Nano-specific alternative methods in human hazard/safety assessment under different EU regulations, considering the animal testing bans already in place for cosmetics and their ingredients

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Final Report

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Abbreviation list

3Rs	Three Rs
7FP	7th Framework Programme
AOP	Adverse Outcome Pathway
ASCCT	The American Society for Cellular and Computational Toxicology
BfR	German Federal Institute for Risk Assessment
BMD	Benchmark Dose
CLP	Classification, Labelling and Packaging Regulation
CTA	Cell Transformation Assays
DA	Defined Approach
DNT	Developmental Neurotoxicity
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EFSA EU-FORA	The European Food Risk Assessment (EU-FORA) Fellowship Programme
ENMs	Engineered Nanomaterials
ESAC EURL ECVAM	EURL ECVAM Scientific Advisory Committee
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FAIR principles	Findable, Accessible, Interoperable and Reusable principles
GD	Guidance Document
GIT	Gastrointestinal Tact
GIVIMP	Good In Vitro Methods Practice
GLP	Good Laboratory Practice
GSH	Globally Harmonized System of Classification and Labelling of Chemicals
HTS	High Throughput Screening
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IATA	Integrated Approaches to Testing and Assessment
IL-4	Interleukin 4
ITS	Integrated Testing Strategy
ISO	International Standard Organisation
JRC	European Commission's Joint Research Centre
KE	Key Event
NAM	New Approach Methodology
NIA	Nano Industries Association
NGRA	Next Generation Risk Assessment
NOAEL	No Observed Adverse Effect Level
NRU	Neutral Red Uptake
NRR	Neutral Red Release
NSC	NanoSafetyCluster
NT	Neurotoxicity
OECD	Organisation for Economic Co-operation and Development
PATROLS	Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment project
PBPK	Physiologically-based pharmacokinetic model
PETA	People for the Ethical Treatment of Animals
QSAR	Quantitative structure-activity relationship
REACH	Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive Oxygen Species
RDT	Repeated Dose Toxicity
SCCS	Scientific Committee on Consumer Safety
SOP	Standard Operating Procedure
TER	Transcutaneous Electrical Resistance
TG	Test Guideline
Three Rs	Replacement, Reduction and Refinement of animal testing
TOXCAST	EPA's Toxicity Forecaster
TSCA	Toxic Substances Control Act
US EPA	US Environmental Protection Agency
WoE	Weight of Evidence
WPMN	Working Party on Manufactured Nanomaterials
WNT	Working Group of the National Coordinators of the Test Guidelines Programme
WHO	World Health Organization

Abstract

The purpose of the study reported here, and performed at the request of the European Chemicals Agency (ECHA) and the European Union Observatory for Nanomaterials (EUON), was to conduct a comprehensive literature review on nano-specific New Approach Methodologies (NAMsin human safety assessment and to prepare an inventory of the available NAMs identifying the validated ones, the ones currently under validation and the most promising methods to undergo validation for nanomaterials' testing.

The starting point was the listing of the endpoints of regulatory relevance to be considered for assessing the human safety of chemicals under different EU regulations. Then, the publicly available sources of information were reviewed with the aim to identify methods that can serve as useful alternatives to animal testing of these specific toxicological endpoints. The possible modification/adaptation of the collected NAMs for nanomaterials was assessed. Additionally, to enable a broader perspective, the literature search was enriched with the survey results from experts from academia, industry and regulatory affairs.

As a result, 220 NAMs were identified (to date 2022-12-30) and assigned to a category that describes the status of their regulatory acceptance, validation, or development. The current data gaps and needs identified through the literature review and identified by experts in the context of NAMs application to fulfil nanomaterials-specific safety testing requirements, were discussed. Additionally, a tabular overview of all collected NAMs was compiled with respect to regulatory-relevant endpoints. The findings evidenced in this Report can be helpful to industry, academia and other stakeholders who are interested to use nano-specific NAMs of regulatory relevance in the human safety assessment.

Executive summary

Background

At the request of the European Chemicals Agency (ECHA) and the European Union Observatory of Nanomaterials (EUON), QSAR Lab Ltd. has performed a comprehensive review of the New Approach Methodologies (NAMs) used for engineered nanomaterials (ENMs) testing for human safety assessment in accordance with various European Union regulations, taking into account the existing ban on animal testing for cosmetics and their ingredients.

The purpose of this report is to provide stakeholders with accurate and transparent information regarding the NAMs available for the human safety assessment and to create a comprehensive inventory of these methods, identifying the validated ones, the ones currently under validation and the most promising methods to undergo validation for nanomaterials' testing. The increasing use of ENMs in various consumer products necessitates the development of efficient and effective nano-specific approaches for assessing the potential hazards of ENMs to human health. Given the ethical, economic, and time-related drawbacks of animal testing and the aforementioned ban on such testing for cosmetics, it is of the highest priority to develop and validate alternative methods. The main aims of presented study were to:

- conduct a systematic literature review of the currently available, nano-specific NAMs for assessing the safety of nanomaterials;
- conduct surveys of experts for nano-specific NAMs in human safety assessment with regulatory relevance;
- conclude on the remaining gaps, requirements and needs, and propose future directions of NAMs in human safety assessment of nanomaterials, under different EU regulations, considering the animal testing bans already in place for cosmetics and their ingredients.

Methodology

The presented report is based on a two-step strategy aimed at acquiring the necessary information on NAMs for nanomaterials and providing relevant data to enhance the transparency of information regarding nano-specific NAMs in human safety assessment. The strategy is as follows:

1. Literature search, analysis, and critical review: (i) development of a map summarizing crucial toxicological endpoints for chemical safety assessment, including nanomaterials, under various regulations established by the European Union, and (ii) a systematic literature search focused on alternative methods for safety assessment of nanomaterials that have been validated, are currently undergoing validation, or have the greatest potential to enter the validation step.

2. Expert survey: (i) experts in the field of NAMs were selected from three sectors (academia, regulatory agencies, and industry) and invited to participate in the survey; (ii) based on the results of step 1, the survey was designed in collaboration with ECHA to collect information regarding nano-specific NAMs in human safety assessment.

The **literature search** was conducted, using a variety of sources including the most frequently utilized multidisciplinary citation databases and indexing services (such as Web of Science, Scopus, and PubMed), CORDIS Result Packs, EU project websites and databases, OECD Test Guidelines and Guidance Documents, ISO Standards, repositories of NAMs from EURL ECVAM, the EPAA website, and the AOP wiki. Inclusion and exclusion criteria were established and applied during the search process. The relevance of each document to toxicological endpoints of regulatory significance was then assessed. The quality of the publications was evaluated using the GuideNano quality scoring system, which assesses the substance characterization (S-score) and methods performance (K-score) based on provided information to ensure transparency and feasibility of the assessment process. The original intention was to only consider papers with K- and S-scores of 1-2 for further analysis, however, as the majority of papers on NAMs did not meet these criteria, it was decided to consider all collected literature in order to maintain a broader perspective. The gathered documents were reviewed to identify NAMs that could be of the highest relevance to the human safety assessment of ENMs.

The survey experts were chosen from three distinct target groups: academia, industry, and regulatory agencies. This approach allowed to provide a comprehensive outlook on the subject of NAMs. The selection process of relevant experts involved two methods: i) data-mining and text processing techniques were utilized alongside a comprehensive literature search, and ii) selection based on expert knowledge - QSAR Lab by participation in nano-safety-related EU projects and other associations, and its scientific and commercial network was able to identify leading experts in the fields of the nano-specific NAMs. After the completion of the literature search, QSAR Lab, in close collaboration with ECHA/EUON, formulated targeted questionnaires (**Annex 1**) the nano-specific NAMs and their impact on the human safety assessments under various EU regulations. The identified experts were then presented with the surveys and the collected responses were analysed. The opinions of the experts were then used to discuss the gaps and needs in the context of NAMs and their application in the safety assessment of ENMs.

Findings

As a starting point, the map of needs that summarizes the toxicological endpoints essential in terms of the safety assessment of chemicals, including nanomaterials, under different EU regulations, was developed. The most relevant regulations were taken into consideration, including the REACH Regulation, the Biocidal Products Regulation, the Cosmetic Products Regulation, and all regulations pertaining to food and the food chain. The purpose of these regulations is to ensure the safe use of substances used as industrial chemicals, in biocidal products, cosmetics, food, and feed ingredients within the European Union. Consequently, a list of endpoints was established corresponding to the information requirements of each regulation. Guidance documents relevant for the regulations have also been analysed in detail. The development of a map of the hazard

endpoints required for regulatory purposes enabled to determine for which endpoints alternative methods are available and might be considered in further assessments. In order to identify alternative methods for evaluating toxicological endpoints relevant to human safety, including ENMs, the available literature was reviewed (i.e., OECD Test Guidelines and Guidance Documents, ISO standards, ECVAM repositories, SOPs, scientific publications, nano-relevant Adverse Outcome Pathways, EU project deliverables and the OECD Working Plans). The adjustment or modification of existing scientifically-justified and inter-laboratory validated testing strategies may prove more advantageous than developing new methods from scratch. This approach would reduce the time required to develop NAMs dedicated solely to ENMs. Therefore, initially methods that have gained regulatory approval or are under validation for conventional chemicals were researched and evaluated for their potential application to ENMs. Subsequently, methods designed specifically for ENMs were collected. In effect, the following categorization of the NAMs was established:

1. non-nano specific regulatory accepted NAMs
2. non-nano specific under validation NAMs
3. nano-specific regulatory accepted NAMs
4. nano-specific under validation NAMs
5. nano-specific under development NAMs

The main findings of the literature analysis:

- In total, 220 NAMs were identified (data gathered to date 30.12.2022). Detailed information on each method with references (including method description) is provided in **Annex 2** (List of NAMs assigned to toxicological endpoints.xlsx).

- There are 68 '**non-nano specific regulatory accepted NAMs**' identified and assigned to specific human health relevant endpoints. These NAMs refer to methods intentionally developed for conventional chemicals and have already gained regulatory acceptance.

- Another 19 '**non-nano specific under validation NAMs**' were identified. These approaches were proposed for conventional chemicals and according to the ECVAM repository status they are currently in the stage of validation or peer-review.

- There is a limited number of '**nano-specific regulatory accepted NAMs'** (our findings show that there are only 8 available so far). Of the few accepted NAMs, most are available only for 3 endpoints, mainly for "Toxicity in vitro testing" (N=5). This number strongly indicates urgent needs for speeding up validation processes for nano-specific NAMs which are currently under development for different endpoints.

- The highest number of identified NAMs was classified as '**nano-specific under development NAMs**' (N=120).

- The endpoints of acute toxicity by inhalation, repeated dose toxicity, and toxicokinetics, which are highly complex, have numerous NAMs under development, albeit with none or only one being '**regulatory accepted**'. This suggests a substantial level of potential for the NAMs, however, it also highlights the requirement for expediting their validation process and ensuring their formal regulatory approval.

- For the endocrine disruption, which is another extremely complex endpoint, 14 '**non-nano-specific under validation NAMs**' have been found. However, for the other complex endpoints such as carcinogenicity, neurotoxicity or reproductive toxicity, only a few '**nano-specific under development**' or '**non-nano-specific regulatory accepted NAMs**' have been identified.

- For the majority of endpoints for which there are currently no '**nano-specific under development NAMs**', such as phototoxicity, eye damage, or skin corrosion/irritation, there are at least several '**non-nano-specific regulatory accepted NAMs**'. This suggests that the pace of current efforts towards application and validation trials of the latter NAMs for ENMs testing is insufficient, and the scientific community needs to re-evaluate its approaches to improve the situation.

- The most complex scenario pertains to the endpoints for which there are currently no '**nano-specific under development NAMs**' nor '**nano-specific regulatory accepted NAMs**' available. These endpoints include acute toxicity by dermal exposure, effects on gut microbiome, hypersensitivity/food intolerance, respiratory sensitisation. Although the complexity of these endpoints may be a contributing factor to this situation, the large population of workers and consumers who may be potentially exposed highlights the urgent need for the development of the nano-specific NAMs for these endpoints.

The main finding of the survey analysis:

- Experts pointed to the usefulness and suitability of several existing alternative methods for assessing the risk of nanomaterials.

- NAMs most commonly used by the industry to assess the hazard associated with nanomaterials are mainly *in vitro* skin sensitization/irritation assays, reconstructed skin models, *in vitro* skin batteries and ISO/TS 21633:2021 (Label-free impedance technology to assess the toxicity of nanomaterials in vitro).

- Experts very clearly indicated the usefulness of commercially available 3D organ models that represent different exposure routes (i.e., MatTek, Epithelix, AlveoliX, ImmuOne). Several respondents indicated use of *in silico* methods in general i.e., read-across, QSAR.

- The necessity of prioritization of specific NAMs for several regulatory-relevant endpoints in terms of both industrial needs and regulatory acceptance was highlighted. These include tests related to specific types of organs such as lung, liver and the gastrointestinal tract. The

endpoints of the highest priority include genotoxicity, carcinogenicity, dermal and oral absorption, inhalation toxicity, neurotoxicity and toxicokinetics.

- According to the experts, the following steps should be taken to fulfil the requirements that may result in increase of the regulatory acceptance of the nano-specific NAMs and their potential transfer to the EU regulations:

  (i) adjustment of the exposure-driven scenarios to consider different routes of exposure to ENMs;
  (ii) adjustment of the test systems to mimic human biology;
  (iii) development of appropriate *in vitro* exposure protocols to take into account the behavior of nanomaterials;
  (iv) development of appropriate methods to characterize nanomaterials, both in pristine forms and in culture media, and,
  (v) reuse of available data and accessible databases to support the development and validation of *in silico* methods.

- In relation to the most promising methods, both from the regulatory point of view and in the context of industry needs, experts pointed out OECD TGs, co-culture and 3D organ models, inhalation/respiratory models, genotoxicity tests. Few mentioned *in silico* methods, including read-across/machine learning techniques and reuse of the existing data on nanomaterials. The experts additionally highlighted the importance of EU Horizon 2020 project outcomes, such as PATROLS and NanoHarmony, in which several nano-specific alternative methods were/are being developed.

Conclusion

In conclusion, the challenge of advancing the nano-specific NAMs is exceedingly complex. The differences in endpoint complexity influence the uneven pace of NAMs development. There are only 8 regulatory accepted nano-specific NAMs, available only for 3 endpoints, mainly for "Toxicity in vitro testing". Thus, there is an urgent need to speed up validation processes for nano-specific NAMs which are currently under development for different endpoints, and to develop new NAMs for complex endpoints (e.g., neurotoxicity or reproductive toxicity). There are a lot of new, promising methods currently under development (the majority of them employing 3D organ models).
It is imperative that further initiatives in this field be grounded in a more constructive and effective dialogue between all relevant parties, particularly regulatory bodies. The most urgent gaps identified in the field which need to be addressed are:

1. the lack of physicochemical characterization of nanomaterials in conditions in which human exposure is likely to occur;
2. gaps in understanding *in vitro* dosimetry for nanomaterials;
3. gaps in understanding the real exposure scenarios of nano-enabled products.

1. Introduction

Over the last few decades, there has been a growing interest in the applications of engineered nanomaterials (ENMs) in a variety of consumer products. This forced the urgent need for the development of comprehensive and functional nano-specific approaches that would allow to assess human hazard of ENMs in a timely manner. Considering the general (not only relevant for ENMs) drawbacks of applying animal testing for this purpose in terms of its ethical, economic, and time limitations, delivering new NAMs is of high priority.[1,2] It is recommended by regulatory and decision-making agencies that non-animal-based alternatives should be applied, whenever possible. Alternative approaches are in line with the strategy published in 2015 by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), known as the 3R principle[3], which is related to the replacement, reduction, and refinement of animal tests. Similar recommendations were also provided by Cosmetic Regulation ((EC) No 1223/2009), as well as by REACH Regulation and other relevant initiatives at the EU and international level (e.g., Organization for Economic Co-operation and Development, OECD) ((EC) No 1907/2006).

The recently coined term of new approach methodology (NAM) is becoming increasingly used, but there is no harmonised definition or common use of the term. NAM is probably differently understood by different researchers, regulators, and organisations.[4] During a Scientific Workshop held by the European Chemicals Agency (ECHA) in 2016[5], "*NAMs were taken in a broad context to include toxicological methods that serve as (replacement, reduction or refinement) alternatives to animal testing (e.g. in silico, in chemico and in vitro methods), as well as the inclusion of information from the exposure of chemicals in the context of hazard assessment. They also include a variety of new testing tools, such as "high-throughput screening" and "high-content methods" e.g., genomics, proteomics, metabolomics; as well as some "conventional" methods that aim to improve understanding of toxic effects, either through improving toxicokinetic or toxicodynamic knowledge for substances*". The US Environmental Protection Agency (US EPA) states in their "Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program" that NAM "*has been adopted as a broadly descriptive reference to any technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of intact animals*",[6] referring to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Strategic Roadmap. In the context of the US Toxic Substances Control Act (TSCA), NAM encompasses any "*alternative test methods and strategies to reduce, refine or replace vertebrate animal testing*".[7] "List of alternative test methods and strategies (or NAMs)", according to TSCA Section 4(h), has been published on the US EPA website.[8] ICCVAM includes Integrated Approaches to Testing and Assessment (IATA) and Defining Approaches (DAs) in the NAM definition. NAMs are also intensively discussed in the context of the Canadian Chemicals Management Plan[9] and include alternative methods that bridge the transition from conventional *in vivo* studies to *in vitro* assays, and that satisfy the 3Rs criteria. Overall, it can be stated that NAMs thus include both established methods, such as Test Guideline *in vitro* tests, as well as newly developed assays and technologies such as 3D-organoids and organ-on-a-chip

devices. However, interpretations vary as to whether certain animal models (e.g., lower vertebrates, e.g. fish embryo), and whether grouping and read-across, are regarded as NAMs.

The usage of cell-based and computational methods allows to understand the mechanism of events that are triggered after the exposure to the material. In effect, such procedures could support the prediction of whether chemicals are harmful to humans. The significant progress in wider application of non-animal approaches in risk/safety assessment could be reached by close collaborations between researchers, industry, and national/international regulators. Thus, understanding the needs from different perspectives can help establishing novel NAMs-based assessment frameworks.

Enrichment and development of nano-specific NAMs was the objective of many international initiatives, including scientific projects founded by European Commission in the frame of 7FP and H2020 projects (https://cordis.europa.eu/projects/en). In effect, many NAM-based strategies were developed. They include tools for grouping and read-across, *in silico* models for predicting hazard, *in vitro* high-throughput and high-content screening. While these nano-specific alternative approaches are gaining scientific acceptance, there is still uncertainty about their use in ENMs risk/hazard assessment. Hence, for NAMs to be widely adopted for regulatory purposes, it is crucial to increase confidence in their use through validation. This could involve comparing NAMs with *in vivo* experimental data, which are still the gold standard in hazard assessment. This solution, however, is associated with many limitations related to uncertainties in toxic dose extrapolation between species due to differences in body physiology. Moreover, as recently pointed out by Doak et al. (2022),[10] there is still limited *in vivo* data generated according to OECD Test Guidelines (TG) that could be used to validate the nano-specific NAMs. This means further extensive animal testing may be required prior to the validation and use of the nano-specific NAMs.

The main objectives of the service contract (Figure 1) are to:

- conduct a systematic literature review of the currently available, nano-specific alternative methods for assessing the safety of nanomaterials;
- conduct surveys amongst experts for nano-specific alternative methods in human risk/safety assessment with regulatory relevance;

and, based on the result to provide information about the actual state of the research, gaps, requirements and needs for nano-specific alternative methods in human risk/safety assessment, under different EU regulations, considering the animal testing bans already in place for cosmetics and their ingredients.

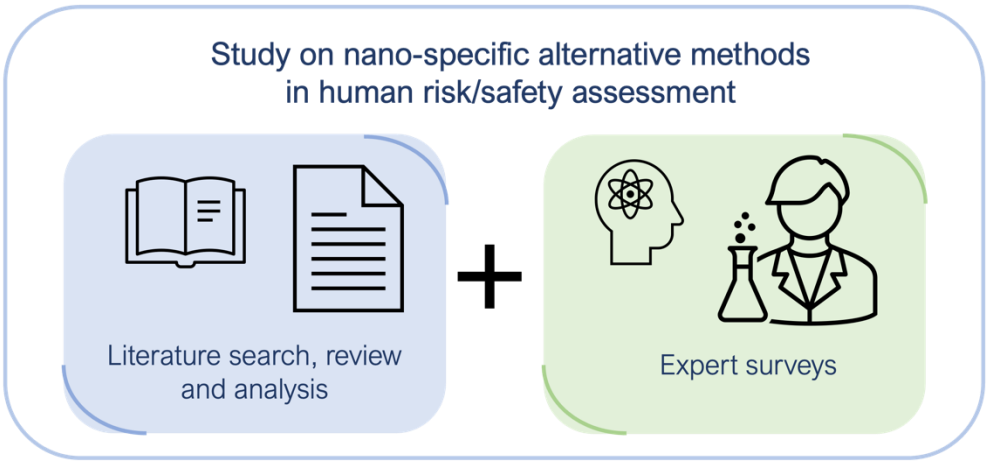

***Figure 1**. Study on nano-specific alternative methods in human risk/safety assessment.*

2. Methodology

Methodology of literature search

The systematic literature search (in order to collect documents for systematic literature review) followed the general reporting format for the data review based on the expanded checklist details elements, abstract checklist, and the revised flow diagrams for original and updated reviews recommended for reporting by PRISMA 2020 guidance.[11] This guidance is suitable for both regular publications and other data source reviews. In the case of protocols, the PRISMA-P (PRISMA for Protocols) was used.[12] The process of searching is presented in Figure 2.

