristine materials, their size and shape looked similar.
- For donor 3, the D9 and D10 samples of the Valida S231 material contained NFC fibres. Simple dilution up to a concentration of ~600 µg/mL worked well to obtain a distribution of NFC fibres on the TEM grids, suitable for descriptive TEM analysis. The fibres observed in the D9 and D10 samples did not show any signs of degradation or digestion. When comparing them to the pristine material, their size and shape looked similar.
- However, the NCV100-NAL90 samples of donor 3 sampled at D9 and D10 did not contain any CNC fibres. To rule out experimental errors, the experiment was replicated at a higher concentration (dilution of 3 times, concentration of ~600 µg/mL). Under these conditions, also no NC crystals were observed. Since crystals were observed in the test sample after 24 h of donor 3, a sampling issue appears likely, although the possibility that the crystals were degraded/digested cannot be ruled out completely.

3.2.4. Fluorescence-detection for studying cell uptake and barrier crossing

In order to assess cell uptake via CLSM, fluorescence staining for NC detection was used and a specific SOP developed (Annex M). Two alternative staining reagents – namely (i) CFW or (ii) GFP-CBM – were used and compared in terms of selectivity (does the reaction occur with all NC types?) and sensitivity (has the staining the ability to detect at least larger fibres/crystals and/or smaller agglomerates?).

Both dyes were able to react with the different NC types (CNC, NFC and BNC) at the test concentration of 30 µg/mL. The signal intensity was high enough to allow detection of larger NC fibres/crystals (based on the length of the maximum external dimension) and smaller agglomerates, i.e. the particles falling within the size detection capability of CLSM, i.e. ≥150 nm. Whereas both reagents enabled sensitive and selective detection of all types of NC investigated in Tier 2, the GFP-CBM dye was preferred when DAPI was used for cellular nuclear staining, since it does not interfere with.

The selected approach for the assessment of NC internalisation suffers from the limitations of the size detection capability of CLSM. Uptake of the smaller crystals, fibres, and fibre fragments, i.e. the particles with the highest likelihood of being internalised by cells, may remain undetected with the approach used, unless these particles agglomerate intracellularly (e.g. in lysosomes). This is especially true for the CNC material, which is composed by the smallest particles (i.e. nanocrystals). Intracellular localization was studied at a magnification of 11500× with LAMP-1 antibody staining of lysosomes, to confirm that the observed agglomerates were actually localised in the lysosomal compartment (see Section 3.3).

On important advantage of the selected method is that it allowed a quantitative approach to be followed for screening NC internalisation. In detail, uptake of NC crystals (CNC) or fibres (NFC, BNC) was measured as percentage of NC-containing cells on the total number of counted cells in ten different frames at low (630x) magnification.[10] A specific SOP was developed for this purpose (Annex M).

Whereas with this methodology a quantitative estimation of the proportion of cells involved in the uptake process could be made, it has to be recognised that the low magnification used may have missed the presence of smaller particles/agglomerates in some cells (i.e. they may have wrongly assigned them to the 'no uptake' group). Therefore, the cell uptake measurements presented below have to be considered as underestimates of the true cell uptake. It has also to be highlighted that such measurements should not be misinterpreted as cell uptake expressed on a mass basis (i.e. as a fraction of the delivered 'dose').

In addition to low-magnification imaging, cellular uptake was characterized by acquiring not less than 8 images with positive identification of NC crystals or fibres (or their agglomerates) at the highest magnification (13000×), which allows detecting particles at the highest possible resolution.

The selected staining approach was also successfully applied to the assessment of barrier crossing and allowed the visualization of NC translocation into the basolateral compartment in the experiment with the triculture intestinal model under repeated exposure conditions (see Section 3.3).

3.2.5. Uptake in Caco-2 and THP-1 cell monocultures

NC uptake was investigated in Caco-2 and macrophage differentiated THP-1 monocultures treated at the test concentration of 30 µg/mL (Annex N). Cells were exposed for 24 h and 72 h and the uptake evaluated by CLSM as detailed in Section 3.2.4.

In Caco-2 monocultures, after 24 h treatment on the 20th day, for all the selected Tier 2 materials, the majority of the detected particles were layered on the cellular surface. However, all the three materials were taken up by Caco-2 cells. Internalisation rate was between 1.4% and 2.4%. After 72 h NC treatment, the internalisation rate remained substantially unchanged for Valida S231 and BNC-02-PT, whereas a noticeable increase (8.7 %) was observed for CNC-PDC.

In macrophage PMA-differentiated THP-1 monocultures a massive uptake of Tier 2 NCs was observed. After 24 h treatment with NCs, CNC-PDC and Valida S231 were internalized at 100%, whereas BNC-02-PT was internalised at 60%. At 72 h, BNC-02-PT internalisation increased to 90%.

3.2.6. Uptake with the triculture model

NC uptake was investigated in the triculture model following exposure at the test concentration of 30 µg/mL (Annex N). Cells were exposed for 24 h and 72 h on the 21st day and the uptake evaluated by CLSM as detailed in Section 3.2.4.

[10] Given the counting approach behind these measurements, the associated uncertainty is considered high, i.e. most likely in the range 20-30%.

Most of the detected particles were observed on the upper surface of the cellular layers. Internalisation rate was in the range 0.9%-3.9% at 24h and increased to 3.3%-4.7% at 72 h. It is worth noting that cell uptake in the triculture model was overall greater than that observed in the Caco-2 monoculture, which is in line with the improved simulation of the barrier properties of the intestinal epithelium by the triculture model. CNC-PDC also with this model showed the highest cell uptake, even though the relatively low internalisation rate at 72 h as compared to the Caco-2 monoculture highlights that an underestimation has probably occurred. This might be the result of the greater challenge associated to CLSM detection of smaller agglomerated particles in the triculture model. Alternatively, secretion of mucus in the triculture model may have hindered permeation of the smaller CNC crystals to higher degree as compared to other NC types.

The triculture model was successfully transferred to the JRC Nanobiotechnology laboratory. A joint SOP devoted to this model was developed by ISS and JRC (Annex O). Cell uptake measurements carried out at JRC were in excellent agreement with those independently performed at ISS.

3.2.7. Barrier function impairment in the triculture model

The triculture model was used to assess if any functional impairment of the intestinal barrier resulted after exposure to Tier-2 materials (Valida S231, CNC-PDC and BNC-02-PT) at a concentration of 30 µg/mL for 24 and 72 hours (Annex P). TEER measurement and paracellular permeability, assessed by Lucifer Yellow (LY) translocation, were the selected endpoints. Values recorded with differentiated Caco-2 monocultures were also included as functional controls to verify the effectiveness of the M phenotype induction, which is associated to a TEER decrease and an increase in permeability. EGTA (2,5 mM) was used as positive control.

TEER was measured to monitor the cell monolayer integrity, both before and after NCs exposure. TEER values on the 20th day of culture before NC treatment (T0) were identical for the different inserts within the experimental error (including biological variability). At 24 h after NC treatment (T24), a marked TEER decrease was detected for all NCs with respect to the negative control (effect size 21-31%, statistically significant decrease for Valida S231 and CNC-PDC). A recovery in the cell monolayer integrity is seen at T72, even though this is only partial in the case of CNC-PDC.

Paracellular permeability, assessed by LY translocation in the basolateral compartment of the triple cultures, mirrored the results obtained for cell monolayer integrity (as measured by TEER) after 24 h exposure to NC. Permeability increases were in the range 33-36%. After 72 h, permeability recovered partially but not completely for Valida S231, whereas a decrease in permeability as compared to control was found for CNC-PDC and BNC-02-PT.

3.2.8. Genotoxicity of Tier 2 materials

The genotoxicity of Tier 2 materials was tested at ANSES with classical and Fpg-modified comet assays in differentiated THP-1 cells. In addition, the phospho Histone H3 S10 (PH3) test, capable of accurately predicting aneugenic mechanisms of genotoxicity, was performed on proliferating Caco-2 cells (Annex T).

A 24 h treatment of differentiated THP-1 cells with the Tier 2 materials at concentrations of 0.3, 3, and 30 µg/mL had no effect in the classical or Fpg-modified comet assays. In addition, no

modifications of PH3 staining were observed in Caco-2 cells. No cytotoxic effects were observed at any concentration as well. Taken together, these data indicate that Tier 2 NC materials do not exert clastogenic or aneugenic effects in *in vitro* intestinal and macrophage models.

3.2.9. Impact of Tier 2 materials on mucus production and secretion

Differentiated HT29-MTX cells treated at ANSES for 24 h with Tier 2 NC materials at concentrations of 0.3, 3, and 30 µg/mL were analysed for mucin production and secretion through direct quantification of mucins by PAS and AB staining, as well as quantification of the expression of genes associated with mucin production by qRT-PCR (Annex T).