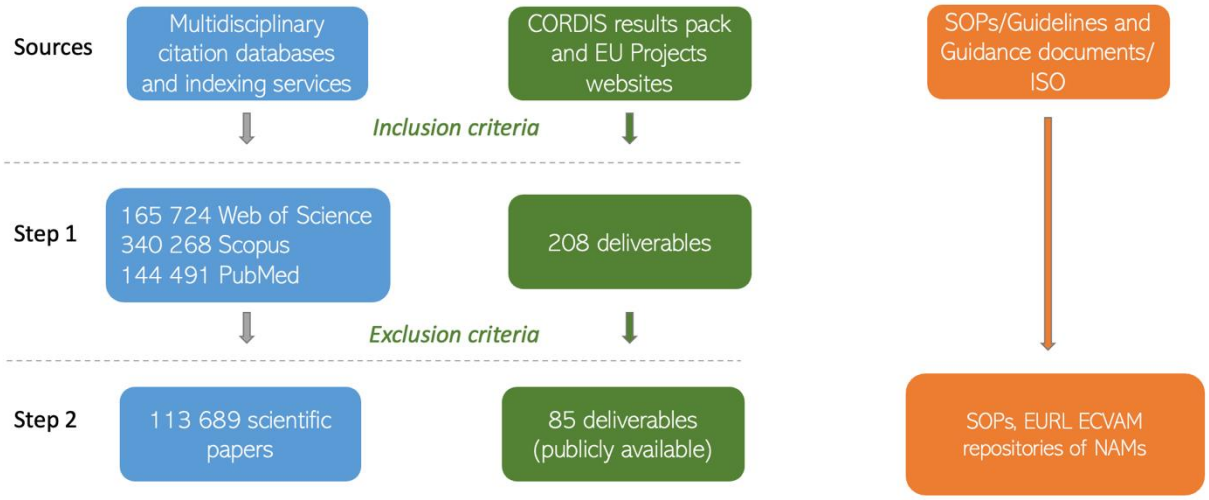

***Figure 2.** Literature searching and selection process*

To generate the most inclusive repositories, we considered various sources of data, such as: most frequently used multidisciplinary citation databases and indexing services (Web of Science, Scopus, PubMed); CORDIS Result Packs and EU projects' websites; EU project cloud platforms and databases; OECD Test Guidelines and Guidance documents, OECD Publications in the Series on the Safety of Manufactured Nanomaterials; ISO Standards; EURL ECVAM repositories of NAMs, EPAA website. The citations platforms were selected according to the criteria of relevance and database coverage as well as technical feasibility of records extraction (including advanced filtering and downloading). We primarily selected Web of Science, Scopus, PubMed and Google Scholar as the basis for the research, however the latter did not allow for efficient extraction of the records for further data mining and processing. Nonetheless, vast majority of the results that was extracted was subsequently confronted with the Google Scholar search results to verify the consistency of our dataset. Any important records that were identified through Google Scholar, and were not present in our database, were also included.

The used sources are summarized in Table 1. In the case of deliverables and other EU project outputs, we have included there the finished and/or ongoing EU projects that were considered as relevant - those that aimed at delivering tools and assess the risk after nanomaterials exposure.

Table 1. *Sources of information*

Scientific articles	Multidisciplinary citation databases and indexing services (Web of Science, Scopus, PubMed)
EU project Deliverables	CORDIS Result Packs, Project websites (Calibrate, CompSafeNano, Diagonal, eNanoMapper, ENPRA, Gov4Nano, Gracious, GuideNano Harmless, Marina, NanoCommons, NanoGenotox, NanoInformaTIX, NanoReg, NanoReg2, NanoRIGO, NanoTEST, NanoSolveIT, NanoSolution, PATROLS, RiskGONE, SmartNanoTOX, SUN)
Guidance's/SOPs/Protocols	OECD Test Guidelines and Guidance documents; ISO Standards, SOPs, EURL ECVAM repositories of NAMs, EPAA website

In the systematic strategy for the review, we have considered any scientific papers, deliverables, guidelines, protocols, and other public repositories that describe the alternative approaches that can be applied for assessing the human hazard/safety of ENMs. It includes available *in silico* models, *in vitro* and *in chemico* assays developed for nanoforms or those that can be easily assigned to be applied for nanomaterials. However, in this Report less importance was given to *in silico* nano-specific NAMs, as these are planned to be assessed in a separate review prepared by other authors.

In the first step as **inclusion criteria**, we have used the following set of keywords as search queries (* due to nomenclature heterogeneity in literature each query was used four times with different variation of first word nano*/ ENMs / MNMs / NMs):

nano/ENMs/MNMs/NMs new approach methodologies OR*
nano/ENMs/MNMs/NMs in vitro testing OR*
nano/ENMs/MNMs/NMs in vitro validation OR*
nano/ENMs/MNMs/NMs hazard assessment regulatory OR*

nano/ENMs/MNMs/NMs toxicity assessment OR*
nano/ENMs/MNMs/NMs risk assessment OR*
nano/ENMs/MNMs/NMs risk assessment regulatory OR*
nano/ENMs/MNMs/NMs in vitro OR*
nano/ENMs/MNMs/NMs in chemico OR*
nano/ENMs/MNMs/NMs in vitro risk assessment OR*
nano/ENMs/MNMs/NMs in chemico risk assessment OR*
nano/ENMs/MNMs/NMs NAMs OR*
nano/ENMs/MNMs/NMs hazard tool* OR*
nano/ENMs/MNMs/NMs risk tool* OR*
nano/ENMs/MNMs/NMs adverse outcome pathway OR*
nano/ENMs/MNMs/NMs AOP OR*
nano/ENMs/MNMs/NMs alternative testing OR*
nano/ENMs/MNMs/NMs in silico OR*
nano/ENMs/MNMs/NMs PBPK OR*
nano/ENMs/MNMs/NMs dose response model OR*
nano/ENMs/MNMs/NMs *omics OR*
nano/ENMs/MNMs/NMs kinetic model* OR*
nano/ENMs/MNMs/NMs BMD OR*
nano/ENMs/MNMs/NMs multicellular 3D model OR*
nano/ENMs/MNMs/NMs hazard assessment OR*
nano/ENMs/MNMs/NMs qsar risk assessment OR*
nano/ENMs/MNMs/NMs qsar regulatory OR*
nano/ENMs/MNMs/NMs grouping read-across OR*
nano/ENMs/MNMs/NMs exposure assessment OR*
nano/ENMs/MNMs/NMs high throughput screening OR*
nano/ENMs/MNMs/NMs HTS OR*
nano/ENMs/MNMs/NMs data* OR*
nano/ENMs/MNMs/NMs database OR*
nano/ENMs/MNMs/NMs database hazard OR*
nano/ENMs/MNMs/NMs human risk assessment OR*
nano/ENMs/MNMs/NMs NGRA* OR*
nano/ENMs/MNMs/NMs next generation risk assessment OR*
nano/ENMs/MNMs/NMs in silico risk assessment OR*
nano/ENMs/MNMs/NMs in silico hazard assessment OR*
nano/ENMs/MNMs/NMs in vivo hazard assessment*
nano/ENMs/MNMs/NMs Point of departure OR*
nano/ENMs/MNMs/NMs multiple path dosimetry model*

Then, as the second step, the initial number of documents to be further reviewed were retrieved by applying the **exclusion criteria**. The considered exclusion criteria were listed in Table 2.

Table 2. *Exclusion criteria used to select documents for further review*

1.	*In vivo* studies unless the *in vivo* conditions described in the document were used in context of the validation of the in vitro studies
2.	Non-English documents
3.	Duplicates
4.	Published before 2010
5.	The document is: Editorial Materials; Retracted Publications; Letters; Corrections; Software Reviews; Reprints; News Items; Publication with expression of concern.

In case of EU projects where QSAR Lab has participated as a Partner, although we have access to all deliverables, we have included only those that are publicly available (documents that are still confidential were not included).

In the next step, the **regulatory relevance** of collected documents was verified. At first, we have prepared an inventory of EU regulations which stipulate conducting the safety assessment of chemicals, Table 3. Based on these regulations, the toxicity endpoints that are used to assess human safety were listed together with the relevant testing guidelines/protocols. Next, text mining tools written in Python were applied for assigning the initially collected documents (i.e., documents that met inclusion/exclusion criteria) to listed endpoints. Finally, the quality of each considered publication was evaluated according to the GuideNano quality scoring system[13]. This procedure allows to assess if all necessary information in terms of substance characterisation (S-score) and methods performance (K-score) are provided to ensure transparency and feasibility of the study. The goal was to consider in further analysis only papers with K- and S-score of 1-2. The GuideNano scoring system is very rigorous in terms of physical and chemical characteristics (requires taking into account a huge vast amount of information). In many cases, the articles did not present some physicochemical properties due to their insignificance for the scope of the entire work or included them in supplementary materials to which direct access could not be obtained. Nevertheless, these articles were of high quality and omitting them from the analysis would have resulted in a significant information gap. Therefore, despite failure to meet the criteria by the majority of papers containing NAMs, it was decided to consider all collected literature to keep a broader perspective.

Table 3. *EU regulations for risk assessment of chemicals*

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals
Regulation (EC) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products
Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products
Regulation (EU) No 2015/2283 of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001
Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives
Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings

Methodology of selection experts for survey and survey performance

The identification of experts for the surveys for nano-specific NAMs in human risk/safety assessment with regulatory relevance started with identifying target groups, since the main assumption in the selection of experts was to obtain a broad perspective of work in the context of NAMs. The goal was to select experts, who would represent groups that participate in: i) development of NAMs, ii) regulatory acceptance of NAMs, and iii) using NAMs in the chemical's registration/approval process. Therefore, three groups of experts were proposed representing**: Academia, Regulatory agencies** and **Industry** (Figure 3).

Figure 3. *Groups of experts for surveys.*

Considering the group of interests (Academia, Regulatory agencies, Industry), the relevant experts were selected using two approaches:

1. Selection based on data mining and text processing studies performed simultaneously with literature searching.
2. Selection based on expert knowledge.

The first approach was based on the results of the literature review, data mining and analysis of all collected documents. Basing on repositories of crucial documents related to nano-specific NAMs (scientific publications, regulatory documents), we have identified key authors and co-authors in strictly defined areas (i.e., regulatory-relevant toxicological endpoint-specific). This approach was applied to select experts representing academia. Here, at first, all gathered documents were assigned to their corresponding 'endpoint group' to further distinguish experts' fields of expertise (such as e.g., mutagenicity, carcinogenicity, acute inhalation). Then, by application of text processing tools, within each defined group of endpoint-specific documents, scientists with the highest number of authorship/co-authorships were selected. Consequently, a pool of academia experts was identified (Figure 4).

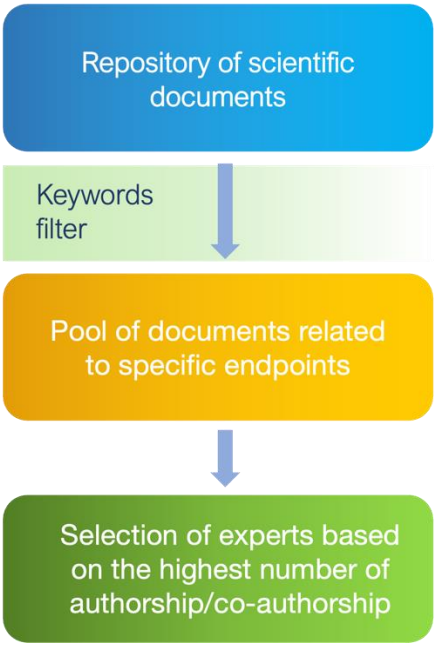

***Figure 4**. Methodology of selecting academia experts*

The second approach was based on QSAR Lab's team expert knowledge and experience gained during participation, management and promotional activities of recent EU projects related to ENMs risk/safety assessment and other nano-related initiatives and clusters (i.e., NanoSafetyCluster, NanoFabNet). Moreover, our expertise is supported by our membership in several EU nano-safety projects as well as in the scientific nano-communities where researchers from QSAR Lab have been actively working for over 15 years. This approach was considered while selecting experts representing regulatory agencies and industry; some additional experts from academia were also found using this strategy (i.e., academia experts from renowned research centres, active in nanosafety communities, clusters, and research projects).
In order to obtain the full range of information and the broadest possible perspective, the main regulatory bodies implementing chemical law in Europe and beyond have been identified. Thus, the regulatory affairs group includes experts from:

- European Chemicals Agency (ECHA)
- European Food Safety Agency (EFSA)
- Scientific Committee on Consumer Safety (SCCS)
- Joint Research Centre of the European Commission (JRC)
- Nano Industries Association (NIA)
- People for the Ethical Treatment of Animals (PETA)
- U.S. Environmental Protection Agency (EPA)
- The Organization for Economic Co-operation and Development (OECD)

Then, from each organization at least 2 to 3 relevant participants were selected.

The group of experts representing industry covers major stakeholders in the chemical industry. Here, an additional factor concerning diversification of industries in the chemical sector was taken

into account. Thus, the group of experts represents producers and manufacturers of various chemical sectors, including cosmetics, pigments, food, paints, household chemicals and more. Experts of each of the companies were selected based on their field of expertise. The group includes mainly toxicologists, and experts working in registration and regulations departments.

As part of the project promotion, we have created a post on LinkedIn, giving the opportunity to apply for candidates that were not selected by using proposed strategies but willing to answer the questionnaires. In effect, two additional experts were added to the industry group.

To carry out the survey, experts were contacted mainly by their publicly available e-mail addresses, or via LinkedIn. The first contact e-mail with an invitation to complete the survey was supplemented with an endorsement letter from EUON and privacy notice survey data. A total of three messages were sent to each expert. The first one introducing the expert to the project's goals with an invitation to participate in the questionnaire and a link to the survey. The second notification was sent after a week, with a reminder to complete the survey. The last notification was sent on the last day of collecting responses. The respondents had roughly 3 weeks from the date of sending the first notification to complete the questionnaire.
The survey was **created using the Microsoft Forms tool**. All privacy rights were preserved. At the beginning of the questionnaire, the experts were informed that their answers will be used to create a report for ECHA and the European Union Observatory for Nanomaterials (EUON), and they were required to give their consent to continue filling out the questionnaire. In addition, the participant could indicate whether they wanted their personal data to be processed by QSAR Lab for the purpose of preparing the report commissioned by EUON, or if they preferred to remain anonymous. All questions are listed in **Annex 1**: Expert Survey – List of questions.

3. Results

Toxicological endpoints required to assess safety under EU Regulations

As a starting point, the map of needs that summarizes the toxicological endpoints essential in terms of the safety assessment of chemicals, including nanomaterials, under different EU regulations (Table 3) was developed (Table 4). In this context, the latest, relevant regulations have been considered. For the purpose of this project, REACH, Biocidal Products Regulation, Cosmetic Products Regulation, and all regulations applying to the food and food chain were taken into account. The considered documents, in general, aim at controlling the marketing of chemicals, biocidal products, cosmetics, food, and feed ingredients in the European Union. Thus, in order to comply with applicable directives and regulations, all products or substances on the market must first be tested, including testing for human health endpoints and environmental impacts. As a result, the list of endpoints that need to be evaluated according to each regulation was established.

For convenience some guidance documents (with links) issued by ECHA and the EC Scientific Committees highlighting ENMs safety testing requirements and risk assessment are listed below:

ECHA documents related to nanomaterials can be found under:

- https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment

EFSA documents related to nanomaterials with links:

- Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2021.6768
- Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2021.6769
- The dedicated EFSA webpage on Nanotechnology https://www.efsa.europa.eu/en/topics/topic/nanotechnology

SCCS documents related to nanomaterials with links:

- 2021_SCCS 1628-21 11th revision NoG https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_250.pdf
- 2019_SCCS_ Guidance on the Safety Assessment of Nanomaterials in Cosmetics https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_233.pdf
- 2021_SCCS_SCIENTIFIC ADVICE on Nanomaterials https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_239.pdf
- 2023_SCCS - Final Opinion on Hydroxyapatite (nano) https://health.ec.europa.eu/system/files/2023-01/sccs_o_269.pdf
- 2021_SCCS_Final Opinion on HAA299 (nano) https://health.ec.europa.eu/system/files/2022-08/sccs_o_256.pdf
- 2021_SCCS_Final Opinion on Gold (nano), Colloidal Gold (nano), Gold Thioethylamino Hyaluronic Acid (nano) and Acetyl heptapeptide-9 Colloidal gold (nano) https://health.ec.europa.eu/system/files/2022-08/sccs_o_251.pdf
- 2021_SCCS_Final Opinion on Platinum (nano), Colloidal Platinum (nano) and Acetyl tetrapeptide-17 Colloidal Platinum (nano) https://health.ec.europa.eu/system/files/2022-08/sccs_o_252.pdf
- 2021_SCCS_Final Opinion on Copper (nano) and Colloidal Copper (nano) https://health.ec.europa.eu/system/files/2022-08/sccs_o_245.pdf
- 2015_SCCS_OPINION on Carbon Black (nano-form) https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_144.pdf

- 2012_SCCS_Opinion on Zinc oxide (nano form)
  https://health.ec.europa.eu/system/files/2016-11/sccs_o_103_0.pdf

SCENIHR documents related to nanomaterials with links:

- 2009 SCENIHR Risk Assessment of Products of Nanotechnologies
  https://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf
- 2015 SCENIHR Nanomaterials Used in Medical Devices
  https://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_045.pdf

Due to fact that the project's objectives are focused on human hazard assessment of nanomaterials, only human health endpoints were considered in further analyses (ecotoxicological endpoints were not included). The validity and importance of nano-specific exposure models as endpoints were not considered. Hence, each considered regulation was analysed in terms of identifying human health relevant endpoints. In effect, a table of 32 endpoints, that had to be evaluated depending on the regulation, was created, Table 4. It needs to be highlighted that some endpoints (e.g., skin corrosion, mutagenicity, repeated dose toxicity) have to be addressed under several regulations, while others are required only in particular ones (e.g., gastrointestinal digestion, developmental neurotoxicity). In other words, some endpoints crucial for the evaluation of e.g., cosmetics products (e.g., dermal adsorption) are not required for the food regulation (gastrointestinal digestion).

Table 4. *The map of endpoints that are crucial for human health safety assessment according to existing EU regulations*

Endpoints	***EU Regulations***						
	REACH (chemicals) [1]	**Biocidal**	**Cosmetic**	**Food**	**Food**	**Food**	**Food**
	Regulation (EC) No 1907/2006	**Regulation (EU) No 528/2012**	**Regulation (EC) No 1223/2009**	**Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001**	**Regulation (EC) No 1331/2008**	**Regulation (EC) No 1333/2008**	**Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003**
Acute toxicity *by oral route*	✓	✓	✓				✓
Acute toxicity *by inhalation*	✓	✓	✓				✓
Acute toxicity *by dermal route*	✓	✓	✓				✓
Carcinogenicity *study*	✓	✓	✓				✓
Dermal absorption *– for dermally applied products*			✓				
Developmental neurotoxicity *Reproductive toxicity*		✓					
Effects on gut microbiome				✓			
Endocrine disruption		✓	✓	✓	✓	✓	
Eye damage/eye irritation *in vitro*	✓	✓	✓				✓
Gastrointestinal digestion *in vitro*				✓			
Hypersensitivity/ Food intolerance				✓	✓	✓	

[1] Requirements for testing endpoints are tonnage-triggered

Immunotoxicity/ developmental immunotoxicity/ allergenicity		✓		✓	✓	✓	✓
In vitro toxicity testing *cytotoxicity/cell viability* *oxidative stress* *(pro-)inflammation* *gastrointestinal barrier integrity*				✓			
Mutagenicity *in vitro cytogenicity study in mammalian cells and/or in vitro micronucleus study*	✓	✓	✓	✓	✓	✓	✓
Mutagenicity *in vitro gene mutation study in bacterial cells*	✓			✓	✓	✓	✓
Mutagenicity *in vitro gene mutation study in mammalian cells*	✓	✓	✓	✓			✓
Neurotoxicity		✓		✓	✓	✓	✓
Phototoxicity		✓	✓				
Repeated dose toxicity *short-term repeated dose toxicity study (28 days)*	✓	✓	✓				✓
Repeated dose toxicity *Sub-chronic toxicity study (90-day)*	✓	✓	✓	✓	✓	✓	✓
Repeated dose toxicity *Long-term repeated dose toxicity (≥ 12 months)*		✓	✓	✓	✓	✓	✓
Reproductive toxicity *screening for reproductive/ developmental toxicity*	✓		✓				
Reproductive toxicity *Developmental toxicity study OECD 414*	✓	✓	✓	✓	✓	✓	✓
Reproductive toxicity *Pre-natal developmental toxicity study*	✓		✓				

Reproductive toxicity *Extended One-Generation Reproductive Toxicity Study*	✓	✓	✓	✓	✓	✓	✓[2]
Respiratory sensitisation		✓					
Skin corrosion *in vitro*	✓	✓	✓				
Skin irritation *in vitro*	✓	✓	✓				✓
Skin sensitisation *in vitro/in chemico*	✓	✓	✓				✓
Toxicokinetics *toxicokinetic behaviour of the substance*	✓	✓	✓	✓	✓	✓	
Water solubility, dissolution rate in relevant biological media (including stability in lysosomal fluid) *in vitro*	✓			✓			

[2] Two generation reproduction toxicity studies can be alternatively used in the weight of evidence approach, if historically available.

Repository of documents for further analysis

The results of the literature search are summarized in Figure 5. The repository included inventory of: EU project deliverables (assigned to NAMs category, project information, dissemination level, sources, and abstract); SOPs and guidance's (sources, NAMs category and description), EURL ECVAM NAMs (description, stage of development, endpoint); OECD Test Guidelines and Guidance Documents, ISO Standards and scientific publications (article details and abstract).

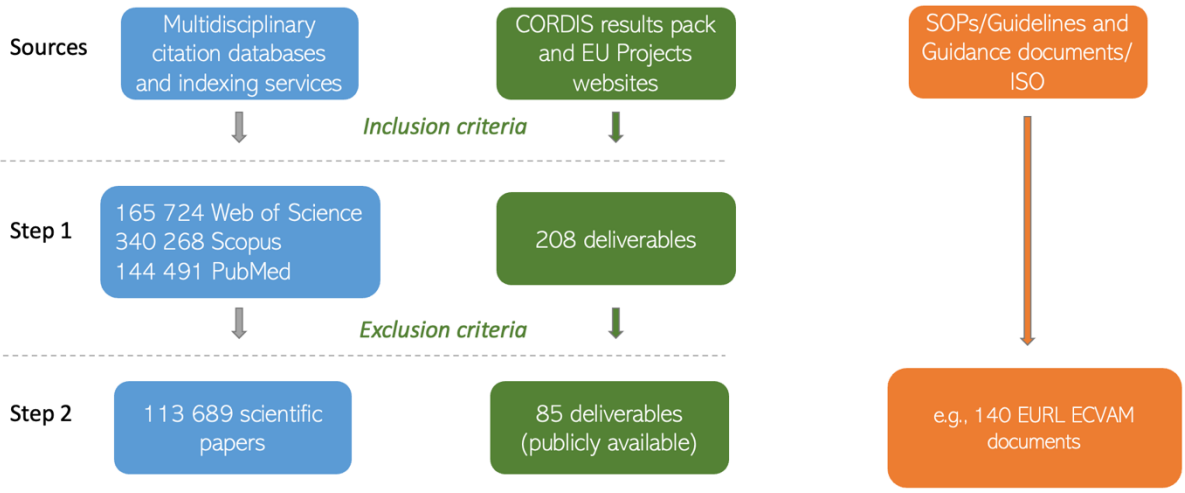

***Figure 5**. Results of initial literature search*

A total of 208 deliverables from the CORDIS and/or project websites was retrieved. We have taken into account here only EU projects that were aimed at delivering tools and/or assessing the safety of nanomaterials. After selection of the titles and abstract of documents and applying the inclusion and exclusion criteria, 85 project deliverables were finally considered for further review. Among these documents, 44 describe various *in silico* approaches that were developed for assessing the risk relevant for ENMs; 22 documents focused on establishment of *in vitro* assays suitable for nanomaterials; 12 present nano-specific methods for characterisation and databases; and 7 describe the risk assessment or risk management frameworks in general, Figure 6.