Analysis of mucin staining in HT29-MTX cells demonstrated no effects on mucin content following exposure to Tier 2 NC materials. However, while no effect was observed through direct quantification of mucins, a significant increase in the expression of MUC3A was observed in HT29-MTX cells treated with CNC-PDC and Valida S231, but not BNC-02-PT.

3.2.10. Toxicity of microbiome-digested materials in intestinal models

HCA of proliferating Caco-2 cells and differentiated THP-1 treated with microbiome-digested NC materials was performed at ANSES (ANNEX L). Cells were exposed 24 h to dilutions of pooled samples from days 8, 9 and 10 of the ARCOL digestion.

For non-differentiated Caco-2 cells, endpoints of cytotoxicity included direct cell counts, nuclear size and nuclear intensity. Furthermore, markers of apoptosis (active Caspase-3), pro-inflammatory effects (NF-κB, IL-8), DNA damage (γH2AX, pATM S1981) were quantified. In Caco-2 cells treated with digested NC materials, only slight effects on cell counts and nuclear intensity were observed at the highest concentration tested. However, such slight cytotoxic effects were observed in control samples in the absence of NC, suggesting that the digestion media alone induced them. Slight increases in the apoptotic marker active Caspase-3 and the DNA damage marker γH2AX were also observed for samples of digested NC from all donors. However, similar increases were also observed in control samples in the absence of NC, suggesting that they were likely due to the digestion media alone. No changes in responses for phospho ATM or NFκB were observed in Caco-2 cells treated with digested NC materials. Results on IL-8 secretion were inconclusive due to bacterial contamination.

As far as THP-1 cells are concerned, preliminary experiments indicated that the control media alone (without NC materials) had significant cytotoxic effects in THP-1 cells and higher dilution had to be applied consequently. Therefore, HCA experiments in THP-1 cells were conducted with 7.5 µg/mL as the highest concentration. Nevertheless, significant toxicity was observed with both control samples and digested materials already at very low concentrations. In addition, very high levels of IL-8 secretion were observed both with control samples and digested materials at the lowest concentration. Since these toxic effects were due to the digestion media alone and were unrelated to the presence of NC, no further experiments were performed in THP-1 cells. Results are thus inconclusive.

In conclusion, either no effects were seen, or inconclusive results were obtained both for THP-1 and Caco-2 cells, the latter due to effects observed with control samples in the absence of NC.

The triculture model was used at ISS to assess if any functional impairment of the intestinal barrier resulted after exposure to ARCOL digested-NFC (pooled samples from days 8, 9 and 10)

at a concentration of 30 µg/mL for 24 hours (Annex L). Cell monolayer integrity, monitored by TEER measurement, was assessed both before and after NCs exposure. However, similarly to HCA, also in this case results were inconclusive due to effects caused by the digestion media alone (controls).

3.3. Tier 3

CNC-PDC was selected as Tier 3 material to investigate whether exposure to this NC may lead to barrier functional impairment in the intestinal triculture model after repeated exposure (Annex P). At ISS, differentiated cells were exposed in the apical (AP) compartment to 30 µg/mL CNC-PDC every 48 h from the 14th until the 21st day of culture in parallel to Raji B induction in the basolateral (BL) compartment. EGTA (2,5 mM) was used as positive control in the last 48 h. In addition, uptake and crossing were investigated by fluorescent staining and detection by CLSM.

CNC-treated tricultures showed a significant decrease in TEER compared to the negative control. The effect size was almost identical to that observed after 24 h from a single exposure to CNC-PDC (24% vs 27%). In agreement with TEER data and with the results obtained with single exposure, LY translocation increased compared to negative triculture controls, although the effect size was smaller than that observed with single exposure (14% *vs* 34%).

Confocal images revealed a 30% of CNC-PDC uptake measured as percentage of NC-containing cells on the total number of counted cells. This represents a substantial uptake, taking into account the considerations (see Section 3.2.4) suggesting that, especially for CNC, the selected detection approach might have led to an underestimation of true cell internalisation. Remarkably, this represents a 10-fold increase compared to single exposure (Section 3.2.6) and indicates potential for CNC accumulation. Localization of CNC in the lysosomes was also demonstrated by CLSM at ISS, whereas TEM analyses at Sciensano were unsuccessful owing to methodological issues.

In order to check potential CNC-PDC translocation across the model barrier, BL media after exposure to CNC-PDC were treated with CFW. Clear evidence of CNC-PDC crossing was obtained, whereas control BL media gave negative results. Whereas the method is by definition qualitative, CLSM images indicate that CNC-PDC crossing was substantial. The smallest detected CNC-PDC agglomerates were in the size range of ca. 200-400 nm.