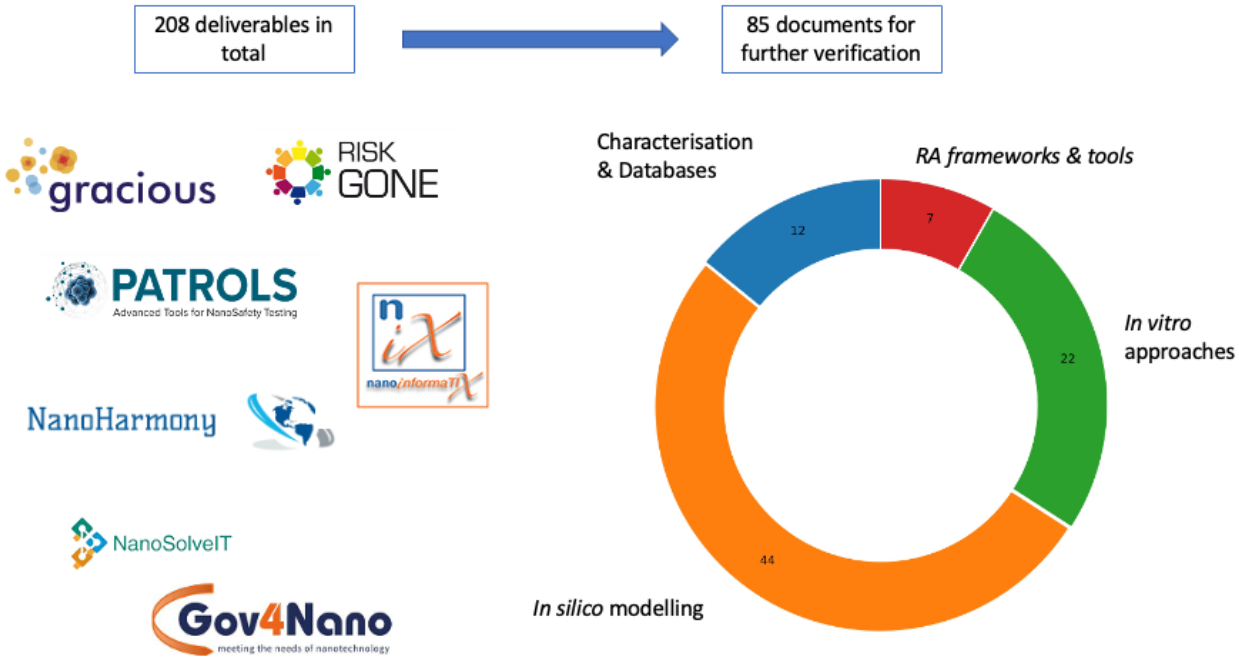

Figure 6. *The results of the analysis of the EU Project deliverables.*

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In the case of scientific publications, we have considered the three most frequently used multidisciplinary citation databases and indexes services (Web of Science, Scopus and PubMed) and initially retrieved more then 140 000 records that cover the inclusion criteria. The found number of records retrieved depended on used source, details are presented in Table 5.

Table 5. *Results of literature searching*

Multidisciplinary citation database and indexes service	**Number of records**
Web of Science	165 724
Scopus	340 268
PubMed	144 491

In the next step, we have applied the exclusion criteria (Table 2) and finally extracted 127 420 records for further consideration (Figure 7). It is noticeable that the number of publications related to risk assessment of nanomaterials is increasing every year (from 2010 till 2021).

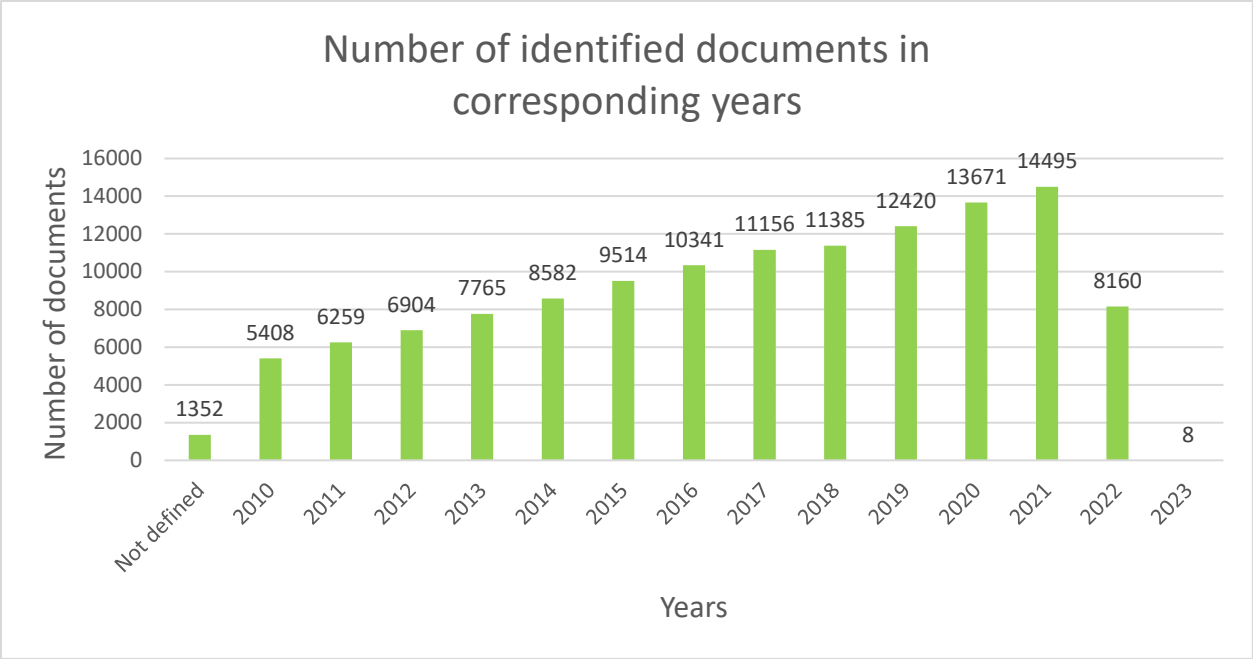


Figure 7. *Number of identified documents (after applying inclusion and exclusion criteria) per each year since 2010. Since 2010, the number of documents relating to NAMs is growing every year.*

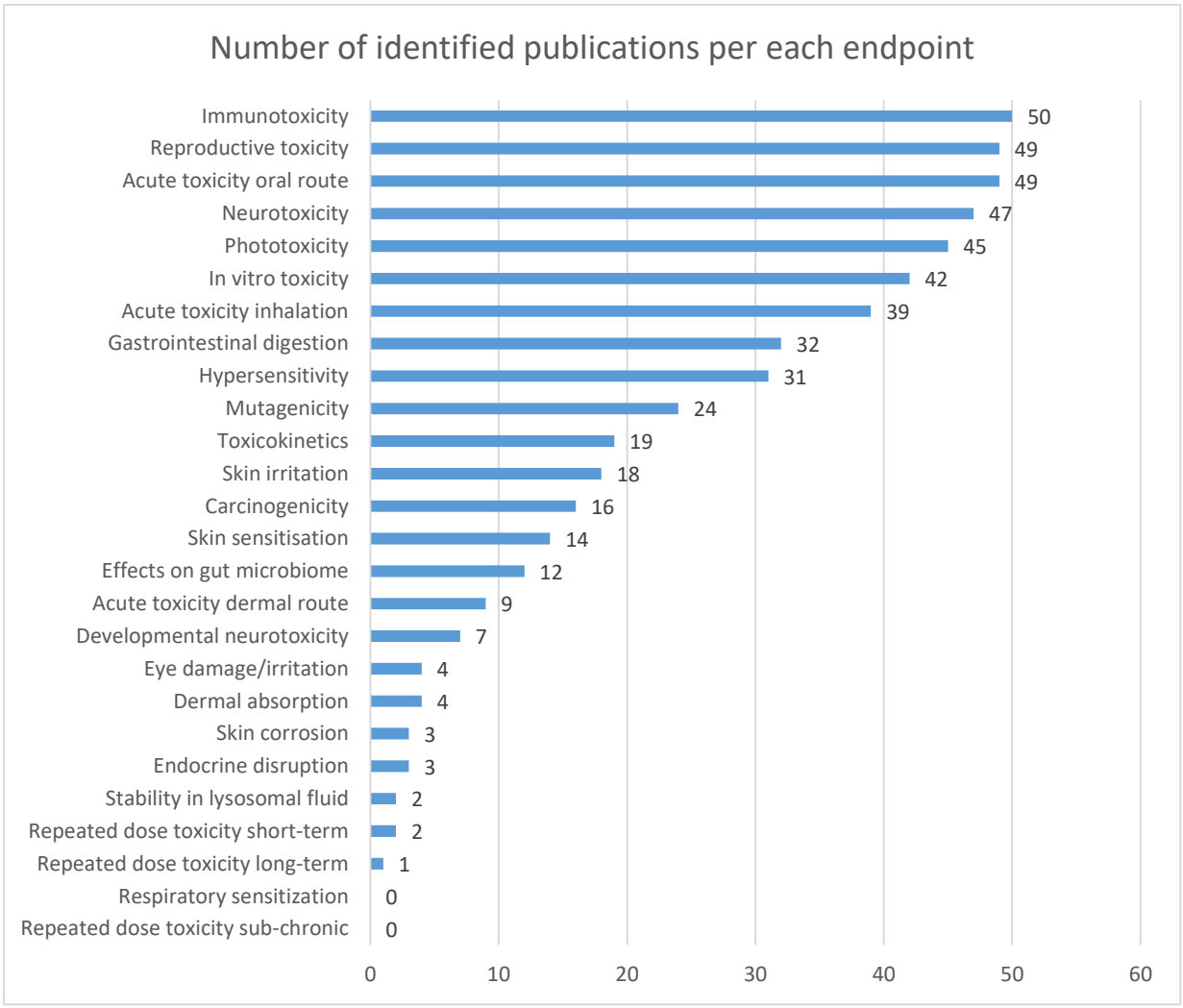
Further analysis was based on the collected documents and regulations. For all identified relevant regulations (Table 3) we tentatively listed toxicity endpoints that are required in the assessment together with recommended testing guidelines/protocols. Next, in order to narrow the search results specifically to articles/documents of relevance, we applied text mining tools to match gathered documents (scientific papers and deliverables) to identified endpoints. We have applied filters to the total number of 127 420 scientific articles and 85 project deliverables in order to retrieve those that consider relevant regulatory toxicological endpoints. In a subsequent step we filtered title and abstracts of the documents using following substrings (parts or whole words that would include specific text, Table 6). The filtering in this case included obligatory keywords (or parts of keywords), i.e. "*in vitro/in silico/in chemico*", "nano", that had to be present in the title or abstract of the paper.

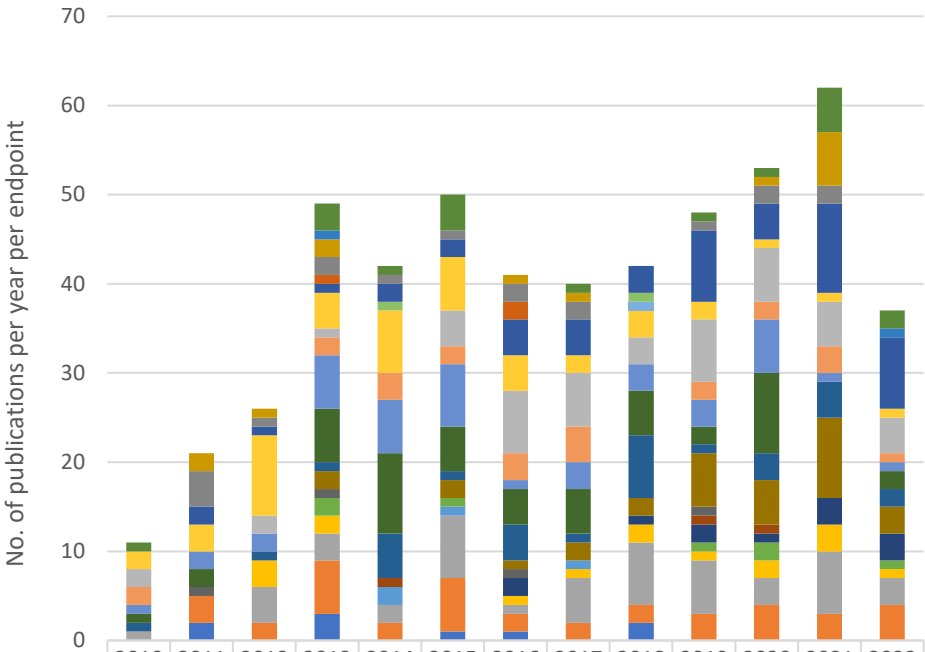
Table 6. *Filter keywords for each identified endpoint*

Endpoint	**Keywords**
Acute toxicity (oral route)	*nano*, acute, toxicity, oral*, in vitro/in silico/in chemico*
Acute toxicity (inhalation)	*nano*, acute, toxicity, inhal*, in vitro/in silico/in chemico*
Acute toxicity (dermal route)	*nano*, acute, toxicity, dermal*, in vitro/in silico/in chemico*
Carcinogenicity	*nano*, carcinogenicity, in vitro/in silico/in chemico*
Dermal absorption	*nano*, dermal, absorption, in vitro/in silico/in chemico*

Developmental neurotoxicity	*nano*, developmental, neurotoxicity, in vitro/in silico/in chemico*
Effects on gut microbiome	*nano*, gut, microbiome, in vitro/in silico/in chemico*
Endocrine disruption	*nano*, endocrine disruption, in vitro/in silico/in chemico*
Eye damage/eye irritation	*nano*, eye, irritation, in vitro/in silico/in chemico*
Gastrointestinal digestion	*nano*, gastrointestinal, digestion, in vitro/in silico/in chemico*
Hypersensitivity, food intolerance	*nano*, hypersensitivity, in vitro/in silico/in chemico*
Immunotoxicity, developmental immunotoxicity, allergenicity	*nano*, immunotoxicity, allergenicity, in vitro/in silico/in chemico*
In vitro toxicity	*nano*, toxicity, test*, in vitro/in silico/in chemico*
Mutagenicity	*nano*, mutagen*, in vitro*
Neurotoxicity	*nano*, neurotoxicity, in vitro/in silico/in chemico*
Phototoxicity	*nano*, phototoxicity, in vitro/in silico/in chemico*
Repeated dose toxicity (short-term)	*nano*, repeated, dose, toxicity, short*, in vitro/in silico/in chemico*
Repeated dose toxicity (sub-chronic)	*nano*, repeated, dose, toxicity, sub-chronic, in vitro/in silico/in chemico*
Repeated dose toxicity (long-term)	*nano*, repeated, dose, toxicity, long-term, in vitro/in silico/in chemico*
Reproductive toxicity	*nano*, reproductive, toxicity, in vitro/in silico/in chemico*
Respiratory sensitization	*nano*, respiratory, sensitization, in vitro/in silico/in chemico*
Skin corrosion	*nano*, skin, corrosion, in vitro/in silico/in chemico*
Skin irritation	*nano*, skin, irritation, in vitro/in silico/in chemico*
Skin sensitisation	*nano*, skin sensitization, in vitro/in silico/in chemico*
Stability in lysosomal fluid	*nano*, lysosomal, fluid, in vitro/in silico/in chemico*
Toxicokinetics	*nano*, toxicokinetics, in vitro/in silico/in chemico*

The search results are shown in Figure 8. Between years 2010 – 2022 we identified a total of 522 articles and 40 SOPs that address the identified endpoints. The endpoints for which most documents were found (>45) were: reproductive toxicity, neurotoxicity, immunotoxicity, acute toxicity (oral) and phototoxicity. On the other hand, hardly any literature was found for long term and sub-chronic repeated dose toxicity and respiratory sensitization.


Number of identified publications per each endpoint
Immunotoxicity 50
Reproductive toxicity 49
Acute toxicity oral route 49
Neurotoxicity 47
Phototoxicity 45
In vitro toxicity 42
Acute toxicity inhalation 39
Gastrointestinal digestion 32
Hypersensitivity 31
Mutagenicity 24
Toxicokinetics 19
Skin irritation 18
Carcinogenicity 16
Skin sensitisation 14
Effects on gut microbiome 12
Acute toxicity dermal route 9
Developmental neurotoxicity 7
Eye damage/irritation 4
Dermal absorption 4
Skin corrosion 3
Endocrine disruption 3
Stability in lysosomal fluid 2
Repeated dose toxicity short-term 2
Repeated dose toxicity long-term 1
Respiratory sensitization 0
Repeated dose toxicity sub-chronic 0
0 10 20 30 40 50 60



	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Toxicokinetics	1	0	0	3	1	4	0	1	0	1	1	5	2
Stability in lysosomal fluid	0	0	0	1	0	0	0	0	0	0	0	0	1
Skin sensitisation	0	2	1	2	0	0	1	1	0	0	1	6	0
Skin irritation	0	4	1	2	1	1	2	2	0	1	2	2	0
Skin corrosion	0	0	0	1	0	0	2	0	0	0	0	0	0
Reproductive toxicity	0	2	1	1	2	2	4	4	3	8	4	10	8
Repeated dose toxicity short-term	0	0	0	0	1	0	0	0	1	0	0	0	0
Repeated dose toxicity long-term	0	0	0	0	0	0	0	0	1	0	0	0	0
Phototoxicity	2	3	9	4	7	6	4	2	3	2	1	1	1
Neurotoxicity	2	0	2	1	0	4	7	6	3	7	6	5	4
Mutagenicity	2	0	0	2	3	2	3	4	0	2	2	3	1
In vitro toxicity testing	1	2	2	6	6	7	1	3	3	3	6	1	1
Immunotoxicity	1	2	0	6	9	5	4	5	5	2	9	0	2
Hypersensitivity	1	0	1	1	5	1	4	1	7	1	3	4	2
Gastrointestinal digestion	0	0	0	2	0	2	1	2	2	6	5	9	3
Eye damage/irritation	0	1	0	1	0	0	1	0	0	1	0	0	0
Endocrine disruption	0	0	0	0	1	0	0	0	0	1	1	0	0
Effects on gut microbiome	0	0	0	0	0	0	2	0	1	2	1	3	3
Developmental neurotoxicity	0	0	0	2	0	1	0	0	0	1	2	0	1
Dermal absorption	0	0	0	0	2	1	0	1	0	0	0	0	0
Carcinogenicity	0	0	3	2	0	0	1	1	2	1	2	3	1
Acute toxicity oral route	1	0	4	3	2	7	1	5	7	6	3	7	3
Acute toxicity inhalation	0	3	2	6	2	6	2	2	2	3	4	3	4
Acute toxicity dermal route	0	2	0	3	0	1	1	0	2	0	0	0	0

Figure 8. *Identified publications for specific endpoints. The upper chart presents the total number of records assigned to each endpoint. The lower chart presents the number of records assigned to each endpoint published in the years 2010 – 2022.*

It has to be highlighted, however, that due to a high number of endpoints, the search methodology was unified which may have resulted in some documents not being found. Additionally, search

engines and tools rely on different databases and algorithms, resulting in different records being returned. Therefore, the designated collection of documents served as the initial base for all subsequent actions within the project, and any additional documents that were found during the duration of the contract were actively incorporated into the final repository of NAMs.

Alternative methods for assessing human safety of nanomaterials

The map of required legal information requirements with the relevant hazard endpoints for regulatory purposes (Table 4) enabled the identification of endpoints for which alternative methods could be considered in further assessments. All listed endpoints were included in the map, regardless of the number of regulations that included them. For example, while gastrointestinal digestion *in vitro* is only required in EU Food regulation (Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001), all recent achievements in nano-specific alternative methods for this endpoint were researched thoroughly.

The only exception from a legal requirement is the case of "Toxicity *in vitro* testing", which referrs to an endpoint recommended by EFSA in "*Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health*"[14] (2021) and relevant for European food regulation. The EFSA[14]Guidance outlines a structured approach for testing of ENMs for identification and characterisation of toxicological hazards. In Step 2, EFSA recommends that all available information should be gathered, and a set of *in vitro* studies carried out to identify the hazards and any need for further testing. In Step 3, "Specific endpoints relevant for *in vitro* testing are: cytotoxicity/cell viability, induction of oxidative stress, (pro-)inflammation and gastrointestinal barrier integrity impairment". For Cosmetics regulation, the SCCS Nanoguidance also recommends assessing in vitro testing, although it is not explicitly mentioned in the regulation.

In the present Report, as majority of the analysed NAMs also can be classified into the category of "Toxicity *in vitro* testing", this endpoint was considered relevant for the other effects as well (e.g., acute toxicity oral or by inhalation) and added as the 'Alternative regulatory relevant endpoint' in the "Annex 2_List of NAMs assigned to toxicological endpoints". Additionally, it needs to be highlighted that in the case of endpoints measuring the same adverse outcomes (mutagenicity, repeated dose toxicity (RDT) and reproductive toxicity), but which may differ in experimental study designs (e.g., different mutagenicity endpoints like gene mutations or chromosomal aberrations, 28-days vs. 90-days exposure RDT, one-generation vs. two-generation reproductive toxicity study), these endpoints were combined in order to search all relevant NAMs used to determine the main adverse effects. In this Report less importance was given to *in silico* nano-specific NAMs, as these are planned to be assessed in a separate review prepared by other authors.