4. Discussion

In line with the EFSA SC Guidance on Nano - Risk Assessment (EFSA Scientific Committee, 2021), the present project addressed the main methodological shortcomings identified in most of the existing *in vitro* studies on NC possible adverse effects as a result of oral exposure, in particular:

- Absent or insufficient physicochemical characterisation
- Absence of suitable dispersion protocols
- Use of extremely high concentrations (>100 µg/mL)
- No confirmation of cellular exposure

Experimental studies were performed in a way to ensure that the results are relevant and reliable in the perspective of their use for regulatory risk assessment. This included: (i) ensuring a high

level of standardisation (establishment of SOPs or internal validation protocols for all experimental procedures); (ii) obtaining results for key NCs under different experimental conditions (e.g. single *vs* repeated exposure); (iii) achieving reproducibility of results in terms of adequate number of experimental replicates and replication of key experiments in different laboratories. The development of best practices and the standardisation of methodology was one constant element of the project. These efforts aimed at increasing inter-comparability of results, decreasing uncertainty and increasing the regulatory relevance of the obtained data.

The physicochemical characterisation of the eight investigated NC materials confirmed they are nanomaterials on the basis of the minimum external dimension (i.e. fibre diameter for NFC and BNC materials), with 100% of particles having such dimension <100 nm. The estimated median diameter is in the range 5-9 nm for all materials. The estimated median length is in the range 169-220 nm in the three CNC materials, whereas it is >1 µm for the fibres composing the NFC and the BNC materials. The chemically-modified (TEMPO-oxidized) NFC, i.e. the TOCN, has a median length of 188 nm. A clear trend was observed for crystallinity: it was highest in the BCN materials (93%), followed by the CNC materials (84-87%), whereas it was the lowest for the NFC materials (43-52%). Chemical modifications in the TOCN material led to the loss of crystalline organization and to a very low crystallinity value (10%). The MCC comparator in the micro-range exhibited an intermediate-to-high crystallinity (82%). In this material, upon sonication, the agglomerated micrometer-sized cellulose fibres were broken down to single nanometre-sized cellulose fibres (already present beforehand as impurities), with a shape similar to the CNC materials, and small agglomerates.

A detailed SOP for sample handling and dilution in testing media before *in vitro* testing was developed. The maximum concentration applicable in *in vitro* studies according to this SOP was 30 µg/mL. It was considered that exceeding this concentration, which is lower than the 100 µg/mL mentioned in the EFSA SC Guidance on Nano - Risk Assessment for nanomaterials in general, would not be relevant from the biological and toxicological point of view, taking into account the carbonaceous nature of NC and the fact that the resulting particle number concentrations are most likely comparable to those achieved at 100 µg/mL by inorganic nanomaterials (such as metal- and oxide-based particles)[11].

In Tier 1, HCA indicated that the eight NC materials had negligible effects on the toxicity endpoints evaluated in undifferentiated Caco-2 cells. Pronounced effects on some toxicity endpoints were observed in macrophage differentiated THP-1 cells. TOCN and the nanofibrillated Valida S231 induced cytotoxic effects in THP-1 cells. CNC-PDC induced increases in the apoptotic marker active Caspase-3 (on the contrary, Valida S231 resulted in decreases in this marker). CNC-PDC induced increases in pro-inflammatory markers at low concentrations. The most remarkable effects observed were associated with IL-8 secretion. With the exception of NCV100-NAL90 and BNC-01-AU, all NC materials induced significant increases in IL-8 secretion according to the following order: CNC-PDC>TOCN>BNC-02-PT>CNF>Valida S231> NG01NC0102. These results on differentiated THP-1 cells were confirmed in a different laboratory using a cell culture approach.

A significant effect of sonication was observed for MCC. A considerable decrease in cell count at high concentrations was observed in THP-1 cells treated with sonicated MCC, whereas no effect

[11] This consideration is also relevant for other carbon-based nanomaterials with low density.

was observed for the non-sonicated material. Sonicated MCC induced significant increases in IL-8 secretion at much lower concentrations compared to the non-sonicated material.