In order to find alternative methods to assess toxicological endpoints relevant to human hazard of chemicals, including nanomaterials, the collected literature was reviewed (i.e., OECD TG/GD, ISO standards, ECVAM repositories, SOPs, scientific publications, Nano-relevant AOPs, EU project deliverables and OECD Working Plans). Modification/adaptation of the already available scientifically justified and interlaboratory validated testing strategies might be more beneficial than starting with developing new methods from the scratch. Such approach would

shorten the time needed for development of NAMs dedicated solely for nanomaterials. Therefore, at first, methods that have already gained regulatory acceptance and/or were under validation for conventional chemicals were searched. The possibilities to apply these NAMs for nanomaterials were assessed. Then, methods developed intentionally for ENMs were collected. In effect, the following categories of NAMs were distinguished, Figure 9:

1. non-nano specific regulatory accepted NAMs
2. non-nano specific under validation NAMs
3. nano-specific regulatory accepted NAMs
4. nano-specific under validation NAMs
5. nano-specific under development NAMs

It is essential to emphasize that while there are numerous valuable publications available, they were not chosen because they do not adequately represent the ENMs testing for the specific endpoints in our NAM database. Consequently, we have exclusively selected publications that we believe to be most representative. We extend our sincere apologies to authors whose publications were not examined by us and consequently not included in the Report.

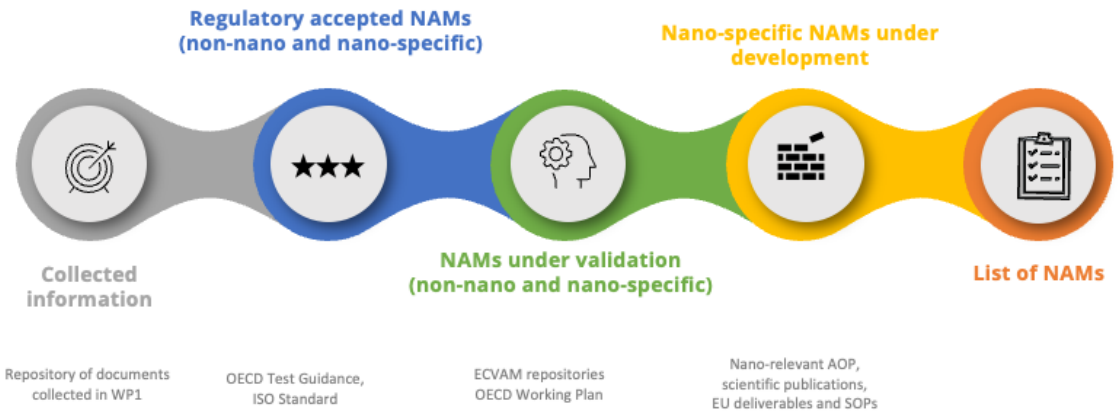


Figure 9. *Strategy for NAMs-relevant information searching*

1. Non-nano specific regulatory accepted NAMs refer to methods that were intentionally developed for conventional chemicals and have already gained regulatory acceptance. In this category, all relevant, available OECD TGs were reviewed. As a result, 68 approaches were identified and assigned to specific human health relevant endpoints, Table 7. The highest number (22) of alternative methods were available for serious eye damage/eye irritation *in vitro*. Some of these NAMs were included in the proposed IATA (OECD GD 263[15]). Based on combination of *in vitro* and *in silico* methods it is possible to assign chemicals or their mixtures to either 1) causing "serious eye damage" or 2) not requiring classification for eye irritation or serious eye damage according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and EU Classification, Labelling and Packaging of Substances and Mixtures regulation (CLP). In 2022, also OECD TG 467: Defined Approaches for Serious Eye Damage and Eye Irritation was adopted in which the prediction from a DA may be used alone to determine eye hazard potential

according to the hazard classes of the UN GHS (Categories 1, 2, or not classified). The second endpoint with the highest number of available regulatory accepted NAMs (11 NAMs) is skin sensitization *in vitro*, for which OECD Guideline No 497[16] containing the defined alternative approaches on skin sensitisation were published in June 2021. This guideline uses the combination of *in chemico* and *in vitro* OECD validated tests combined with *in silico* approaches for estimating potential dermal sensitization hazard. Similarly, to eye irritation/damage, the chemicals are categorized to a class defining their sensitization potential according to GHS. The obligation to reduce the risk for consumers after exposure to cosmetics ingredients, raised the need to develop reliable methods for assessment of potential harmful skin effects of such ingredients. The development of alternative non-animal testing strategies for skin relevant endpoints was additionally motivated since 2013 by a ban on animal testing applying to both the final formulation and the ingredients of the product.
Nevertheless, for several regulatory relevant endpoints (e.g., acute toxicity dermal, acute toxicity inhalation, developmental neurotoxicity, effect on gut microbiome, hypersensitivity, immunotoxicity, neurotoxicity, repeated dose toxicity, respiratory sensitisation, stability in lysosomal fluid) so far regulatory accepted NAMs are not available even for the conventional compounds. Since in all available regulations these endpoints are covered only by *in vivo* studies, it seems that for these endpoints there is an extensive need for developing alternative approaches in the future.

2. Non-nano specific under validation NAMs collect methods proposed for conventional chemicals that, according to ECVAM repository status, are currently in the stage of validation or peer-reviewed process. Here, 19 methods were identified. However, it needs to be stressed that within this category were also included methods that, according to EURL ECVAM (Scientific Advisory Committee, ESAC) have been already validated but not yet implemented in OECD guideline/guidance. The majority of these NAMs consider endocrine disrupting activity or developmental toxicity and one has been proposed for eye damage (Table 7).

3. Nano-specific regulatory accepted NAMs describe methods that have already gained regulatory acceptance and were intentionally developed for nanomaterials. In this category, the ISO standards were reviewed (up to now, regulatory accepted nano-specific NAMs recommended by OECD are not available), and 1 method was identified and assigned to a relevant endpoint (ISO Standard for the phototoxicity induction by nanomaterials, Table 7) although ISO standards are available for testing for example cytotoxicity or ROS generation by nanomaterials. These regulatory accepted methods can be applied to test key events which are identified as integral parts of various adverse outcomes.

4. Nano-specific under validation NAMs refer to several methods developed for nanomaterials that according to OECD Working Plan are currently under validation.

5. Nano-specific under development NAMs is a category that covers all recently proposed methods that have the potential to be applied for assessing human safety of nanomaterials and are presented to the public area through scientific publications, EU project deliverables, SOPs developed within projects, individual labs, etc. and nano-relevant AOPs. Many methods were

assigned to this category. Most of the methods were dedicated to mimic acute or repeated dose toxicity testing of nanomaterials following inhalation exposure.

It needs to be highlighted here that there are NAMs that were assigned to the several endpoints simultaneously. For example, the same NAMs are assigned to acute toxicity inhalation, toxicity *in vitro*, carcinogenicity and repeated dose toxicity because the same protocols can be used for evaluating different effects, after some adjusting for duration of exposure, or set of endpoints measured. It means that within 50 methods applied to assessing toxicity *in vitro* there are 24 NAMs already assigned to other endpoints.

Table 7. *The number of identified NAMs for each endpoint and classification of the NAMs according to their stage of regulatory acceptance and development*

Regulatory relevant endpoint	*Nano-specific regulatory accepted NAMs*	*Nano-specific under development NAMs*	*Nano-specific under validation NAMs*	*Non-nano-specific regulatory accepted NAMs*	*Non-nano-specific under validation NAMs*
Acute toxicity oral	-	1	-	1	-
Acute toxicity inhalation	-	26	-	-	-
Acute toxicity dermal	-	-	-	-	-
Carcinogenicity	-	1	-	3	-
Dermal absorption	-	-	-	1	-
Developmental neurotoxicity	-	2	-	-	-
Effects on gut microbione	-	-	-	-	-
Endocrine disruption	-	-	-	5	14
Eye damage/eye irritation in vitro	-	-	-	22	1
Gastrointestinal digestion	-	12	1	-	-
Hypersensitivity/Food intolerance	-	-	-	-	-
Immunotoxicity/developmental immunotoxicity/allergenicity	-	8	-	-	-
In vitro toxicity testing* *cytotoxicity/cell viability* *oxidative stress* *(pro-)inflammation* *gastrointestinal barrier integrity*	5	26	-	-	-
Mutagenicity/genotoxicity	-	11	2	6	-
Neurotoxicity	-	2	-	-	-
Phototoxicity	1	-	1	3	-
Repeated dose toxicity	-	17	-	-	-
Reproductive toxicity/Endocrine disruption/Developmental toxicity	-	3	-	3	3
Respiratory sensitization	-	-	-	-	-
Skin corrosion in vitro	-	-	-	6	-
Skin irritation in vitro	-	-	-	6	-
Skin sensitisation in vitro/in chemico	-	-	1	11	-
Toxicokinetics	-	11		-	1
Water solubility and dissolution in biological media (including stability in lysosomal fluid)	2	-	-	1	-
Total number of NAMs:	**8**	**120**	**5**	**68**	**19**

* In the case of '*in vitro* toxicity testing', this endpoint is recommended by EFSA (2021: Guidance on risk assessment of nanomaterials) and relevant for the food regulations (Regulation (EU) No 2015/2283 amending Regulation (EU) No 1169/2011 and repealing Regulation (EC) No 258/97, Regulation (EC) No 1852/2001), and refers to testing cytotoxicity/cell viability, oxidative stress, (pro-)inflammation, gastrointestinal barrier integrity after exposure to different food components. However, many

of the NAMs included in the table for 'Toxicity *in vitro* testing' can be useful in assessment of other endpoints (e.g., acute toxicity inhalation or oral).
There is also ongoing work for a new OECD TG on toxicokinetics specifically for ENMs for all routes of exposure (New Test Guideline on toxicokinetics to accommodate testing of nano-particles), however this methodology cannot be classified as a NAM.

In total 220 NAMs were identified (data gathered to date 30.12.2022). Detailed information on each method with references (including method description) is provided in **Annex 2** (List of NAMs assigned to toxicological endpoints.xlsx). Each of the NAM has an individual ID, the same IDs are used in the Report where NAMs are being described. In this Report in cases of the NAMs under development we assessed only those NAMs which were intentionally proposed for nanomaterials (irrespective of their type) testing. If an original NAM description (not necessarily used for nanomaterials) was available in a previous source publication, a reference to such publication was provided in the NAMs description column in the source Excel File (**Annex 2**). If a review paper or other document (e.g., website of manufacturer) was found on a NAM or group of NAMs relevant for conventional chemicals but not yet used specifically for nanomaterials, a reference to such source was additionally included in the description of a nano-specific NAM from similar category.
In case of NAMs classified as non-nano specific, their possible adaptation for nanomaterials was verified. For this purpose, the scientific publications where those methods were applied for nanomaterials were searched and critically analysed. Each scientific publication considered was evaluated according to the GuideNano quality scoring system.[13] This procedure allowed to evaluate if all necessary information in terms of substance characterisation as well as methods performance are provided to ensure transparency and feasibility of the process. The goal was to consider in further analysis only papers with K- and S-score of 1-2. However, due to failure to meet these criteria by the majority of papers containing NAMs, it was decided to consider all collected literature to keep a broader perspective.

A. Documents/repositories highly relevant for NAMs but not discussed in detail in the report

The Authors of the Report are aware of the whole set of extremely valuable assay endpoints developed in the TOXCAST project.[17] However, for clarity of the document and due to the fact that these assays are still under development, and that their applicability for ENMs testing has not yet been proven, these tests methods have not been assessed in this Report.
Other relevant documents/repositories:

- More and more very relevant cell-based and exposure systems are being developed by different manufacturers, especially related to respiratory toxicity:
    - AlveoliX[18]
    - Epithelix Sárl[19]
    - Emulate[20]
    - ImmuONE[21]
    - MatTek Corporation[22]
    - TissUse [23]
    - Cultex(R) Laboratories[24]
    - MedTec[25]
    - Vitrocell Systems GmbH[26]

- Series of Case Studies on the Use of Integrated Approaches for Testing and Assessment for different toxicological endpoints are available as the OECD publications.[27]
- Webinars presented during meetings organized by The American Society for Cellular and Computational Toxicology (ASCCT) which is dedicated to the promotion of toxicology testing and research that reduces and replaces the use of animals are available on the Society website.[28]
- Databases on Alternative Methods Validated For Regulatory Use are available (e.g., by PETA Science Consortium International e.V.[29]
- OECD (2018), Guidance Document on Good In Vitro Method Practices (GIVIMP), OECD Series on Testing and Assessment, No. 286, OECD Publishing, Paris.[30]
- Review publications on regulatory aspects of NAMs when used for safety testing,[31,32] or reflecting a US regulatory perspective.[33]

It is important to acknowledge that the adoption of FAIR (findable, accessible, interoperable and reusable) principles in the development of NAMs (and nano-specific NAMs) is of paramount significance. Since 2016, there has been a growing endorsement of the application of FAIR principles in the field of nanosafety,[34] which are now considered a standard for data and metadata management and stewardship. The FAIR principles emphasize improved findability, accessibility, interoperability, and reuse of digital objects through the FAIRification process, with the aim of making scientific data more machine-actionable, thereby enhancing data handling by both humans and computer systems.[35] These principles and guidelines are not only relevant in the field of data science, but can (and should) be standardized and then applied wherever possible. Unfortunately, given the intricate nature of developing, validating, and obtaining regulatory acceptance for NAMs, the implementation of FAIR principles will require a significant amount of time and effort to be realized.

B. NAMs assigned to toxicological endpoints

Detailed information about all collected NAMs can be found in **Annex 2**, while below, a summary for each endpoint is provided. While referring to a particular NAM, its ID is provided. By using this ID, further information about this method can be found in **Annex 2**. Current data gaps and needs in the context of NAMs application in order to fulfil nanomaterials-specific testing requirements are listed in the tables that summarize findings about each regulatory relevant endpoint. For general considerations on *in vitro* testing of ENMs, which should be applied to increase reliability and relevance of the studies, the reader is referred to e.g., reviews by Drasler et al., 2017[36] and Elespuru et al., 2022[37].

Acute toxicity

In terms of human health safety assessment, the possibility to detect effects which may occur following accidental or deliberate short-term exposure to a chemical is needed. In general, acute toxicity refers to the adverse effects observed after the oral or dermal exposure to a single dose of a chemical or to multiple doses administrated within 24 hours, or inhalation exposure for 4 hours. According to the REACH[38] Guidance on Information Requirements (page 359 of the Guidance) the acute toxicity can be defined as "*The adverse effects that can be seen as mortality, clinical signs of toxicity (for animals, refer to OECD Guidance Document 19*[39]*), abnormal body*

weight changes, and/or pathological changes in organs and tissues. In addition to acute systemic effects, some substances may have the potential to cause local irritation or corrosion of the gastrointestinal tract, skin, or respiratory tract." In this context, three main types of toxic effects can be considered: (i) general basal cytotoxicity, (ii) selective cytotoxicity, and (iii) cell-specific function toxicity. Additionally, acute toxicity may refer to disturbances of extracellular processes. The information on this endpoint is required under REACH Regulation ((EC) No 1907/2006); Biocidal Regulation ((EU) No 528/2012); Cosmetics Regulation ((EC) No 1223/2009); and Feed Additives Regulation ((EC) Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003).

The majority of testing strategies available so far to assess this endpoint, include animal testing. In case of acute toxicity by the oral route, several testing strategies to assess this endpoint *in vivo* have already been developed (e.g., OECD TG 420[40], 423[41], 425[42]). Although, there is one regulatory accepted non-animal-based approach (OECD GD 129[43]), it can be used exclusively to estimate the starting doses for acute oral systemic toxicity and does not fully replace animal testing. It can be used in an overall Weight-of-Evidence (WoE) approach.

According to OECD TG 402[44] (Acute Dermal Toxicity), the study aimed at assessing the acute toxicity of substance by the dermal route also requires animal testing. For this endpoint no NAMs were found in the available literature. However, it is recommended that performing *in vivo* acute dermal toxicity should be only considered after other potential alternative dermal toxicity studies (e.g. irritation or corrosion) have been evaluated in a WoE analysis.

There are a number of OECD documents that assist in evaluation of the potential of chemicals to induce acute toxicity by inhalation (e.g., OECD TG 403[45], 412[46], 413[47], 433[48] and 436[49]) and all of them are based on animal testing. Since inhalation is one of the main routes of exposure to nanomaterials, there is an urgent need in developing or adapting non-animal-based methods for ENMs testing. Recently, in effect of the discussion between the Working Party on Manufactured Nanomaterials (WPMN) and the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) the revision of existing TGs has been accomplished in order to accommodate nanomaterials and reduce the animal numbers.[50] The OECD GD 39[51] was also updated in the view of nanomaterials' testing. However, because it still includes the necessity of employing the battery of animal tests, there is an urgent need to develop non-animal testing strategies for assessing this endpoint. This is also strengthened in Europe by initiatives within the Malta initiative [52] and NanoHarmony projects[53].

Recently, a number of different NAMs for testing acute toxicity of nanomaterials were proposed. A summary of NAMs considered as relevant for acute toxicity with their possible adaptation/limitations for nanomaterials is provided in Table 8. Presented NAMs were subject of scientific publications but also considered or developed as SOPs within the EU sponsored PATROLS project. In the GRACIOUS project grouping hypotheses and tailored IATA for respiratory toxicity of inhaled ENMs were proposed. These strategies can be used to support decision making regarding Safe(r)-by-Design product development or adoption of precautionary measures to mitigate potential risks.[54] The IATAs can also be used to support read-across of adverse effects such as pulmonary inflammation and subsequent effects such as lung fibrosis and lung tumour formation after long-term exposure.

Table 8. Summary of NAMs relevant for acute toxicity

<table>
<tr><th colspan="4">Endpoint: Acute Toxicity</th></tr>
<tr><th>Advancement status of the NAMs in ENMs testing</th><th>Potential limitations of the NAMs for adaptation for ENMs testing</th><th>Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)</th><th>Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)</th></tr>
<tr><td colspan="4">Non-nano specific regulatory accepted NAMs
By oral route
• 3T3 Neutral Red Uptake cytotoxicity assay to estimate starting doses for acute oral systemic toxicity tests[55–57][ID_28]</td></tr>
<tr><td>• The methods have been validated for conventional chemicals, and no official validation trials for ENMs are ongoing.
• The method allows to determine the in vitro basal cytotoxicity of test substances using NRU assays in order to apply this in vitro data to determine starting doses for in vivo acute oral systemic toxicity tests.
• This test cannot replace the animal testing, but it may be used in WoE approach.</td><td>Further studies are needed for better understanding the complex mechanisms of acute toxicity after different routes, and how these mechanisms can be translated into NAMs.</td><td>Low

Validated NAMs ready for regulatory testing are not available.

The 3T3 NRU test is probably not used for regulatory testing of ENMs.</td><td>High

The acute toxicity endpoint is crucial for many regulations, however there are no validated NAMs methodologies are not available.

3T3 NRU method has the potential to be included in international standards and guidance on Colony-forming efficiency assays to assess cytotoxicity of nanomaterials. It can be stated that after slight modifications (mainly preparation of suspensions, assuring dosing, etc.) the already available method for conventional chemicals might be used for nanomaterials testing.</td></tr>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs

<u>By inhalation route:</u>
Submerged single cultures
• Cell culture of lung epithelial cell-line (A549)[58] [ID_17]
• An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials[59] [ID_25]

Double co-cultures
• Lung tissue model: epithelial cell line (H441) and the endothelial cell line (ISO-HAS-1)[60] [ID_1]</td></tr>
</table>

- Co-culture: human lung epithelial (NCI-H441)/ macrophages derived from a monocytic cell line (dTHP-1) cell lines[61] [ID_8]
- Co-culture: human small airway epithelial/ human microvascular endothelial cells[62, 63][ID_4]
- Co-culture: human lung epithelial cells (A549)/ differentiated human macrophages[64] (THP-1) cell lines [ID_6]
- Co-culture: lung epithelial (Calu-3)/ macrophages derived from a monocytic cell line[65] (dTHP-1) [ID_16]

Triple co-cultures

- 3D *in vitro* triple human cell co-culture model: epithelial cells (16HBE14o-)/monocyte-derived macrophages/monocyte-derived dendritic cells[66] [ID_26]

Air Liquid Inteface

Air Liquid Interface (ALI): EpiAirway™ cytotoxicity model

- Air Liquid Interface (ALI): EpiAirway™ cytotoxicity model[67] [ID_3]
- Air Liquid Interface: monoculture of human lung epithelial cells (lung cancer A549) using VITROCELL System[64] [ID_5]
- Air Liquid Interface: co-culture: bronchial epithelial (Calu-3)/ primary macrophages using VITROCELL® Cloud12 system[65,68] [ID_7]
- Air Liquid Interface: triple co-culture: epithelial (A549)/ human peripheral blood monocyte-derived dendritic/ macrophage cells[69–72] [ID_9]
- Air Liquid Interface: triple co-culture: epithelial (A549)/ endothelial (EA. Hy 926)/ macrophage (dTHP-1) cell lines[73] [ID_105]
- Air Liquid Interface: tetra-culture: alveolar type II cell line/ differentiated macrophage-like cells/ mast cells/ endothelial cells[74] [ID_10]
- Air Liquid Interface: P.R.I.T.® ExpoCube®[75] [ID_11]
- Air Liquid Interface: EpiAlveolar model (Mattek™)[76] [ID_12]
- MucilAir™ Reconstituted primary human airway epithelial model with Vitrocell® Cloud exposure system[77] [ID_13]
- VITROCELL® Cloud System for aerosolization[78] [ID_14]
- Air Liquid Interface: human lung epithelial (NCI-H441) cell line[58] [ID_18]
- Quasi-Air Liquid Interface exposure of nanoparticles[79] [ID_19]
- 3D *In Vitro* inflammatory lung co-cultures[80] [ID_20]
- Air-Liquid Interface: moving bioreactor (MALI)[81] [ID_23]
- Air-Liquid Interface: Dynamic Model for the Alveolar Interface (DALI)[82] [ID_24]

Body-on-a-chip system (also referred to as Multi-tissue microphysiological system or Micro Cell Culture Analog)[83,84] [ID_21, ID_27]

Other

- Constrained drop surfactometer to test the function of lung surfactant *in vitro*[85,86] [ID_2]
- Air Liquid Interface: Captive Bubble Surfactometry[87] [ID_22]
- ENMs lung dosing consideration based on *in silico* analysis for Dörntruper quartz (DQ12), barium sulphate (BaSO4), cerium oxide (CeO2), and titanium dioxide (TiO2), and multi-walled carbon nanotubes (MWCNT) [ID_15]

By oral route:

Submerged single cultures

- SOP - 3D *In Vitro* HepG2 Spheroid Model[88] [ID_107]
- SOP - 4401-PATROLS-3D high throughput screening of HepG2 cells[89] [ID_179]

Double co-cultures - SOP - 3D In Vitro HepG2, Kupffer cell co-Culture spheroid model[90] [ID_178] ***Triple co-cultures*** - SOP - Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells[91] [ID_193] - Culture and characterisation of mono and multi-cellular models of the gastrointestinal system[92] [ID_194] ***Tissue model*** - SOP - Tissue characterisation, nanomaterial treatment and toxicological assessment in 3D primary human liver microtissues[93] [ID_60] - SOP - 4203-PATROLS Evaluation-of-nanomaterial-induced-hepatotoxicity-in-a-primary-human multi-cellular microtissue model with emphasis on physiologically meaningful toxicological end-points[94–98] [ID_106]			
Advanced 3D lung and intestinal cultures *in vitro* provide a physiologically relevant assessment of the hazards associated with ENM exposures over both an acute and chronic, repeated dose regime.	Standard 2D model systems have their limitations, and it is widely accepted that they do not adequately represent the biological matrix *in vivo*. Advanced, 3D models in this sense have received credibility and pose a potential valid alternative to invasive *in vivo* approaches. There are many publications or other documents indicating successful usage of 2D or 3D lung or intestinal models for ENMs testing. However, some more adaptations and improvements in line with the general requirements of nanotoxicology is still needed (e.g., suitable physicochemical characterisation of pristine ENMs, in suspensions, dosing, etc.). Especially further standardization with improved repeatability is needed.	Limited The NAMs as a group show great promise however, they still need comprehensive validation tests before gaining acceptance in scientific and regulatory community. The use of a cell line does not cover the complexity of the lung, several of the characteristics of primary cells, or interindividual variability. Some cells (e.g., NCI-H441) cultured at an air-liquid interface will only remain stable for ENM exposures until few days after being switched to an air-liquid interface (ALI). There are also restrictions placed on some of the cell lines which state the cells are only permitted for use for research purposes and proposed commercial uses must be negotiated with the supplier (e.g., National Cancer Institute). Complex cell co-cultures and ALI systems require extensive training and experience in using them. Most of the models currently use static media without flow, and do not include membrane flexing to simulate peristalsis or bronchial movement.	High The lung and gastrointestinal cell models are amongst the most extensively developed and required for regulatory testing.

		The 3D HepG2, Kupffer cell spheroid model system is only viable for 14 days in culture (to date) as continued proliferation results in the formation of a necrotic core in the centre of the spheroid. As a result, longer term or repeated exposures of up to 7-10 days can be conducted on this 3D model system as the viability of cells within the spheroid during this period, according to the Trypan Blue Assay, remain above 80%. Primary Human Kupffer Cells are expensive, cannot be sub-cultured and with limited stocks can introduce donor to donor variation, reducing the reproducibility and cost-effective nature of the hepatic spheroid model. Some endpoints like detection of the cytokines (e.g., IL-1β and TNF-α at both, the protein secretion and gene expression levels), is at the lower detection limit when 12-well plates are used (as opposed to 6-well plates). This may limit high throughput methodology.	

Carcinogenicity

The multiple-step process of transition of normal cells into cancer cells is recognized as crucial under various EU regulations related to establish safety criteria for human exposure (Regulation (EC) No 1907/2006; Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009; Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003). According to REACH[36] Guidance on Information Requirements (page 585 of the Guidance) "*chemicals are defined as carcinogenic if they induce tumours, increase tumour incidence and/or malignancy or shorten the time to tumour occurrence*." The route of exposure (by inhalation, ingestion, dermal application or injection), patterns and duration of the exposure may impact the carcinogenic potential of chemicals. The process of transition of normal cells into cancer cells involves several steps that may involve both genetic and non-genetic changes. For this reason, ECHA has divided chemicals causing different changes leading to carcinogenesis into non-genotoxic and genotoxic. Non-genotoxic substances may induce epigenetic changes that do not alter the DNA, however, may affect e.g., gene

expression, or cell-cell communication, whereas genotoxic substances chemically interact with the DNA and change the primary DNA structure. The approaches for evaluation of carcinogenicity of chemicals are based on careful observation of the animals exposed to stressor allowing the identification of a point of departure for risk assessment (e.g. the NOAEL and/or BMD), as well as characterisation of the tumour dose-response relationships and extrapolation of carcinogenic effects to low human exposure levels as indicated in majority of regulations (e.g., OCED TG 451[99], 452[100], 453[101]). Proposed *in vivo* strategies rely on the assessment of genotoxic carcinogens, and in spite of many efforts currently undertaken even at the OECD level, there is a lack of regulatory accepted approaches applicable for testing non-genotoxic carcinogens. Due to different (in many cases still unknown) mechanism of carcinogenesis, conducting the non-animal studies for efficient testing of the carcinogenic potential of chemicals is not a trivial task. So far, two *in vitro* cell transformation assays (CTA) (OECD GD 214[102], OECD GD 231[103]) were proposed as alternative methods to *in vivo* approaches.

Recently, one NAM for assessment the carcinogenicity of nanomaterials was proposed and is available as SOP on the PATROLS project website (Table 9).

Table 9. *Summary of NAMs relevant for carcinogenicity*

Endpoint: Carcinogenicity			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • Cell Transformation Assay in vitro on Syrian hamster embryonic (SHE) cells at pH 6.7[104] [ID_29] • Cell Transformation Assay in vitro on the Bhas 42 cells[105] [ID_30] • Cell Transformation Assay in vitro using Syrian hamster embryonic (SHE) cells at pH 7[106] [ID_31]			
• The methods have been validated for conventional chemicals, however official validation trials for ENMs have not started up to now. • Quite many publications are already available on CTAs with ENMs, showing both positive and negative results.	• The general biological limitations of the CTAs apply also for ENMs testing.	High It seems that the CTAs can be successfully applied for ENMs testing after slight adaptations, in line with the general requirements of nanotoxicology (e.g., providing suitable physicochemical characterisation of pristine ENMs, in suspensions, dosing, etc.)	High Carcinogenicity testing is required by many regulations, hence besides CTAs which are at the moment the only useful alternative method for assessing carcinogenicity, development of other NAMs is of great importance for regulatory purposes. NAMs for testing non-genotoxic carcinogens are urgently needed, as well. For conventional chemicals there are initiatives started at the OECD level to develop IATAs for non-genotoxic carcinogens.

<table>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs
Quantitative reverse transcription polymerase chain reaction (RT-qPCR) with Hepatocarcinoma PCR Array plates (Bio-Rad 10034966)[107] [ID_32]</td></tr>
<tr><td>• The NAM has been developed for nanomaterials; however official validation trials have not yet started.</td><td>• The protocol has been developed based on predefined disease pathway PCR array plates, hence may be representative of only a part of relevant genes.
• The PCR array plates can be costly and therefore it may not be feasible to run each sample in triplicate</td><td>Limited

The NAM represents gene expression changes associated with cell transformation induction. However, the set of the genes has not been formally validated (especially in HepG2 cancer cell spheroids), hence its relevance is not certain at the moment.</td><td>High

Carcinogenicity testing is required by many regulations, hence NAMs for assessing carcinogenicity are of great importance for regulatory purposes.</td></tr>
</table>

Dermal absorption

Dermal absorption is an important parameter that should be assessed under the Cosmetic Regulation ((EC) No 1223/2009) that permits only using non-animal testing strategies. According to the SCCS [108] (page 6 of the Opinion) "*the percutaneous/dermal absorption process is a global term which describes the passage of compounds across the skin. This process can be divided into three steps: 1. penetration, which is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum; 2. permeation, which is the penetration through one layer into another, which is both functionally and structurally different from the first layer; 3. resorption which is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment*." Up to now, there is one accepted and recommended *in vitro* method (OECD TG 428[109]) that allows to measure the diffusion of the substances into and across skin. Skin from different mammalian species, including humans and pigs, can be applied for the purpose. Non-vital skin is applied to measure only the diffusion, while fresh, metabolically active skin enables investigating skin metabolism as well.
Within the GRACIOUS project, to develop the grouping hypotheses and IATAs for dermal toxicity, the authors gathered and analysed existing information on skin irritation, skin sensitization, and dermal penetration of ENMs from the open published literature and performed experimental work to generate data on ENMs dissolution in sweat simulant fluids.[110] A summary of NAMs for dermal absorption is provided in Table 10.

Table 10. Summary of NAMs relevant for dermal absorption

Endpoint/ Parameters: Dermal absorption			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • Skin Absorption: *In Vitro* Method[109] [ID_33]			
• The method has been validated for conventional chemicals, however official validation trials for ENMs have not yet started.	Further studies are needed for better understanding the relationship between physicochemical properties of ENMs and the permeation through intact skin. It is generally accepted that dermal absorption is low for ENMs, especially for ENMs of higher than several nm in size, but may be significantly changed after using coatings or after impairing the integrity status of the skin. ENMs might be specifically designed for high dermal penetration, or as delivery systems (example of encapsulated materials).	High The OECD TG 428 on dermal absorption is relevant for testing ENMs after slight modifications of the protocol, by e.g., including proper physicochemical characterisation of ENMs, and performing careful analysis of nanoparticle translocation to skin layers and through the skin. In addition, the duration of the observation time, the sampling time, the ENMs solubility and the compatibility of the receptor fluid with ENMs need to be further explored. Also selection of the most appropriate analytical method for quantification may be an issue (see example of the SCCS Opinion on carbon black (nano)[108]).	High The endpoint is relevant for Regulation (EC) No 1272/2008 (Regulation on classification, labelling and packaging of substances and mixtures). Its assessment is required according to the SCCS Guidance on the safety assessment of nanomaterials in cosmetics. The NAM validated for conventional chemicals can be used for ENMs after adaptation for nanotoxicology basic rules (general considerations for in vitro testing of ENMs, which should be applied to increase reliability and relevance of the studies are reviewed in Drasler et al., 2017[36] and Elespuru et al., 2022[37]). Appropriate analytical techniques for quantification should be considered.
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs Not available			

Endocrine disruption

Exposure to the chemicals may lead to possible adverse effects connected with the endocrine system (e.g., developmental malformations, disorders of immune and nervous systems functions or increased cancer risk). This endpoint has to be considered in each regulation including the biocidal products, cosmetic and food additives (Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009; Regulation (EC) No 2015/2008, Regulation (EC) No 1331/2008, Regulation (EC) No 1333/2008, Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001) and has recently been taken up into Regulation (EC) No 1272/2008 (Regulation on classification, labelling and packaging of substances and mixtures; CLP regulation). Among methods that are currently accepted for testing the endocrine disruption are NAMs allowing for detection of substances with estrogen/androgen receptor binding affinity (OECD TG 455[111], OECD TG 456[112], OECD TG 458[113], OECD TG 493[114]). These methods provide mechanistical information and can be used for screening and prioritization purposes. There are also many methods which are currently under validation process supervised by the EURL ECVAM. They are focused on thyroid receptors (measuring e.g., inhibition of the metabolism and excretion of the thyroid hormones, inhibition of thyroid hormones deiodination, active transport of thyroid hormone T3 across the plasma membrane by monocarboxylate transporter 8, and others), however they have not yet been used/validated for ENMs.

Although there are studies indicating that nanomaterials can act as endocrine disruptors[115], validated alternative methods for assessing this potential are not available. Within this Report the possibility of using/adapting the accepted methods developed for conventional compounds and their application for nanomaterials was considered (Table 11).

Table 11. *Summary of NAMs relevant for endocrine disruption*

Endpoint: Endocrine disruption			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • Human Estrogen Receptor-α Transactivation Assay (ERα-HeLa-9903 cell line)[116] [ID_45] • The Freyberger-Wilson (FW) In Vitro Estrogen Receptor (ER) Binding Assay Using a Full-Length Human Recombinant ERα[117] [ID_54] • Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR-EcoScreen™ cell line)[118] [ID_55] • Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR-CALUX)[118,119] [ID_56] • H295R Steroidogenesis Assay[120] [ID_57]			
• The methods have been validated for conventional chemicals, however no official validation trials for ENMs are ongoing.	• Usefulness and limitations of the NAMs for ENMs testing are not fully understood at the moment. • It is very likely that most of the NAMs can be successfully applied	High It is very likely that most of the NAMs are relevant for ENMs regulatory testing after considering necessary nanotoxicological requirements.	High The endpoint is required by all major regulations and considering its complex nature development of NAMs for testing both conventional chemicals

	for ENMs testing after considering necessary nanotoxicological requirements (general considerations for in vitro testing of ENMs, which should be applied to increase reliability and relevance of the studies are reviewed by Drasler at al., 2017[36] and Elespuru et al., 2022 [37]).		and ENMs is of great importance. There are still major gaps in understanding the basic mechanisms of the endpoint and development of suitable NAMs is urgently needed.

Non-nano specific under validation NAMs

- Thyroid hormone disruption: In vitro human adipose stromal cell—human umbilical vein endothelial cell (hASC-HUVEC) vasculogenesis/angiogenesis method[121] [ID_39]
- Thyroid hormone disruption: Thyroxine-binding prealbumin (TTR) binding using fluorescence displacement (T4-FITC)[122] [ID_40]
- Thyroid hormone disruption: Inhibition of thyroid hormones sulfation using liquid chromatography[123] [ID_41]
- Thyroid hormone disruption: Inhibition of monocarboxylate transporter 8 (MCT8) based on Sandell-Kolthoff reaction[124] [ID_42]
- Thyroid hormone disruption: Tyrosine iodination measured using liquid chromatography[125] [ID_43]
- Thyroid hormone disruption: PATHHUNTER® BETA-ARRESTIN thyrotropin-releasing hormone (TRH) receptor activation (beta-galactosidase)[126] [ID_44]
- Thyroid hormone disruption: Deiodinase 1 activity based on Sandell-Kolthoff reaction[127] [ID_46]
- Thyroid hormone disruption: Activation of the sodium iodide symporter (NIS) based on Sandell-Kolthoff reaction[128] [ID_47]
- Thyroid hormone disruption: Thyroxine-binding prealbumin (TTR) / thyroxine-binding globulin (TBG) binding using fluorescence displacement (ANSA)[129] [ID_48]
- Thyroid hormone disruption: Measurement of proliferation of rat pituitary-derived cell line GH3[130] [ID_49]
- Thyroid hormone disruption: Human thyroid hormone receptor beta (TRβ) reporter gene transactivation assay (TRβ CALUX®)[131] [ID_50]
- Thyroid hormone disruption: Human thyroid hormone receptor alpha (TRα) and Human thyroid hormone receptor beta (TRβ) reporter gene transactivation assays[132] [ID_51]
- Thyroid hormone disruption: Thyroperoxidase (TPO) inhibition assay based on oxidation of Amplex UltraRed[133] [ID_52]
- Thyroid hormone disruption: Thyrotropin-stimulating hormone (TSH) receptor activation based on cAMP measurement[134] [ID_53]

Nano-specific regulatory accepted NAMs
Not available

Nano-specific under validation NAMs
Not available

Nano-specific under development NAMs
Not available

Mutagenicity/genotoxicity

Information about the ability of chemicals to cause genetic alterations in somatic and/or germ cells is required within safety assessment under a variety of EU regulations ((EC) No 1907/2006; (EU) No 528/2012; (EC) No 1223/2009; (EC) No 429/2008, (EC) No 1831/2003). According to REACH[38] Guidance on Information Requirements, mutagenicity is a component of genotoxicity and refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms[38] (page 551 of the Guidance). These may lead to transmissible changes in the amount or structure of the genetic material of cells or organisms and may involve single gene or gene segments, a block of genes or chromosomes. Genotoxicity is defined broader and also includes alterations in the structure of DNA that are not permanent. Definition of genotoxicity provided by REACH[38] (page 551 of the Guidance) indicates that it "*refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity.*" Genotoxicity may be tested by using methods measuring the induced DNA damages, e.g., DNA strand breaks, DNA adduct formation or mitotic recombination, as well as tests for mutagenicity.

According to REACH, the standard information required for assessing mutagenicity includes *in vitro* gene mutation study in bacteria (OEDC TG 471[135]), *in vitro* cytogenotoxicity study in mammalian cells or *in vitro* micronucleus study (OECD TG 487[136]), *in vitro* gene mutation study in mammalian cells (tests using the Hprt and xprt genes, OECD TG 476[137] and/or tests using the thymidine kinase gene, OECD TG 490[138]) and *in vitro* mammalian chromosomal aberration test (OECD TG 473[139]). Currently the Bacterial Reverse Mutation Test (OECD TG 471[135]) is not recommended for ENMs testing. According to REACH, in case of positive results in any of the *in vitro* genotoxicity study, *in vivo* studies (OECD TGs 474[140], 483[141]) are necessary.

A summary of NAMs considered as relevant for mutagenicity/genotoxicity with their possible adaptation/limitations for ENMs is provided in Table 12. The listed NAMs were subject of scientific publications but were also considered or developed as SOPs within the EU sponsored projects: PATROLS and DaNa. For a recent review of selected NAMs useful for genotoxicity testing of ENMs (e.g. the Alamar Blue assay, the colony-forming efficiency assay, the expression of anti-oxidative enzymes under the control of the nuclear erythroid 2-related factor 2 (NRF2) transcription factor) please refer to the special issue on "Methods and protocols in nanotoxicology" published in Frontiers in Toxicology (2022).

Table 12. *Summary of NAMs relevant for mutagenicity/genotoxicity*

Endpoint: Mutagenicity/genotoxicity			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • *In vitro* mammalian cell micronucleus test[142] [ID_81] • Bacterial Reverse Mutation Test[143] [ID_93] • Mouse lymphoma assay (MLA)[144] [ID_94] • TK6 test using the thymidine kinase (TK) locus[144] [ID_95] • *In vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes[145] [ID_96] • *In vitro* Mammalian Chromosomal Aberration Test[146] [ID_97]			

• The methods have been validated for conventional chemicals, there is one validation trial for ENMs ongoing at the OECD level. • Many publications are available in the open literature using the NAMs for ENMs, with both positive and negative results reported.	• Lack of reference ENMs available which have well established mutagenic properties. However, there are some ENMs which consistently yielded positive or negative results. • There is a general consensus that the NAMs validated for conventional chemicals can be successfully applied for safety assessment of ENMs but after slight adaptations specific for nanoparticles (e.g., using cytochalasin B after the exposure to ENMs, assuring nanoparticle cell internalisation in case of negative results, etc.).	High It is generally recognized that the NAMs validated for conventional chemicals can be used for genotoxicity testing safety assessment of ENMs but after implementation of relevant adaptations [37] .	High Mutagenicity testing is required by all relevant EU regulations. The validated NAMs are already available, but they require some adaptations before using.

Non-nano specific under validation NAMs
Not available

Nano-specific regulatory accepted NAMs
Not available

Nano-specific under validation NAMs

- Guidance Document on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials[147,148] [ID_82]
- 3D skin models: reconstructed skin micronucleus test and reconstructed skin comet assay[148] [ID_83]

• 3D skin models are ready to be internationally validated at the OECD level for the conventional chemicals, however not for ENMs.	• 3D skin models are mainly dedicated for testing conventional chemicals, but for ENMs they would need a validation step. It is well known that ENMs generally very poorly translocate through intact stratum corneum of the skin.	High Until now, 3D skin models have been mainly developed for cosmetic ingredient testing, especially as follow-up tests in case of positive results in basic testing. However, for ENMs, the situation is more complex considering their generally poor dermal absorption. Considering resemblance of the 3D skin models to real exposure conditions their relevance comparing to standard monocultures is superior.	High 3D skin models are relatively well characterised when used for conventional chemicals, however for ENMs testing they need to undergo a formal validation process. Development of more complex cell models mimicking real exposure conditions comparing to standard monocultures is needed.

Nano-specific under development NAMs

- V.I.G.O. "Alkaline single cell gel electrophoresis in A549 cells v1.0"[149] [ID_79]
- V.I.G.O. "Alkaline single cell gel electrophoresis in THP-1 cells v1.0"[150] [ID_80]
- Whole-genome sequencing (WGS) to analyse the mutagenicity of NPs[151] [ID_84]
- CD59 gene loci mutation assay *in vitro*[152] [ID_85]
- FE1 Muta™ Mouse Lung Epithelial Cell Line gene mutation assay[153,154] [ID_86]
- ToxTracker assay[155,156] [ID_87]
- Secondary genotoxicity testing by using in vitro methods based on conditioned media or co-cultures[157–159] [ID_88]
- High content imaging of DNA double strain breaks DSBs (phosphorylated H2AX staining)[160] [ID_89]

- Comet assay on 3D HepG2 spheroids[161] [ID_90]
- Micronucleus assay on 3D HepG2 spheroids[162] [ID_91]
- 3D reconstructed skin micronucleus (RSMN) assay (EpiDerm™)[163] [ID_92]

Repeated dose toxicity

The assessment of general toxicological effects that may be induced by repeated exposure to a substance is required under REACH, Biocidal and Cosmetics Regulation and all Food and Feed Additives EU Regulations (Regulation (EC) No 1907/2006; Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009; Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011; Regulation (EC) No 258/97; Regulation (EC) No 1852/2001; Regulation (EC) No 1331/2008; Regulation (EC) No 1333/2008; Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003). According to REACH[38] Guidance..(page 415 of the Guidance) "*the term repeated dose toxicity comprises the general toxicological effects occurring as a result of repeated daily dosing with, or exposure to, a substance for a part of the expected lifespan (sub-acute or sub-chronic exposure) or for the major part of the lifespan, in case of chronic exposure*". The investigation of repeated dose toxicity includes a multitude of organs and tissues as listed in OECD TG 407, 408, 410, 411, 412, 413. The expected adverse effect may be related to morphology, physiology, height or lifespan, growth, development as well as reproduction of the organism, system or (sub)population, which may result in impairment of "*functional performance or ability to compensate for additional stress or increases vulnerability to other environmental influences*"[38] (page 420 of the Guidance). Two main types of effects that can be observed after repeated exposure of chemical substance were defined as: (i) local effects - observed at the point of first contact, caused regardless of whether the substance is systemically available, (ii) systemic effects - observed away from the site of the first contact, i.e., after the substance has passed through the physiological barrier and achieved systemic availability.