In Tier 2, CNC-PDC and Valida S231 were submitted to complete testing and BNC-02-PT to partial testing. In the studies on colonic NC digestion/degradation by human microbiome, CNC-PDC was replaced with NCV100-NAL90, an almost identical CNC material. These studies, performed in a preliminary way with batch assays and then with the ARCOL model, an *in vitro* model of the human colon, showed that gut microbiota activity was not markedly affected exposure to CNC and Valida S231, whereas both NC materials did impact gut microbiota β-diversity. Characterisation via TEM of the NC fibres or crystals submitted to fermentation in the ARCOL system did not show any signs of degradation or digestion. Inconclusive results were obtained with one donor. Toxicity studies with these microbiome-digested materials showed either absence of effects or inconclusive results (the latter due to methodological issues. i.e. effects observed with control samples in the absence of NC) in all the cell models used.

A quantitative approach for cell uptake measurements was developed based on fluorescence staining with two alternative and equally effective dyes (CFW and GFP-CBM) and determination of the percentage of NC-containing cells on the total number of counted cells in ten different frames at low (630x) magnification. This approach had the advantage to be quantitative and the limitation of detecting only relatively large agglomerates, i.e., of providing underestimates of the true cell uptake. It was considered the most suitable approach for screening purposes, with the option to resort to higher magnification in case of negative results. As a matter a fact, positive results (i.e., measurable cell uptake levels) were obtained for all the materials tested in the different cell lines. In Caco-2 monocultures, after 24 h treatment, internalization of the three selected Tier 2 materials was between 1.4% and 2.4%. After 72 h, the internalization rate remained substantially unchanged for Valida S231 and BNC-02-PT and increased to 8.7% for CNC-PDC. In macrophage -differentiated THP-1 monocultures a massive uptake of Tier 2 NCs was observed. After 24 h treatment with NCs, CNC-PDC and Valida S231 were internalized at 100%, whereas BNC-02-PT was internalized at 60%. At 72 h, BNC-02-PT internalization increased to 90%.

In the triculture model, Internalization rate was in the range 0.9%-3.9% at 24 h and increased to 3.3%-4.7% at 72 h. Cell uptake in the triculture model was overall greater than that observed in the Caco-2 monoculture, which is in line with the improved simulation of the barrier properties of the intestinal epithelium by the model. CNC-PDC also with the triculture model showed the highest cell uptake. Cell uptake measurements were independently carried out in another laboratory and results were found to be in excellent agreement.

The triculture model was used to assess if any functional impairment of the intestinal barrier resulted after exposure to Tier-2 materials. TEER measurement showed that cell monolayer integrity was compromised at 24 h after NC treatment. A recovery was seen at 72 h, even though this was only partial in the case of CNC. Paracellular permeability at 24 h, assessed by LY translocation, increased after NC exposure mirroring the results obtained by TEER measurements.

Genotoxicity studies with Tier 2 materials in differentiated THP-1 cells and in proliferating Caco-2 cells indicated that Tier 2 NC materials do not exert clastogenic or aneugenic effects in the two selected *in vitro* intestinal and macrophage models. No impact of Tier 2 materials on mucus

production and secretion after 24 h exposure of differentiated HT29-MTX cells was detected via direct quantification of mucins following exposure to Tier 2 NC materials. However, a significant increase in the expression of MUC3A was observed with CNC-PDC and Valida S231, whereas no effect was seen with BNC-02-PT.

CNC-PDC was selected as Tier 3 material to investigate whether exposure to this material may lead to barrier functional impairment in the intestinal triculture model after repeated exposure. Differentiated cells were exposed in the apical compartment to 30 µg/mL NC from the 14th day until the 21st of culture (in parallel to Raji B induction in the basolateral compartment). CNC-treated tricultures showed a significant decrease in TEER; the effect size was almost identical to that observed after 24 h from a single exposure to CNC (24% vs 27%). LY translocation in the basolateral compartment of the triculture increased, although the effect size was smaller than that observed with single exposure (14% vs 34%). CNC-PDC uptake rate after repeated exposure was 30%, with a 10-fold increase compared to single exposure and indicated potential for CNC accumulation. CNC crystals were shown to be localized in lysosomes. As expected on the basis of the detected cell uptake, CNC was shown to cross the intestinal model barrier.

5. Assessment

The findings of the present study provided evidence for addressing some key knowledge gaps in the assessment of the hazards potentially associated to NC oral exposure. The NAM-based IATA focused on (i) the assessment of the uptake and potential crossing of the intestinal barrier by NC; (ii) the assessment of local effects, including inflammation and genotoxicity, of NC on the gastrointestinal epithelia; (iii) the assessment of any digestion or degradation of NC by the human microbiome.