Testing strategies proposed so far to assess this endpoint include animal testing and are divided into three categories depending on the length of the exposure to the substance. Repeated dose 28-day studies evaluate toxic effects that may be caused by the exposure of the young adult animals to the chemical substance for 28 days. There are three regulatory accepted tests to evaluate repeated dose toxicity depending on the route of administration: oral (OECD TG 407[164]), dermal (OECD TG 410[165]) and inhalation (OECD TG 412[46]). Another set of tests refers to "*general toxicological effects arising from subchronic exposure (a prolonged period of the animals' life span) covering post-weaning maturation and growth well into adulthood, on target organs and on potential accumulation of the substance*".[38] Several already accepted test designs for 90-days studies are in place covering the different routes of administration: oral (e.g., OECD TGs 408[166]), dermal (OECD TG 411[167]) and inhalation (OECD TG 413[47]). A third category, the chronic toxicity studies address adverse effect of repeated exposure to chemical over a major part of animals' life span. The chronic toxicity studies should last at least 12 months. This category includes also combined chronic toxicity study/carcinogenicity study (OECD TG 453[168]). Chronic studies should provide information on the general toxicity effects, e.g., biochemical, physiological and haematological effects. However, they should also inform on neurotoxicity, immunotoxicity, reproductive disorders or carcinogenic effects. There are two carcinogenicity OECD test guidelines (TG 451[99] and 452[169]), reproduction/developmental toxicity screening test (OECD TG

422[170]), guideline for testing neurotoxicity (OECD TG 424[171]) or testing delayed neurotoxicity of organophosphorus substances (OECD TG 419[172]).
Currently, available alternative methods to animal testing that are considered acceptable for regulatory purposes for assessing the toxicity after repeated exposure are not available.

Recently, a number of different NAMs for testing repeated dose toxicity of nanomaterials were proposed. The summary of NAMs under development which are relevant for assessing repeated dose toxicity along with their possible adaptation/limitations for nanomaterials testing is provided in Table 13. The listed NAMs were subject of scientific publications but were also considered or developed as SOPs within the EU sponsored PATROLS project.

Table 13. *Summary of NAMs relevant for repeated dose toxicity*

<table>
<tr><th colspan="4">Endpoint: Repeated dose toxicity</th></tr>
<tr><th>Advancement status of the NAMs in ENMs testing</th><th>Potential limitations of the NAMs for adaptation for ENMs testing</th><th>Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)</th><th>Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)</th></tr>
<tr><td colspan="4">Non-nano specific regulatory accepted NAMs
Not available</td></tr>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs

Lung models
Co-cultures
• Co-culture: human epithelial cell line (NCI-H441)/ endothelial cell line (ISO-HAS-1)[60] [ID_1]
• Co-culture: human epithelial cell line (NCI-H441)/ macrophages (dTHP-1)[61] [ID_8]
• Triple co-culture: human epithelial cells (16HBE14o-)/ monocyte-derived macrophages/monocyte-derived dendritic cells[66] [ID_26]
Air Liquid Interface (ALI)
• Air Liquid Interface: co-culture: bronchial epithelial (Calu-3)/ primary macrophages using VITROCELL® Cloud12 system[173,174] [ID_110]
• Air Liquid Interface: co-culture: bronchial epithelial (Calu-3)/ THP-1 macrophages using VITROCELL® Cloud12 system [65,68][ID_7]
• Air Liquid Interface: triple human co-culture model: epithelial (A549)/ endothelial (EA.hy 926)/ macrophage (THP-1)[74] [ID_105]
• Air Liquid Interface: tetra-culture: alveolar type II cell line/ differentiated macrophage-like cells/ mast cells/ endothelial cells[74] [ID_10]
Lung fibrosis
• Biomarkers of lung fibrosis in human lung fibroblasts[175] [ID_112]
• Increased production of TGF-1β by bronchial epithelial cells in vitro[176,177] [ID_113]
• NLRP3 inflammasome and pro-fibrotic markers induction in bronchial epithelial and fibroblast cells in vitro[178] [ID_114]
• Framework of an adverse outcome pathway (AOP) for lung fibrosis to identify key biological events linking MWCNT exposure to lung fibrosis [179] [ID_115]
• In vivo 17-gene biomarker panel (PFS17) applicable to the assessment of lung fibrosis[180] [ID_116]</td></tr>
</table>

- Gene expression biomarkers for lung toxicity models[181] [ID_109]

Liver models

- 3D *In Vitro* HepG2 Spheroid Model[88] [ID_107]
- 3D InSight™ Microtissue of liver [94–98] [ID_106]

Dysregulation of fibrinolysis/coagulation system

- Whole human blood model for testing the dysregulation of fibrinolysis[182–184] [ID_108]
- Calibrated thrombin generation test (cTGT) [185] [ID_119]
- Platelet aggregation using single cell counts [186] [ID120]
- Hemolysis test *in vitro*[187,188] [ID_117, ID_118]

Other models

- Atherosclerosis in vitro model caused by serum amyloid A response in lungs after exposure to ENMs[189] [ID_111]
- Toll-like receptors (TLRs) activation by ENMs on monocytes[190] [ID_71]
- Neuronal-like cells derived from human umbilical cord lining membranes mesenchymal stem cells as a tool for the neurotoxicity and developmental neurotoxicity testing[191] [ID_35]
- RNA-sequencing (RNA-Seq) and genome-wide DNA methylation analysis[192] [ID_121]

Advancement stage of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
All the listed NAMs have been satisfactorily used for ENMs, therefore they show some promise for adaptation for ENMs safety testing.	It is virtually impossible to present limitations for all the NAMs. Some of the limitations have already been described in other chapters, and for the most other endpoints it is too early to convincingly identify limitations.	High Some of the NAMs have high relevance for ENMs testing (e.g., repeated exposures of lung cells in ALI systems, lung fibrosis models), while other NAMs may have less relevance (e.g., disturbances of coagulation system). Although, in overall the relevance of the NAMs for ENMs testing can be classified as high, the NAMs are generally still at rather early stage of development.	High Repeated dose toxicity is one the most important endpoints for which reliable NAMs and IATAs are lacking, and therefore are urgently needed.

Respiratory sensitisation

The potential of chemicals to induce the hypersensitivity of the airways after inhalation exposure is an important endpoint in Biocidal Regulation (EU) No 528/2012). According to REACH[38] Guidance on Information Requirements (page 271 of the Guidance) „*respiratory sensitization (or hypersensitivity) is a term that is used to describe asthma and other related respiratory conditions (rhinitis, extrinsic allergic alveolitis), irrespective of the mechanism (immunological or non-immunological) by which they are caused.*" The mechanisms behind the respiratory sensitization are still under investigation; however, it is hypothesized that they involve a Th2-type immune response, which is characterized by the production of cytokines such as IL-4

and IL-5, and IgE antibodies. Moreover, the AOP for respiratory sensitization (AOP 39[193]) for small chemicals (with low molecular weights) is now under development within the OECD Working Plan. Validated or regulatory accepted methods for assessing this endpoint are not available. The application of several alternative methods for assessing respiratory sensitization have been published for conventional chemicals however, they are not regulatory accepted. Since relevant NAMs for conventional chemicals are still under development, their potential adaptation for nanomaterials will not be evaluated in this Report. It is obvious that development of nano-specific NAMs for respiratory sensitization is urgently needed.

Skin corrosion/irritation

Changes induced by substances at the site of first contact (such as skin or eye) are considered as part of the health safety assessment required under several EU Regulations, including Regulation (EC) No 1907/2006, Regulation (EU) No 528/2012, and Regulation (EC) No 1223/2009. These refer to local effects and may be observed after single or repeated exposure. Substances can be classified as inducing irritation and/or corrosion. According to REACH[38] (page 184 of the Guidance) "*corrosive substances are those which may destroy living tissues with which they come into contact*". Thus, while considering the skin as the first contact tissue, skin corrosion is defined as: "*the production of irreversible damage to skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test substance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars*" [38] (page 185 of the Guidance). In contrast to corrosive substances, irritants are "*non-corrosive, and through immediate contact with the tissue under consideration may cause inflammation*". According to REACH[38] (page 184 and 185 of the Guidance) skin irritation refers to "*the production of reversible damage of skin following the application of a test substances for up to 4 hours*."

Although, evaluation of skin corrosion/irritation potential of substances has traditionally employed laboratory animals (OECD TG 404[194]), there are regulatory validated and accepted procedures allowing to distinguish non-corrosive (non-irritative) and corrosive (irritative) chemicals in accordance with UN GHS. For example, for this purpose, OECD TG 431[195] (*in vitro* skin corrosion) and OECD TG 439[196] (*in vitro* skin irritation) recommend using the reconstructed human epidermis (RhE) models since they mimic the histopathological, morphological, and biochemical properties of human skin. The OECD TG 430[197] utilizes rat skin discs to measure the skin transcutaneous electrical resistance (TER) aimed at identifying the corrosives by their ability to produce a loss of normal *stratum corneum* integrity and barrier function. The *in vitro* membrane barrier test method commercially available as Corrositex, allowing identifying skin corrosive substances, is also recommended by OECD TG 435[198]. The use of these *in vitro* test methods and their integration with other non-test data is described in the OECD Guidance document on IATA for Skin Irritation/Corrosion (GD 203)[199,189] well as in a Cristo et al.[110]. According to the OECD GD 203[189], "*the positive results of in vitro test methods can be used to classify a chemical as corrosive/irritative without need for animal testing*". The SCCS in Nanoguidance (2019) noted that: "*The alternative tests proposed for skin corrosion and irritation are based on colorimetric assays (such as sulforhodamine B dye, MTT assay). These techniques may not be suitable for certain NMs because of possible interactions. Thus, additional controls need to be included to*

avoid possible interference of NMs with the detection system. Some NMs may themselves disperse/absorb light and therefore interfere with colorimetric measurements. These aspects need to be considered when spectrophotometric methods are applied".

As there are several, already accepted non-animal methods to assess skin corrosion/irritation within this Report their possible usage/adaptation for nanomaterials was considered. In Table 14 and 15 the summaries of NAMs for these endpoints with their possible adaptation/limitations for nanomaterials are discussed.

Table 14. *Summary of NAMs relevant for skin corrosion*

<table>
<tr><th colspan="4">Endpoint: Skin corrosion</th></tr>
<tr><th>Advancement status of the NAMs in ENMs testing</th><th>Potential limitations of the NAMs for adaptation for ENMs testing</th><th>Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)</th><th>Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)</th></tr>
<tr><td colspan="4">Non-nano specific regulatory accepted NAMs
EpiDerm™ Skin Corrosion Test[200] [ID_151]
EpiSkin™ Skin Corrosion Test[201] [ID_152]
LabCyte EPI-MODEL24 Skin Corrosion Test[202] [ID_153]
epiCS (previously named EST-1000) Skin Corrosion Test[203,204] [ID_154]
Corroxites® (in vitro membrane barrier test method) [205] [ID_155]
Rat Skin Transcutaneous Electrical Resistance (TER) [206] [ID_156]</td></tr>
<tr><td>The methods have been validated for conventional chemicals, however official validation trials for ENMs have not yet been initiated.
Some publications are available in the open literature which were performed on ENMs using some of the NAMs, with negative results reported. The tests were performed usually as a part of panel of basic safety testing requirements.</td><td>The skin corrosive potential is usually characteristic for compounds with very low or high pH, and in general, ENMs used in different applications do not have such properties.
Reference ENMs with well described skin corrosive properties are not available, hence, any validation round-robin tests cannot be performed at the moment.
Nevertheless, the skin models and in silico methods seem appropriate for ENMs testing, after slight adaptations (e.g. selecting suitable exposure times, assay interference, ENMs dosing, etc.).</td><td>Limited

Considering biological properties of ENMs generally indicating low, if any, dermal toxicity, the development and validation efforts towards this group of NAMs for regulatory purposes is rather of lower priority.</td><td>Limited

The endpoint is required by all relevant EU regulations. Validated NAMs are already available but require official validation for ENMs. The intrinsic skin corrosive potential of ENMs is low, if any.</td></tr>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
Not available</td></tr>
</table>

Nano-specific under validation NAMs Not available
Nano-specific under development NAMs Not available

Table 15. *Summary of NAMs relevant for skin irritation*

Endpoint: Skin irritation			
Advancement stage of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • epiCS (previously named EST-1000) Skin Irritation Test[203,204] [ID_157] • EpiDERM™ Skin Irritation Test (SIT)[207] [ID_158] • EpiSkin™ Skin Irritation Test[208] [ID_159] • SkinEthic™ Reconstructed Human Epidermis model[209] [ID_160] • LabCyte EPI-MODEL24 Skin Irritation Test[210] [ID_161] • Toxtree Module: A decision tree for estimating skin irritation and corrosion potential[211] [ID_162]			
• The methods have been validated for conventional chemicals, however official validation trials for ENMs have not yet been initiated. • There are some publications available in the open literature which were performed on ENMs using some of the NAMs, with usually negative results reported. The tests were performed usually as a part of panel of basic safety testing requirements.	• The skin irritating potential is usually characteristic for compounds with very low or high pH, and in general, ENMs used in different applications do not have such properties. • ENMs usually have very low potential for dermal absorption. • Reference ENMs with well described skin irritating properties are not available, hence, any validation round-robin tests cannot be performed at the moment. • Nevertheless, the skin models and *in silico* methods seem appropriate for ENMs testing, after slight adaptations (e.g., selecting suitable exposure times, assay interference, ENMs dosing, etc.).	Limited Considering biological properties of ENMs generally indicating low, if any, dermal toxicity, the development and validation efforts towards this group of NAMs for regulatory purposes is rather of lower priority.	Limited The endpoint is required by all relevant EU regulations. The validated NAMs are already available but require official validation. The intrinsic skin irritating potential of ENMs is low.

Non-nano specific under validation NAMs Not available
Nano-specific regulatory accepted NAMs Not available
Nano-specific under validation NAMs Not available
Nano-specific under development NAMs Not available

Serious eye damage/eye irritation

Besides the skin, the first tissue chemicals can come into contact with is the eye. Therefore, the potential for serious eye damage/eye irritation needs to be assessed and it is required under several EU Regulations (Regulation (EC) No 1907/2006; Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009). According to REACH[38] Guidance on Information Requirements (page 185 of the Guidance) the eye irritation refers to "*the production of changes in the eye following application of a test substance to the anterior surface of the eye, which are fully reversible within 21 days of application*", while serious eye damage is described as "*the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application*".

Historically, the evaluation of serious eye damage and eye irritation was performed with laboratory animals as recommended by OECD TG 405[212]. However, currently, there are available validated and regulatory recommended *in vitro* testing strategies for identifying if chemicals induce serious eye damage/irritation. For example, in the proposed IATA (OECD GD 263[213]), based on combination of *in vitro* and *in silico* methods it is possible to assign chemicals or their mixtures to either 1) causing "serious eye damage" or 2) not requiring classification for eye irritation or serious eye damage according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and EU Classification, Labelling and Packaging of Substances and Mixtures regulation (CLP). In 2022, also OECD TG 467[214]: Defined Approaches for Serious Eye Damage and Eye Irritation was adopted in which the prediction from a DA may be used alone to determine eye hazard potential according to the hazard classes of the UN GHS (Categories 1, 2, or not classified). OECD TGs 437[215], 438[216], and 492[217] describe different *in vitro* procedures that allow to distinguish substances not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. For example, OECD TG 492[217] recommends using reconstructed human cornea-like epithelium (RhCE) which closely mimics the histological, morphological, biochemical, and physiological properties of the human corneal epithelium.

Because nano-specific methods to assess serious eye damage/irritation are not available, within this Report the possible use/adaptation of regulatory accepted methods developed for conventional chemicals for safety assessment of nanomaterials was considered. In Table 16 the summaries of NAMs and discussion for this endpoint are provided.

Table 16. Summary of NAMs relevant for serious eye damage/eye irritation

<table>
<tr><th colspan="4">Endpoint: Serious eye damage/eye irritation</th></tr>
<tr><th>Advancement status of the NAMs in ENMs testing</th><th>Potential limitations of the NAMs for adaptation for ENMs testing</th><th>Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)</th><th>Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)</th></tr>
<tr><td colspan="4">Non-nano specific regulatory accepted NAMs
Bovine Corneal Opacity and Permeability (BCOP OP-KIT) test method[218] [ID_128]
Isolated Chicken Eye (ICE) test[219] [ID_129]
Fluorescein leakage (FL) assay[220] [ID_130]
Short Time Exposure In Vitro Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage[221] [ID_131]
BfR structural alerts rule-based decision support system (DSS)[222,223] [ID_132]
ACD/Percepta: Eye Irritation[224] [ID_133]
Toxtree Module: A decision tree for estimating eye irritation and corrosion potential[225] [ID_134]
DEREk Nexus[226] [ID_135]
LabCyte CORNEA-MODEL 24 Eye Irritation Test[227] [ID_136]
EpiOcular™ Eye Irritation Test[228] [ID_137]
Eye irritation test with a 3D-reconstructed human cornea epithelium (MCTT HCE™-EIT)[229] [ID_138]
SkinEthic™ Human Corneal Epithelium Eye Irritation Test[230] [ID_139]
Defined Approaches for Serious Eye Damage and Eye Irritation[231] [ID_140]
The Vitrigel-Eye Irritancy Test (Vitrigel EIT)[232] [ID_141]
Ocular Irritection[233] [ID_142]
Cytosensor Microphysiometer[233] [ID_143]
Neutral Red Release (NRR) test[233] [ID_144]
Red Blood Cell (RBC) test[233] [ID_145]
Isolated Rabbit Eye (IRE) models[233] [ID_146]
Hen’s Egg Test on the Chorio-Allantoic Membrane (HETCAM) test[233] [ID_147]
Chorio-Allantoic Membrane Vascular Assay (CAMVA) test[233] [ID_148]
EPA testing strategy for antimicrobial cleaning products[234] [ID_150]</td></tr>
<tr><td>The methods have been validated for conventional chemicals, however official validation trials for ENMs have not yet been initiated.
There are some publications available in the open literature which were performed on ENMs using some of the NAMs. The tests were performed usually as a part of panel of basic safety testing requirements.</td><td>The eye models and in silico methods seem appropriate for ENMs testing, after slight adaptations (e.g., selecting suitable exposure times, ENMs dosing, assuring suspension stability, etc.)
Some ENMs present in opacity measurements may affect the result, and these should be avoided to allow consistent interpretation of results. Possible artifacts due to absorption of the fluorescent dyes to ENMs should be verified and eliminated.</td><td>High

The panel of available NAMs for testing the eye damage potential is rather broad and includes both in vitro and in silico methods.
Majority of the NAMs are ready to be used for ENMs testing after slight adaptations in line with standard nanotoxicology requirements.</td><td>High

The endpoint is required by all relevant EU regulations.
The validated NAMs are already available but require official validation for ENMs.
Considering potentially ubiquitous exposure to ENMs validation of the NAMs for ENMs testing is urgently needed.</td></tr>
</table>

	• Some tests based on colorimetric assays (such as sulforhodamine B dye, MTT assay), may not be suitable for certain NMs because of possible interactions. Thus, additional controls need to be included to avoid possible interference of NMs with the detection system. Some NMs may themselves disperse/absorb light and therefore interfere with colorimetric measurements. These aspects need to be considered when spectrophotometric methods are applied. • The *in vivo* data on eye irritation/damage for ENMs are scarce, therefore the reliable correlation with *in vitro* data is difficult.		
Non-nano specific under validation NAMs • SkinEthic™ Human Corneal Epithelium model[235] [ID_149]			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs Not available			

Skin sensitization

The potential of chemicals to cause an allergic reaction is a crucial feature and must be assessed according to REACH Regulation (EC) No 1907/2006); Biocidal Regulation (EU) No 528/2012); Cosmetics Regulation (EC) No 1223/2009); and Feed Additives Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003. According to REACH[38] Guidance on Information Requirements (page 271 of the Guidance) "*skin sensitizer is an agent that will lead to an allergic response in susceptible individuals following skin contact. As a consequence of a secondary - usually organ-specific - subsequent re-exposure, adverse health effects on the skin (allergic contact dermatitis) can appear*". The lipophilic, reactive substances with low molecular weight (<500-100 Da) may be included in the group of allergens. The mechanism leading to the skin sensitization is still under investigation, however there is an established AOPs[236] that describes the phenomenon initiated by covalent binding to skin proteins. Within this AOP four key events are described: 1) covalent binding to the skin proteins, 2) release of pro-inflammatory cytokines and induction of cyto-protective pathways in keratinocytes; 3) activation and maturation of dendritic cells, and their

migration to the local lymph nodes; 4) activation of the lymph node cells. This approach is not appropriate for metals or allergens of biological origin for which the mechanism of skin sensitization is not completely understood. In June 2021 an official guideline (OECD TG 497[237]) that contains several defined alternative approaches to skin sensitisation that covers the covalent binding to skin proteins was published. For each defined key event there are proposed *in chemico* (under OECD TG 442C[238]) and *in vitro* (under OECD TG 442 D[239] and OECD TG 442E[240]) approaches have been proposed to assess the potency of sensitization. According to OECD TG 497[237] the entire analysis must also be supplemented with an *in silico* approach assuming the similarity of the test substance with others, showing similar structural features, and hence the sensitization potential. The limitations of the defined approaches are associated with the limitations of the individual *in chemico/in vitro/in silico* information sources. In accordance with OECD TG 497[237] the chemicals of interest can be categorized into classes defining their sensitization abilities according to GHS. Within this group of tests, 11 methods have already been regulatory accepted for estimating the skin sensitization potential for conventional chemicals (Table 11).

For nanomaterials, there is one particular NAM currently undergoing a validation process according to the OECD Working Plan. "Applicability of the key event based TG 442D for *in vitro* skin sensitisation testing of nanomaterials" was recently discussed.[239] Within this Report the possibility of adaptation of non-nano specific regulatory accepted NAMs for nanomaterials was considered. In Table 17 the summaries of NAMs for these endpoints with their possible adaptation/limitations for nanomaterials testing are provided.

Table 17. *Summary of NAMs relevant for skin sensitization*

Endpoint: Skin sensitization			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs *In chemico* • The Direct Peptide Reactivity Assay (DPRA)[241] [ID_166] • The Amino Acid Derivative Reactivity Assay (ADRA)[241] [ID_167] The kinetic Direct Peptide Reactivity Assay (kDPRA)[241] [ID_168] *In silico* • Defined Approaches on Skin Sensitisation: 2 out of 3[242] [ID_164] • Defined Approaches on Skin Sensitisation: ITS v1 or v2[242] [ID_165] *In vitro* • KeratinoSens™ test method[243] [ID_169] • LuSens™ test method[243] [ID_170] • Human Cell Line Activation (h-CLAT) test[244] [ID_171] • U937 cell line activation (U-SENS™) Test[244] [ID_172] • Interleukin-8 Reporter Gene Assay (IL-8 Luc assay)[244] [ID_173] • Genomic Allergen Rapid Detection for assessment of skin sensitisers (GARD™skin)[244] [ID_174]			
• The methods have been validated for conventional chemicals, however no official validation trials for ENMs are ongoing.	• The skin sensitization models and *in silico* methods seem appropriate for ENMs testing, after slight adaptations (e.g., selecting suitable	High The group of NAMs covers all identified so far key events in the sensitization process.	High The endpoint is required by all relevant EU regulations.