With regard to the first question, cell uptake was demonstrated with intestinal models for all the three materials investigated, belonging to the three main NC types (NFC, CNC and BNC). Cell uptake was greater in the triculture model, which better simulates the barrier properties of the human intestinal epithelium as compared to Caco-2 monolayers, and was the greatest in repeated exposure conditions. Under these conditions, intestinal barrier crossing was demonstrated. Based on the detected cell uptake for all the NC materials investigated, NC translocation across the intestinal barrier can generally be expected to occur.

As far as the second question is concerned, the results obtained confirmed indications in the literature with regard to inflammatory responses indicative for immunotoxicological effects (Stoudmann et al., 2020, Ventura et al., 2020; Brand et al., 2022). In the present study, increased cytokine production was supported by the massive NC uptake in macrophages. This highlights the immunotoxic potential of NCs and especially CNC. In addition, indications of barrier function impairment for intestinal epithelia exposed to NC consistently emerged. On the contrary, no indications for genotoxicity were obtained.

With regard to the third question, although with a considerable degree of uncertainty, no indications of digestion or degradation of NC by the human microbiome itwith formation of smaller NC particles were obtained. Toxicity tests with NC undergone colonic fermentation were largely inconclusive due to methodological issues, but when this did not happen results were negative (i.e. no effects were detected).

The integration of these results in regulatory hazard assessment of NC after oral exposure requires consideration of specific human exposure scenarios. Use of NC as novel food (ingredient) or as food additive appears relevant, as these are the most likely applications leading to direct exposure of consumers (Brand et al., 2022).

In this regard, one first element to be considered is whether markedly diverging behaviours can be expected for the three types of NC materials (i.e. BNC, NFC, and CNC). CNC consists in rod-shaped crystals, which are generally 50–350 nm long (Brand et al., 2022); the median length of the materials investigated in the present study was <250 nm. On the contrary, NFC consists in fibrils composed of fibres with a length up to 2-3 µm; nanofibers are even longer in BNC and organized in networks. For all the NC types, the diameter can be very small (<10 nm for the eight materials investigated here). In the present study, consistent differences were observed in other physicochemical parameters (e.g. see crystallinity). Notwithstanding these differences, in the present research no clear boundaries could be identified in terms of cell uptake and adverse effects, albeit CNC was confirmed as the material type of higher concern. Also, differences in toxic responses were noted within each type of materials, highlighting that complex relationships exist between physicochemical and (adverse) biological effects.

The variability in toxic responses within each class of NC material observed in this study may have consequences for read across. Unless physicochemical determinants of such variability are clearly identified and well understood, options for read across might be limited.

The NC physical form (solid, liquid suspension) is another relevant aspect to be considered in terms of human exposure scenarios via the diet. NC is commonly commercialised as a liquid suspension, which turns into gels or flexible aerogels at higher concentrations. It is also found in sheet form or as powders. BNC-01-AU (Nata de coco) used in this study was provided as dry sheets and was dispersed in the laboratory following the instructions of the manufacturer, i.e. similarly to industry practices. It is conceivable that in many applications NC is uniformly dispersed in the final product for human consumption as a liquid suspension. Direct ingestion of NC dispersed in a liquid (as it happens, e.g., in nata de coco beverages) is also possible.

When NC is incorporated in a food matrix, this is not expected to change its fate of indigestible nanofibre that reaches the small intestine essentially intact. However, the presence of a matrix may stabilise the fibres (or crystals) and favour their deagglomeration in the small intestinal tract. The opposite might happen if NC is used as the sole (or main component), e.g., in tablets. Insufficient information exists in this respect and consideration of worst-case scenarios in terms of particle agglomeration is warranted in risk assessment (EFSA Scientific Committee, 2021; Schoonjans et al., 2023).

In terms of use levels, compared to their non-nanosized food-grade cellulose counterparts, NC materials, given the greater specific surface areas and the higher viscosities, are not expected to be used in suspensions greater than 1.0% by weight; reported efficacy in improving properties of foams, emulsions, and solid food products is reached at concentrations ranging from 0.2 to 1.0% by weight (DeLoid et al., 2019). Based on this, in the present study, the concentration used in the microbiome digestion experiment was based on a worst-case calculation of a single meal with 200 g of a food item where NC is present at 1% by weight (i.e. 2 g). The considered volume of GIT secretions was 2 L for adults and 1 L for infants and children for ingestion related

to one instant (e.g. one meal). This led to a concentration of 1 g/L for adults and 2 g/L for infants and children; the latter concentration was used in the ARCOL experiment.