• There are some publications available in the open literature which were performed on ENMs using some of the NAMs. The tests were performed usually as a part of panel of basic safety testing requirements.	exposure times, ENMs dosing, etc.) • Recent publications indicate that KeratinoSens™, h-CLAT and USENS™ assays can be useful for evaluating the skin sensitization potential of ENMs. • Generally, there is limited number of relevant nanomaterials for validation testing as well as limited availability of *in vivo* skin sensitisation data for nanomaterials.	Most of them are suitable for ENMs testing, however they need to go through a formal validation process to gain regulatory acceptance.	The validated NAMs are already available but require official validation for ENMs.
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs • Study Report Applicability of the key event based TG 442D for in vitro skin sensitisation testing of nanomaterials[237] [245] [ID_163]			

Toxicity in vitro testing

Testing of the endpoint is recommended by EFSA "*Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health*"[14] (2021) and is relevant for the food regulation (Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001). EFSA recommends testing of cytotoxicity/cell viability, oxidative stress, (pro-)inflammation, gastrointestinal barrier integrity after exposure to different food components.

Most of the NAMs described below cover the endpoint, however, are neither sufficiently developed nor validated for ENMs testing. Therefore, there is an urgent need for developing reliable NAMs in this field.

Many of the NAMs can be used for assessment of other toxicity endpoints, but because of their wide range of specificity and different levels of development, their limitations and relevance are virtually impossible to be concisely discussed (Table 18).

Table 18. *Summary of NAMs relevant for toxicity in vitro testing*

Endpoint: Toxicity *in vitro* testing			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs			
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs • *In vitro* ROS generation in RAW 264.7 macrophage cells[246] [ID_175] • 3D cellular high throughput screening method for cytotoxicity[247] [ID_176] • Label-free cell impedance technology for toxicity assessment (xCELLigence System, ACEA Biosciences)[248] [ID_177] • *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles[249,250] [ID_191] • Endotoxin contamination testing I[251] [ID_204]			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs Monoculture models • nanOxiMet "Cellular viability – WST-1 assay Protocol for adherent cells v2.0"[252] [ID_180] • nanOxiMet "Cellular viability – WST-1 assay in NR8383 macrophages v1.0"[253] [ID_181] • nanOxiMet "Cellular DCF-DA assay v1.0"[254] [ID_186] • V.I.G.O. "Detection of reactive oxygen species in THP-1 cells v1.0"[255] [ID_187] • V.I.G.O. "Detection of reactive oxygen species in A549 cells v1.1"[256] [ID_188] • V.I.G.O "Flow cytometric Annexin V/Propidium Iodide measurement in A549 cells v1.0"[257] [ID_182] • V.I.G.O. "MTS assay in A549 cells v1.1"[258] [ID_183] • V.I.G.O. "MTS assay in THP-1 cells v1.1"[258] [ID_184] • Stimulation of fibroblasts *in vitro*[176,248,259,260] [ID_190] Macrophage differentiation from THP-1 cells [261][ID_192] • Mono-culture: lung epithelial cell-line (Cl-hAELVi) model[262] [ID_196] • Fibroblast proliferation and expression of pro-fibrotic biomarkers[175] [ID_195] • 2D epithelial tissue in-vitro model of in situ detection of ENM elemental distribution[263] [ID_199] Co-cultures/ 3D models • Guidance Document for the induction of the inflamed alveolar epithelial model[80] [ID_198] • Mono and multi-cellular models of the gastrointestinal system[92] [ID_194] • Isolation and differentiation of peripheral blood monocytes and further assembly into co-culture models with epithelial cells[264] [ID_197] • 3D *In Vitro* HepG2, Kupffer Cell Co-Culture Spheroid Model[90] [ID_178] • 3D kidney organoid proximal tubule culture model[265] [ID_200] • 3D colon cell spheroids in micropatterned agarose hydrogel platform[266] [ID_201] • 3D cell spheroids from human adipose-derived mesenchymal stem cells (hAD-MSCs)[267] [ID_203] • Triple culture of the inflamed-like intestine: human Caco-2/ HT29-MTX-E12/ THP-1 cell lines[91] [ID_193] Advanced HTS techniques • Advanced mechanism-based high throughput *in vitro* screening[89] [ID_179]			

- Proteomics and metabolomics to assess proteins and metabolites related to the production of reduced glutathione[268] [ID_189]
- Cell impedance technology (xCELLigence System, ACEA Biosciences)[248] [ID_177]

Advanced microscopy methodologies

- Advanced mechanism-based high throughput *in vitro* screening[89] [ID_179]
- Digital holographic microscopy[269] [ID_202]

Other

- Endotoxin contamination testing II[269,270] [ID_205]

In vitro gastrointestinal digestion

The assessment of behaviour of ENMs in the gastrointestinal lumen (digestion process) is recommended by the EFSA "*Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health*"[14] (2021) and is relevant for the food regulation (Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001). In the digestive tract, food components e.g., proteins and fats are broken down into absorbable units and afterwards, together with vitamins, minerals and water, are absorbed into the blood or lymph. Recently, various *in vitro* models were developed to study novel food and feed before conducting *in vivo* studies. Such models simulate digestion of food and are based on the *in vivo* physiological functions and conditions. Proposed approaches were divided into two categories: (i) static models – fast, simple, however applicable only to limited digestion conditions (gastric and/or intestinal step) and (i) dynamic models - more complex but much more physiologically relevant and applicable for complex digestion studies. Although very intensive research is being conducted on this topic, the improvement of the proposed model is needed for closer mimicking of the *in vivo* conditions. To this end, more details about the biochemistry, physiology, morphology, and anatomical structure of the entire human digestive tract need to be taken into account. Also, combination of *in vitro* methodologies, bioinformatic and artificial intelligence technology may be necessary for standardization of proposed approaches and including them into validation/ or acceptance process.

Currently, regulatory accepted NAMs for assessing the gastrointestinal digestion are not available. Nevertheless, a number of different well advanced NAMs for testing this endpoint using nanomaterials have been developed. A summary of relevant NAMs under development for nanomaterials is provided in Table 19. Presented NAMs were subject of scientific publications but also were considered or developed as SOPs within the EU sponsored PATROLS project. One of the proposed NAMs in now under validation process according to the OECD Working Plan.

Table 19. *Summary of NAMs relevant for gastrointestinal digestion*

Endpoint: In vitro gastrointestinal digestion			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs Not available			

<table>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under validation NAMs
• Integrated In Vitro Approach for Intestinal Fate of Orally Ingested Nanomaterials[271] [ID_70]</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs
• Biotransformation in the Oral–Gastro–Intestinal Tract[272] [ID_58]
• Interaction between nanoparticles and food proteins (albumin, casein, and zein)[273] [ID_59]
• Nanomaterial pre-treatment in vitro with simulant fluids to mimic oral and inhalation exposures for hazard assessment using 3D liver models in vitro[93] [ID_60]
• In vitro simulated human digestive system I[272] [ID_61]
• In vitro simulated human digestive system II[274] [ID_62]
• In vitro simulated human digestive system III[275] [ID_63]
• In vitro simulated human digestive system IV[276] [ID_64]
• In vitro simulated human digestive system V[277] [ID_65]
• In vitro simulated gastrointestinal digestion system in either a fasting food model, a standardized food model, or a high fat food model[278] [ID_66]
• The In vitro dynamic gastrointestinal simulator (simgi®)[278] [ID_67]
• The integrated methodology for dissolution of NPs along the gastrointestinal tract[279,280] [ID_68]
• Dynamic Nanoparticle Digestion Simulator (DNDS)[281] [ID_69]</td></tr>
<tr><td>• Most of the NAMs are in rather early stage of development, in spite they offer well standardized procedures with satisfactory physicochemical analysis of ENMs undergoing transformations in simulated, sequential gastrointestinal fluids.</td><td>• The models still do not simulate the complexity of the GIT, considering both secreted glandular enzymes, and consumed food constituents, which may significantly influence the nanoparticle fate in GIT.</td><td>Limited

The NAMs are still at an early development phase.</td><td>High

The NAMs are urgently needed for the food regulations.</td></tr>
</table>

Reproductive toxicity/Developmental toxicity/Endocrine disruption

During the safety assessment of substances their reproductive hazards should be considered. Determination of this endpoint is required under REACH, Biocidal and Cosmetics Regulation and all Food and Feed Additives EU Regulations (Regulation (EC) No 1907/2006; Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009; Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001; Regulation (EC) No 1331/2008; Regulation (EC) No 1333/2008; Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003). According to the CLP Regulation "*reproductive toxicity is characterized by multiple diverse endpoints, which relate to impairment of male and female reproductive functions or capacity (fertility), the induction of non-heritable harmful effects on the progeny (developmental toxicity), and effects on or via lactation*". According to REACH[38] Guidance on Information Requirements (page 469 of the Guidance): „*adverse effects on sexual function and fertility include any effect of a substance that has the potential to interfere with sexual*

function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive (oestrus) cycle normality, sexual behaviour, fertility, gestation length, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive system".

According to REACH[38] Guidance on Information Requirements (page 469 of the Guidance): "*Developmental toxicity includes, in its widest sense, any effect interfering with normal development of the organism, before or after birth and resulting from exposure of either parent prior to conception, or exposure of the developing organism during prenatal development, or postnatal development, to the time of sexual maturation – thus generally speaking, these effects can be manifested at any point in the life span of the organism*".[38]

Currently, the assessment of the endpoint is possible using laboratory animals (e.g., OECD TG 421[282], 422[170]). Due to complexity of the reproductive toxicity the alternative approaches to *in vivo* testing are very challenging. Up to now, three *in vitro* tests relevant for reproductive toxicity have been officially adopted at OECD level. Two of them allow to measure the oestrogenicity (OECD TG 455 [283] and OECD TG 457[284]) and another one allows to measure the steroidogenesis (OECD TG 456[112]). Other developed tests focus on measuring the binding and activating or inhibiting a steroid (or a thyroid) hormone receptor (OECD TG 458[285], ToxCast model). Three NAMs have been developed for embryotoxicity testing which successfully passed the validation process at the ECVAM level but have not yet gained acceptance at OECD level.

Recently, different NAMs for testing reproductive toxicity of nanomaterials were proposed. A summary of NAMs under development relevant for assessing this endpoint with their possible adaptation/limitations for nanomaterials is provided in Table 20. Because of different levels of NAMs development, their limitations and relevance are virtually impossible to be concisely discussed.

Table 20. *Summary of NAMs relevant for reproductive toxicity/developmental toxicity/endocrine disruption*

Endpoint: Reproductive toxicity/Developmental toxicity/Endocrine disruption			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (22Rv1/MMTV_GR-KO cell line) [286][ID_122] • Estrogen Receptor Transactivation Assay (ERalpha-CALUX)[287] [ID_123] • Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR-CALUX)[118,119] [ID_56] • ToxCast estrogen receptor (ER) pathway mathematical model[288] [ID_124]			
• The methods have been validated for conventional chemicals, however official validation trials for	• Usefulness and limitations of the NAMs for ENMs testing are not fully understood yet. • It is very likely that most of the NAMs can be	High It is very likely that most of the NAMs are relevant for ENMs regulatory	High The endpoint is required by all major regulations and considering its

ENMs have not yet started. • Some publications available in the open literature which were performed on ENMs using some of the NAMs.	successfully applied for ENMs testing after considering necessary nanotoxicological requirements. • Considering the complex nature of the endpoint any *in vitro-in vivo* extrapolations for ENMs are very uncertain, e.g., due to translocation of NPs across the lung and placenta, or dosing regiments. • It is necessary to understand if inflammation is the driving force for developmental effects and whether the inflammatory response is different between the pregnant and the non-pregnant state. • As indirect mechanisms have also been described to be responsible for reproductive effects by ENMs (e.g. release of inflammation mediators distant from reproductive organs but acting in reproductive organs) the question arise how these effects can by addressed by NAMs	testing after considering necessary nanotoxicological requirements.	complex nature development of NAMs for testing, both for conventional chemicals and ENMs is of great importance. To assess the endpoint a panel of NAMs is required, combined in IATAs. There are still major gaps in understanding the basic mechanisms of the endpoint and development of suitable NAMs is urgently needed.
Non-nano specific under validation NAMs • Embryonic Stem Cell Test (EST) - an *In Vitro* Test for Embryotoxicity[289] [ID_36] • Micromass Test - an *In Vitro* Test for Embryotoxicity[289] [ID_37] • Postimplantation Rat Whole-Embryo Culture Assay - an *In Vitro* Test for Embryotoxicity[289] [ID_38]			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs • Immortalized human trophoblast cell line (HTR-8/SVneo)[290] [ID_125] • GC-1 cells stably transfected with low expression piR-mmu-32362259 lentiviral vector[291] [ID_126] • Chicken Developmental Embryonic Assay[292] [ID_127]			

Immunotoxicity/Developmental immunotoxicity/Allergenicity

During the safety assessment process, it is important to take into account the adverse effects of chemicals on the structure and function of the immune system. Immunotoxicity can be defined as any adverse effect on the immune system that can result from exposure to a range of environmental

agents, including chemicals. Because the immune system has numerous effector and regulatory cell functions that operate at local, regional and systemic levels, exposure to xenobiotics has the capability of producing any combination of the following recognized adverse outcomes: 1) focused or more extensive immunosuppression, 2) increased propensity for allergic disease, including atopy, food allergies and asthma, 3) hypersensitivity reactions directed at the chemical itself, 4) increased risk of autoimmune disease and 5) dysfunctional responses of innate immune cells producing tissue or organ damage or dysfunction (WHO, 2012[293]).

In case of immunotoxicity two aspects should be considered: the immunotoxicity for adult organisms and the developmental immunotoxicity, which provides information on the potential hazard to the immune system arising in the offspring after exposure of the mother during pregnancy and lactation period.

Immunotoxicity has to be considered within the Biocidal and all Food and Feed Additives EU Regulation (Regulation (EU) No 528/2012; Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001; Regulation (EC) No 1331/2008; Regulation (EC) No 1333/2008; Regulation (EC) No 1223/2009; Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003). No OECD or EU test method is currently available to specifically investigate immunotoxicity/developmental immunotoxicity. However, the "Health Effects Test Guidelines OPPTS 870.7800 Immunotoxicity" (EPA, 1998[294], 2013[295] can be referred to. Additional guidance on immunotoxicity is available from the WHO/IPCS Guidance on Immunotoxicity for risk assessment (WHO, 2012[293]). Usually, immunotoxicity is addressed as an additional (sub)endpoint when performing other animal based toxicological studies (e.g., repeated dose toxicity, carcinogenicity, or reproductive toxicity testing according to e.g., OECD TG 408[166], 452[169], OECD 443[296]). For developmental immunotoxicity studies some information is provided in REACH[38] Guidance on Information Requirements (Section R.7.6.4.2.7).

At present, there are no validated *in vitro* methods specifically dedicated to the evaluation of the immunotoxicity. In case of ENMs, because of their potential to be immunotoxic, immunogenic or antigenic, but also based on their availability to transport the immunogens (bound to the particle surface) into close contact with the immune cells, the evaluation of ENMs immunotoxic potential is very important. In Table 21, the summary of NAMs that are under development together with the limitations of these methods is presented.

Table 21. *Summary of NAMs relevant for immunotoxicity/developmental immunotoxicity/allergenicity*

Endpoint: Immunotoxicity/Developmental immunotoxicity/Allergenicity			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs Not available			
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs Not available			

<table>
<tr><td colspan="4">Nano-specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs
• Toll-like receptors (TLRs) activation by ENMs on monocytes[190] [ID_71]
• Lymphocyte stimulation indices and cytokine profiling: peripheral blood mononuclear cells[297] [ID_72]
• Complement activation in pig whole blood[298] [ID_73]
• Complement activation in human whole blood[298] [ID_74]
• Complement activation (CH50) test[299] [ID_75]
• Colony Forming Unit-Granulocyte Macrophage (CFU-GM) assay[300] [ID_76]
• Endocytosis and phagocytosis assay I[301] [ID_77]
• Endocytosis and phagocytosis assay II[302] [ID_78]</td></tr>
<tr><td>• All the NAMs found are at very early stage of development. Only single papers are available where ENMs were tested.</td><td>• It is likely that most of the NAMs can be successfully applied for ENMs testing after considering necessary nanotoxicological requirements.</td><td>Low

The available NAMs cover only a small area of potential immunotoxic effects of ENMs. Hence, their regulatory relevance is negligible at the moment.</td><td>High

The NAMs can be used to test only few basic mechanisms of immunotoxicity.
Considering complex potential interactions between ENMs and immunocompetent cells a possibility to develop regulatory useful NAMs or IATAs within few years is very low.
Nevertheless, there is an urgent need for development of such NAMs.</td></tr>
</table>

Phototoxicity

The potential of substances to induce phototoxicity is relevant for safety assessment of biocidal and cosmetic products (Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009). According to Regulation (EU) No 283/2013 concerning the placing of plant protection products on the market [303] (page 34 of the Regulation) phototoxicity study *"shall provide information on the potential of certain active substances to induce cytotoxicity in combination with light, for example active substances that are phototoxic in vivo after systemic exposure and distribution to the skin, as well as active substances that act as photo irritants after dermal application. A positive result shall be taken into account when considering potential human exposure".*

In general, phototoxicity refers to a "*toxic response elicited by topically or systemically administered photoreactive chemicals after the exposure of the body to environmental light."*[304] Depending on the type of observed response, phototoxicity can be divided into: (i) photoirritation (acute light-induced skin response), (ii) photoallergy/photosensitization (an immune-mediated reaction in which light may cause a structural change in a substance so that it acts as a hapten, possibly by binding to proteins in the skin), and, (iii) photogenotoxicity (genotoxic response after exposure to a chemical by two mechanisms: either directly by photoexcitation of DNA or indirectly by excitation of photoreactive chemicals).

OECD TG 498[305] addresses photoirritation and recommends using the *in vitro* reconstructed human epidermis phototoxicity test (RhE PT) to identify phototoxic potential of chemicals, while OECD TG 432[305] adopts for this purpose the *in vitro* 3T3 Neutral Red Uptake test. In both cases,

substances identified as positive might be phototoxic *in vivo* after application to the skin, however, they are not considered photosensitizers.

Assessing the potential for the phototoxicity of nanomaterials is important since some of them can be used as ingredients in cosmetic products. In Table 22, the summary of NAMs and their possible application for nanomaterials testing is presented.

Table 22. *Summary of NAMs relevant for phototoxicity*

<table>
<tr><th colspan="4">Endpoint: Phototoxicity</th></tr>
<tr><th>Advancement status of the NAMs in ENMs testing</th><th>Potential limitations of the NAMs for adaptation for ENMs testing</th><th>Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)</th><th>Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)</th></tr>
<tr><td colspan="4">Non-nano specific regulatory accepted NAMs
• In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method[306] [ID_100]
• In vitro 3T3 NRU phototoxicity test[307] [ID_101]
• Reactive Oxygen Species Assay for Photoreactivity[308] [ID_104]</td></tr>
<tr><td>• The methods have been validated for conventional chemicals, however no official validation trials for ENMs are ongoing.</td><td>• The phototoxicity NAMs seem appropriate for ENMs testing, after slight adaptations (e.g., selecting suitable exposure times, ENMs dosing, suspension stability, etc.).
• Generally, there is limited number of relevant nanomaterials for validation testing as well as limited availability of in vivo phototoxicity data for nanomaterials.
• ENMs in general have rather low potential for dermal penetration.</td><td>High

The NAMs seem to be very relevant, however they would require a validation trials for ENMs.</td><td>Limited

The endpoint is required by biocidal and cosmetic regulation. The validated NAMs are already available but require official validation for ENMs. Considering general properties of ENMs indicating low dermal penetration, the development and validation efforts towards this group of NAMs for regulatory purposes seems of rather lower priority.</td></tr>
<tr><td colspan="4">Non-nano specific under validation NAMs
Not available</td></tr>
<tr><td colspan="4">Nano-specific regulatory accepted NAMs
• Ultraviolet (UV)-induced nicotine adenine dinucleotide hydrogen (NADH) oxidation[309] [ID_103]</td></tr>
<tr><td colspan="4">Nano-specific under validation NAMs
• Phototoxicity testing[147] [ID_102]</td></tr>
<tr><td colspan="4">Nano-specific under development NAMs
Not available</td></tr>
</table>

Neurotoxicity/Developmental Neurotoxicity

While assessing the safety of chemicals, it is important to consider their potential neurotoxic effects. Neurotoxicity (NT) is listed as relevant endpoint to be evaluated in several EU

Regulations (Regulation (EU) No 528/2012; Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001; Regulation (EC) 1331/2008; Regulation (EC) 1333/2008; Regulation (EC) No 429/2008, Regulation (EC) No 1831/2003). Neurotoxicity is defined as any adverse effect on the nervous system that results from exposure to potentially toxic substances.[310] Besides the adult neurotoxicity, it is crucial to consider the developmental neurotoxicity (DNT) as well. This covers a critical period, during which the nervous system is more susceptible to the exposure to toxicants or stressful events. In general, DNT and NT can lead to dysfunctions, possibly causing alterations in brain and behaviour. Currently, a reliable assessment of this endpoint is possible by in vivo testing (e.g., OECD TG 424[171]). OCED TG 426 [311] covers the clinical observations, and behavioural and neuropathological endpoints. Although a few NAMs have recently been published which are at an early stage of development, their potential application for ENMs testing is not certain at the moment. The Guidance on Evaluation of Data from the DNT *in vitro* testing battery (DNT IVb) provides[312] the criteria for evaluation of the relevance of the data to DNT and "*assist in the determination of the degree of certainty in any positive or negative findings to better inform use of DNT in vitro data in regulatory hazard determinations*". This DNT IVb can be used for screening and prioritizing, however, further effort is needed to gain its international acceptance for hazard characterization and risk decisions.

In Tables 23-24, methods developed recently for assessing NT/DNT for nanomaterials are summarized. Because of different levels of NAMs development, their limitations and relevance are virtually impossible to be concisely discussed.