NC can also be considered as a potential nanosized 'impurity' in food products, e.g. as a fraction of the microcellulose or other celluloses used as food additives. In the present study, the MCC material was found to contain a fraction of single nanometre-sized cellulose fibres. Upon sonication, the agglomerated micrometer-sized cellulose fibres were broken down to such nanofibres, with a shape similar to the CNC materials, and small agglomerates. Whether a similar process may at least partially happen during human GI digestion is a relevant question to be addressed in future studies, also considering that adverse effects were observed with the sonicated material. All this considered, investigating the presence of a nanofraction in food-grade celluloses appears advisable.

Assessment of potential risks associated to chronic exposure to NC from different sources (e.g. direct use in various applications, presence as impurity in other food-grade celluloses) requires all the above considerations to be taken into account. NC is a non-digestible carbonaceous nanomaterial for which, based on the results obtained in this project, a potential for biopersistence and bioaccumulation cannot be excluded upon chronic exposure. The indications for adverse effects described above highlight that further studies are warranted.

6. Conclusions and recommendations

The EFSA SC Guidance on Nano - Risk Assessment recommends the development of NAM-based IATAs for covering the nano-specific considerations based on mechanistic understanding of processes at the nanoscale (EFSA Scientific Committee, 2021). Human-relevant NAMs appear as the best option to generate the needed information for the hazard assessment of NC oral exposure, also considering the limitations of laboratory animals as models because of the differences with humans in digestive physiology, microbiome and rate of fibre degradation. The variability in the physicochemical properties of NC and the fact that the needed studies are technically easier to implement using *in vitro* methods than *in vivo* studies (especially the investigation of intestinal uptake and crossing) make NAMs ideal for efficient testing. NAM-based data can be then integrated with animal (and, when existing, human) data according to the relevant IATA. Based on the available information, current knowledge gaps, and expectations from the nanoscale properties of the different NC forms, a NAM-based IATA for addressing data gaps in the assessment of hazards associated to NC oral exposure and for getting mechanistic understanding was identified.

The assessment of the uptake and potential crossing of the intestinal barrier by NC was the first element considered. The *in vitro* studies performed with different intestinal models demonstrated cell uptake for all the three materials selected for in depth-testing after a first tier of high throughput toxicity testing and physicochemical characterisation, conducted on eight NC materials and a micro-sized comparator. These three materials belonged to the three main NC types (NFC, CNC and BNC). Cell uptake was greater in the triculture model, which better simulates the barrier properties of the human intestinal epithelium as compared to Caco-2 monolayers and was the greatest in repeated exposure conditions. Under these conditions, intestinal barrier crossing was demonstrated for CNC. Based on the detected cell uptake for all the NC materials investigated, NC translocation across the intestinal barrier can generally be expected to occur.

The assessment of potential local effects of NC on the gastrointestinal epithelia, including inflammation and genotoxicity, was the second element considered. The results obtained confirmed indications in the literature with regard to inflammatory responses indicative for immunotoxicological effects. In the present study, increased cytokine production was supported by massive NC uptake in macrophages. This highlights the immunotoxic potential of NCs and especially CNC. In addition, indications of barrier function impairment for intestinal epithelia exposed to NC consistently emerged. On the contrary, no indications for genotoxicity were obtained.

The assessment of possible digestion or degradation of NC by the human microbiome, with formation of (potentially more toxic) smaller particles was the third element considered. Although with a considerable degree of uncertainty, no indications of colonic digestion or degradation of NC with formation of smaller NC particles were obtained. Toxicity tests with NC undergone colonic fermentation also did not support the tested hypothesis.

Integration of the evidence obtained in this research with existing *in vivo* data is possible if the limitations of, e.g., the existing subchronic toxicity studies is considered. These studies showed no indications of toxicity for specific NC materials but were conducted at high doses in the absence of suitable dispersion protocols (Ede et al., 2020, Ong et al., 2020). This is not in line the basic principle that the test conditions must ensure worst case scenarios, which means exposure to the most dispersed form (EFSA Scientific Committee, 2021). As suggested by Brand et al. (2022), these studies may have missed certain early or nanospecific toxic effects, such as inflammation potential, for which other, subacute studies provide some indications (DeLoid et al., 2019; Khare et al., 2020).

For the integration of these results in regulatory hazard assessment of NC after oral exposure, prospective use of NC as novel food or as food additive appears relevant, as these are the most likely applications leading to direct exposure of consumers. NC is a non-digestible carbonaceous nanomaterial for which, based on the results obtained, a potential for biopersistence and bioaccumulation cannot be excluded upon chronic exposure.

Notwithstanding in the present study marked differences in several physicochemical parameters were observed among the different NC materials, no clear boundaries could be identified in terms of cell uptake and adverse effects, albeit CNC, composed of smaller particles, was confirmed as the material type of higher concern. Also, differences in toxic responses were noted within each type of materials, highlighting that complex relationships exist between physicochemical and (adverse) biological effects.

The variability in toxic responses within each class of NC material observed in this study may have consequences for read across. Unless physicochemical determinants of such variability are clearly identified and well understood, options for read across might be limited.

All these elements should be considered in future studies on NC hazards as well as in the assessment of the risks associated to NC oral exposure.

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Annexes A-S

- Annex A: Harmonised conditions used in *in vitro* testing
- Annex B1 to B9: Dispersions of NC samples for TEM analysis and for *in vitro* testing
- Annex C: Physicochemical characterization of Tier-1 NC samples via TEM
- Annex D: Physicochemical characterization via XRD, XPS, FTIR and ToF-SIMS
- Annex E: SOP for Tier-1 NC samples handling
- Annex F: High content analysis (HCA) of Tier-1 NC samples
- Annex G: Endotoxin analysis
- Annex H: Confirmatory experiments on differentiated THP-1 cells
- Annex I1 and I2: Assessment of differentiation protocols for THP-1 cells
- Annex J: Effect of NC in an *in vitro* model of the human colon
- Annex K1 to K3: Evaluation of NC degradation by human microbiome using descriptive TEM
- Annex L: Toxicity of microbiome-digested materials in intestinal models
- Annex M: SOP for the evaluation of NC cell uptake and barrier crossing by CLSM
- Annex N: Assessment of uptake and crossing via fluorescence-detection
- Annex O: SOP for the triculture model as applied to the assessment of NC uptake and crossing
- Annex P: Barrier function impairment in the triculture model
- Annex Q: Quantitative TEM analysis of pristine Valida S231 and CNC from UMaine
- Annex R: Morphological features of the triculture model and CNC uptake after repeated exposure via TEM
- Annex S: Genotoxicity and mucus production & secretion.

In the above Annexes, often reference is made to the original project structure in Workpackages, Tasks and Subtasks.

This is summarised hereunder:

Workpackage A: Developing the IATA and designing the NAM-based studies for assessing nanocellulose oral exposure hazards

- Task A.1. Digestion or degradation of nanocellulose by the human microbiome
  - Subtask A.1.1. NC digestion/degradation in an in vitro model of the human colon
  - Subtask A.1.2. Characterization of NC modifications
- Task A.2. Assessment of nanocellulose uptake and crossing in human intestinal epithelia
  - Subtask A.2.1 Sample handling and dilution in testing media: evaluation of the dispersion of NC fibres
  - Subtask A.2.2. Fluorescence-detection of Tier 2 NCs
  - Subtask A.2.3. Uptake in Caco-2 and THP-1 cell monocultures with Tier 2 NCs
  - Subtask A.2.4. Uptake of Tier 2 NCs using a tri-culture model
- Task A.3. Assessment of local effects, including inflammation, of nanocellulose on the gastrointestinal epithelia
  - Subtask A.3.1. High throughput toxicity testing on Tier 1 NCs
  - Subtask A.3.2. Barrier function impairment in tricultures of Caco-2/HT29-MTX/Raji-B cells
  - Subtask A.3.3. Genotoxicity of Tier 2 materials

- Subtask A.3.4. Impact of Tier 2 materials in mucus production and secretion
- Subtask A.3.5. Toxicity of microbiome-digested materials in intestinal models
- Subtask A.3.6. Tier 3: functional barrier impairment, uptake and crossing in the triculture model using repeated exposure

Workpackage B: Selecting test materials and conducting the NAM-based studies for assessing nanocellulose oral exposure hazards

- Task B.1. Selection of the test materials
- Task B.2. Physicochemical characterization and in situ visualization of NC
- Task B.3. Proposal for ensuring that the results can be used in the regulatory context

Workpackage C: Integrating the results in the regulatory hazard assessments of nanocellulose after oral exposure.

Annex T – Raw data

The raw data generated under this Project are uploaded on the Knowledge Junction platform with the following doi: 10.5281/zenodo.8260849