Table 23. *Summary of NAMs relevant for NT*

Endpoint: Neurotoxicity (NT)			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs Not available			
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs • 3D-spheroids from human D384 astrocyte- and SH-SY5Y neuronal-like cells[313] [ID_98] • 3D LUHMES human neuronal precursor cells and BrainSpheres from neural progenitor cells[314] [ID_99]			

Table 24. Summary of NAMs relevant for DNT

Endpoint: Developmental neurotoxicity (DNT)			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs Not available			
Non-nano specific under validation NAMs Not available			
Nano-specific regulatory accepted NAMs Not available			
Nano-specific under validation NAMs Not available			
Nano-specific under development NAMs • Developmental Neurotoxicity *in vitro* testing battery[315] [ID_34] • Neuronal-like cells derived from human umbilical cord lining membranes mesenchymal stem cells as a tool for the neurotoxicity and developmental neurotoxicity testing[191] [ID_35]			

Hypersensitivity/Food intolerance

While assessing safety of food according to Regulation (EC) 1331/2008; Regulation (EC) No 1333/2008; Regulation (EC) No 2015/2283, the potential of substances to induce hypersensitivity/food intolerance should be considered. Food allergy differs from food intolerance in the type of reaction of the body. Food allergy is the reaction that is mediated by the immune system, particularly involving IgE antibodies, cellular mechanisms, or both. An adverse reaction that does not involve the immune system directly is considered as food intolerance. Details of hypersensitivity or food intolerance is provided in the "Draft Guidance for submission for food additive evaluations" by EFSA.[316]

NAMs relevant for this endpoint have not yet been described in the open literature. Considering that the mechanisms of food allergy/intolerance are still unclear and that most probably complex interactions are occurring in the GIT mucosa, development of relevant NAMs seems to be not possible within the next couple of years.

Water solubility, dissolution rate in relevant biological media (including stability in lysosomal fluid)

Annex VII, section 7.7. column 1, of the REACH Regulation specifies, that (beside the water solubility testing) "*for nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered*". Knowledge on dissolution rates may help to predict the toxicokinetic behaviour of particles. Some toxicological considerations and advice on information regarding solubility and dissolution of nanoforms in

biological media are described under Section 2.1.1 of the ECHA Guidance on information requirements and chemical safety assessment.[317] For the inhalation route of exposure, it is important to assess the dissolution rate in both, simulated lung lining fluid and phagolysosomal fluid, while for the oral route of exposure, dissolution of nanomaterials in simulated gastric fluid and macrophage phagolysosomal fluid is relevant.

According to ECHA the applicability of the dynamic method based on ISO TR 19057:2017[318] for lung and gastrointestinal fluids, has been successfully demonstrated by Koltermann-Jully et al.[319] and Bove et al.[320]Alternatively, OECD TG 105 with specific considerations for nanoforms[321] can be applied to determine dissolution rates.

While assessing the human safety of ENMs under the food EU Regulation (EC) No 2015/2283, there is the necessity to evaluate at first their rate of degradation under conditions that represent the gastrointestinal environment. Risk assessment of ENMs that have a high dissolution/degradation rate in biological fluids and, in effect, can be expected not to behave as nano-species, could follow the standard procedures as for conventional chemicals. Otherwise, the degradation tests under conditions representative of the human gastrointestinal tract and simulated lysosomal conditions that represent conditions after phagocytosis of the nanomaterials by macrophages are required. According to EFSA: "*Assessment of the stability in lysosomal conditions is important to screen the potential of nanomaterials for biopersistence and intracellular accumulation. Lysosomal conditions are considered a suitable model as lysosomal fluid is where nanomaterials generally distribute to and where degradation in lysosomal fluid can occur due to the acidic conditions and presence of enzymes*." [322] The procedure of assessment of the stability in lysosomal fluid is provided in the "Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. Chapter 7.2.2. Stability in lysosomal fluid" by EFSA. [322]

In Table 25, methods developed recently for assessing this endpoint for nanomaterials were summarized.

Table 25. *Summary of NAMs relevant for water solubility, dissolution rate in relevant biological media (including stability in lysosomal fluid)*

Endpoint: Water solubility, dissolution rate in relevant biological media (including stability in lysosomal fluid)			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs • OECD TG 105: Water Solubility [321] [ID_219]			
• The method has been validated for conventional chemicals, however official validation trials for ENMs have not yet started.	• The NAM is appropriate for ENMs testing, after slight adaptations.	High The NAM is relevant, however it requires some adaptations for ENMs testing.	High The endpoint is required by ECHA and food regulations. The validated NAM is already available but requires official validation for ENMs.

Non-nano specific under validation NAMs Not available
Nano-specific regulatory accepted NAMs • ISO TR 19057:2017[318–320] [ID_218] • Stability in lysosomal fluid EFSA[322] [ID_220]
Nano-specific under validation NAMs Not available
Nano-specific under development NAMs

Effects on gut microbiome

While assessing safety of food according to EU Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001, the potential role of hazardous substances on the gut microbiome variability and dysbiosis should be considered. The EFSA "*Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health*"[14] (2021) contains some relevant recommendations. Due to the human microbiota that lives on and in the human body plays an important role in maintaining the health homeostasis or eubiosis, the possible alteration of microbiota patterns can cause serious health effects.

Recently, in the framework of the European Food Risk Assessment (EU-FORA) promoted by EFSA, the program entitled '*Microbiota analysis for risk assessment of xenobiotics and its potential impact on dysbiosis and endocrine pathogenesis: microbiota learning by doing' was performed (EFSA EU-FORA – The European Food Risk Assessment (EU-FORA) Fellowship Programme, Cycle 2021-2022)*[323] and published. The main outcome of this initiative and other studies is that chemicals, including nanomaterials can affect microbial composition in the gut, although the underlying mechanism of this process and the health implications remain unclear. It is important to consider this effect for insoluble/persistent nanomaterials, especially in case they have antimicrobial effects. Although the number of *in vitro* studies exploring how the materials affect gut microbiome is increasing, the wider application of NAMs for assessing this endpoint is limited. According to EFSA: "*Defining the human risk related to the cocktail effect of unintentional mixtures of xenobiotics in the farm-to-fork chain is still a challenge for scientists. Adoption and integration of new approach methodologies (NAMs) into the next generation risk assessment (NGRA) is still under development as animal studies become less relevant with time and integrated approaches to testing and assessment (IATAs) are required (Escher et al., 2022*[324]*). However, the vision for 2030 is to develop and implement a harmonized approach for the assessment of human health risks resulting from both dietary and non-dietary exposure to multiple chemicals*". [323] In line with this perspective, recently 3D *in vitro* models of the human gut microbiota have been developed.[325] Beneficial, in this context would be also the application of extended co-cultures of living human intestinal epithelium with stable communities of aerobic and anaerobic human gut microbiota, designed as microfluidic intestine-on-a-chip devices.[326] However, since these methods are still at the early stage of development for conventional chemicals, their potential adaptation for nanomaterials will not be evaluated in this Report.

Toxicokinetics

According to REACH[38] Guidance on Information Requirements (page 453 of the Guidance) "*toxicokinetic data should be considered in the light of other toxicity data (i.e., repeated dose toxicity) to assist in the estimation of internal exposure to the substance and/or its metabolites and the correlation of the effects observed with internal dose estimates. This is of particular importance for characterizing a dose-response relationship and determining whether administered doses caused saturation kinetics resulting in a non-linear dose-response*." Thus, toxicokinetic parameters provide information on the possible accumulation of the test chemicals in tissue and/or organs, as well as the potential for induction of biotransformation because of exposure to the substance. These parameters need to be assessed under the following EU Regulations: Regulation (EC) No 1907/2006; Regulation (EU) No 528/2012; Regulation (EC) No 1223/2009; Regulation (EU) No 2015/2283, Regulation (EU) No 1169/2011, Regulation (EC) No 258/97, Regulation (EC) No 1852/2001; Regulation (EC) No 1331/2008; Regulation (EC) No 1333/2008. Procedure of conducting *in vivo* toxicokinetic studies is provided in OECD TG 417.[327] This protocol describes how to obtain adequate information on the chemical substance absorption, distribution, biotransformation, and excretion and how to link this data with the concentration or dose to the observed toxicity. However, it is explicitly mentioned in paragraph 9 of that guideline, that it is not intended for the testing of nanomaterials. The ISO/TR 22019:2019[328] "Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials" document describes the background and principles for toxicokinetic studies relevant for nanomaterials. There is also ongoing work for a new OECD TG on toxicokinetics specifically for ENMs for all routes of exposure (New Test Guideline on toxicokinetics to accommodate testing of nano-particles[147]), however this methodology cannot be classified as a NAM. One NAM "Cytochrome P450 (CYP) enzyme induction in vitro on human HepaRG™ cells" has been validated by ECVAM, however its regulatory acceptance is ongoing.

Recently, physiologically based kinetic models (PBK) were proposed to estimate the doses that cause the toxic effect. Such models allow to describe the distribution of chemicals by describing the body as a set of compartments that represent biological tissues and/or organs. Adaptation of these models for the nano-specific exposure was one of the objectives of EU project PATROLs. As a result, the PBPK models to be used in the domain of inhalation exposure to nanomaterials was proposed. There are also few recently developed models for testing translocation of ENMs through different barriers. Details are provided in Table 26. Because of different levels of NAMs development, their limitations and relevance are virtually impossible to be concisely discussed.

Table 26. *Summary of NAMs relevant for toxicokinetics*

Endpoint/Parameter: Toxicokinetics			
Advancement status of the NAMs in ENMs testing	**Potential limitations of the NAMs for adaptation for ENMs testing**	**Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)**	**Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)**
Non-nano specific regulatory accepted NAMs Not available			

Non-nano specific under validation NAMs Cytochrome P450 (CYP) enzyme induction *in vitro* on human HepaRG™ cells[329] [ID_206]
Nano-specific regulatory accepted NAMs Not available
Nano-specific under validation NAMs Not available
Nano-specific under development NAMs • PBPK Model for nanomaterials[330] [ID_207] • *In Vitro* Dosimetric model for nanomaterials[331] [ID_208] • Intestinal Transport: Follicle-associated epithelial model mimicking microfold (M) cells[273] [ID_209] • Intestinal Transport: Caco-2 monoculture[273] [ID_210] • Fluorescence Quenching of Food Proteins by NPs[273] [ID_211] • VenaFlux™ Platform (Cellix Ltd): studying endothelial cells under regulated shear stress conditions[332] [ID_212] • Endothelialized microfluidic device to probe nanoparticle translocation over a permeable microvessel[333] [ID_213] • Blood-Brain Barrier: the triple co-culture model: ALT/ bEend.3/ N2a cell lines[248] [ID_214] • Blood-Brain Barrier: static or flow microfluidic model: bEnd.3 cells[334] [ID_215] • Blood-Brain Barrier: multicellular 3D spheroids: 6 brain cell types: astrocytes, pericytes, endothelial cells, microglia cells, oligodendrocytes, and neurons[335] [ID_216] • Synthetic microvascular networks (SMNs): immortalized RBE4 cells[336] [ID_217]

Survey analysis

The goal of this report is to present a broad perspective on NAMs in the context of nanomaterials risk and human safety assessment. To achieve this goal, the report includes a survey of experts from different fields of expertise. These experts include those with experience in different methodologies (such as *in vitro*, *in silico*, and *in chemico*) for toxicological endpoint-specific assessments, as well as those with different levels of experience in NAMs. This includes experts representing regulatory and industry perspectives, as well as academia. The outputs from the interviews brought a valuable and critical insight into the project, revealing the actual advancements on alternative testing methods, and a practical perspective with regard to the already existing regulations and available data for nano-specific NAMs.

As a result of selection of the experts from three targeted groups (Academia, Regulatory agencies, and Industry), 262 experts in total were chosen for further surveys. The most representative group was academia (119 experts), next to industry (81 experts), and 60 experts representing regulatory agencies, Figure 10.

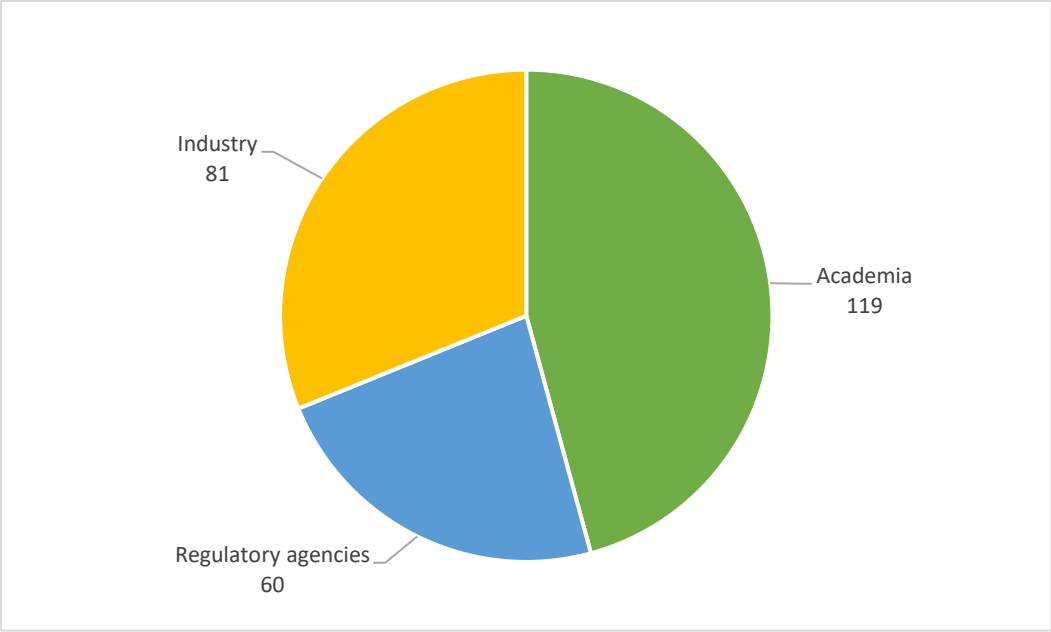


***Figure 10**. Structure of preliminary list of experts for surveys*

The initially selected experts represented various centres and research institutions in Europe and beyond, as well as regulatory agencies and industries. In effect, experts represented different nano-enabled product relevant industry sectors, i.e. pigment industries, household chemicals, polymers and coatings, Figure 11.

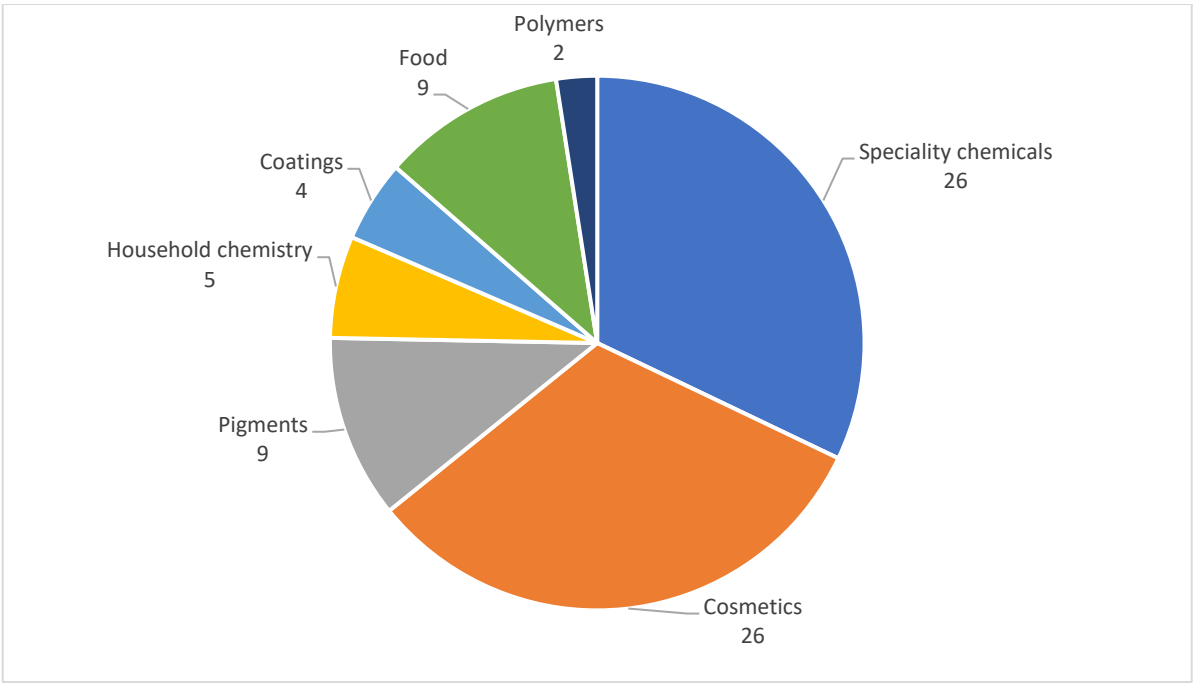


***Figure 11**. Structure of industry group of experts*

All selected experts were contacted via mail or LinkedIn. However, we were able to reach 219 experts out of a preliminary list of 262 names. This was due to the fact that some of the experts had inaccessible/outdated contact information or due to technical difficulties (i.e., spam/junk e-mail filers in internal networks). The invitation was accepted only by 25 experts, representing a

percentage of 11.4% of responses to all emails sent. In effect, the responses to the survey were gathered from 11 representatives from Academia, 10 representatives from Industry and 4 experts from Regulatory Agencies (Figure 12). The list of experts that took part in the survey is attached to the report as Annex 3.

The selected experts were informed about the expected duration of the survey, which was approximately 20-30 minutes. Considering best practices, respondents were given a relatively long time to complete the survey. The extended timeframe was due to the inclusion of open-ended questions, which required the expert to share their experience and personal analysis. Despite this, the responses received were satisfactory and the respondents demonstrated their expert knowledge and shared their own experiences.

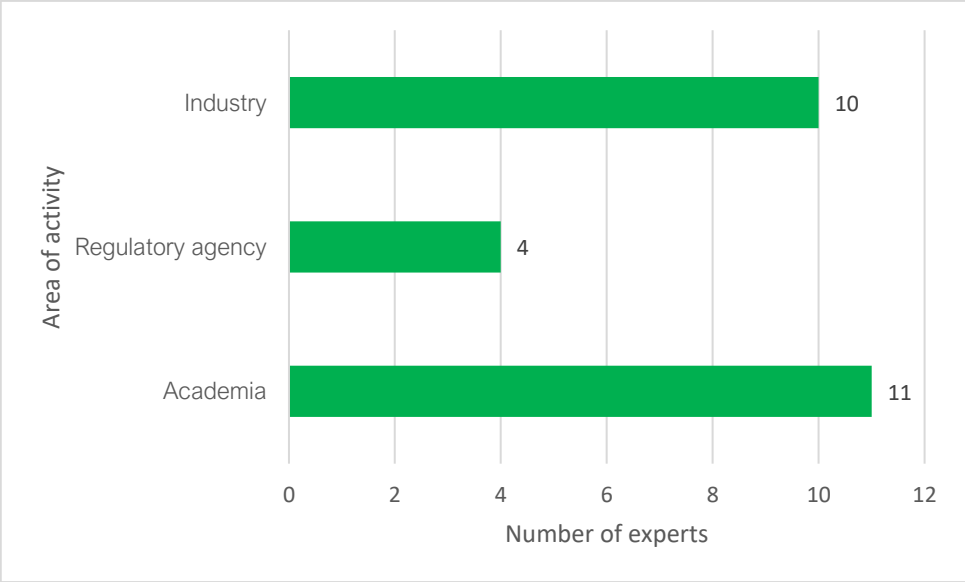


***Figure 12**. Representation of working area of the experts that took part in the survey*

The experts were asked 12 questions: 1) three introductory and close-ended questions; and 2) nine open-ended questions. All asked questions are listed in **Annex 1**: Expert survey - List of questions. The summary of the provided answers to each question is provided below.

Introductory and close-ended questions

In order to identify the experts' field of expertise and to specify what alternative testing methods they are specialized in, respondents were asked to select the listed regulatory relevant endpoints that they are familiar with (additionally within each endpoints they were asked to indicate the specific methods). Experts were asked about their experience and work with the following endpoints:

- Gastrointestinal digestion,
- Stability in lysosomal fluid,
- *In vitro* toxicity testing,
- Skin corrosion,
- Skin irritation,

- Serious eye damage/eye irritation,
- Skin sensitization,
- Phototoxicity,
- Dermal absorption,
- Respiratory sensitization,
- Acute toxicity,
- Repeated dose toxicity,
- Neurotoxicity,
- Reproductive toxicity,
- Developmental toxicity (including neurotoxicity),
- Endocrine disruption,
- Immunotoxicity,
- Hypersensitivity,
- Toxicokinetics,
- Carcinogenicity,
- Mutagenicity.

Of all the respondents' answers, 64% indicated that they were specialized in *in vitro* research, 34% operated in the field of *in silico* methods, and only 2% of the respondents had experience with *in chemico* methods (Figure 13).

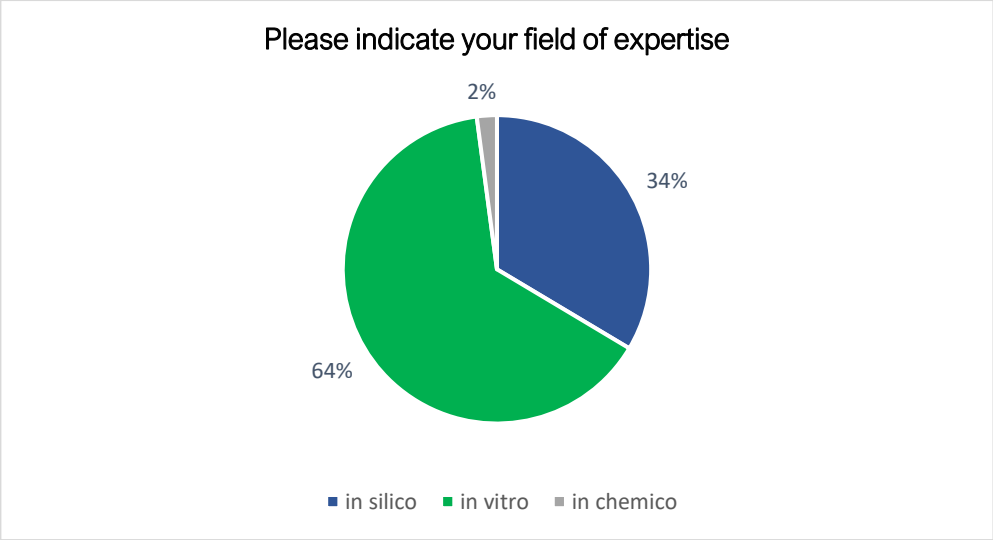


Figure 13. *Pie chart presenting experts field of expertise*

To verify whether the selected group of experts was relevant for this project, the respondents were asked questions verifying and checking their familiarity with NAMs and their regulatory relevance:

Q1: Are you familiar with European regulatory requirements in assessing human safety of chemicals?

24 out of 25 survey participants answered yes. Respondents considered the REACH Regulation to be their best-known regulation, as many as 21 of them confirmed that they were familiar with this legal framework. Subsequently, 14 respondents were familiar with the Regulation on Cosmetics Products, 11 with the Regulation on Food/Feed additives, and 9 with the Regulation on usage of Biocidal products (Figure 14).

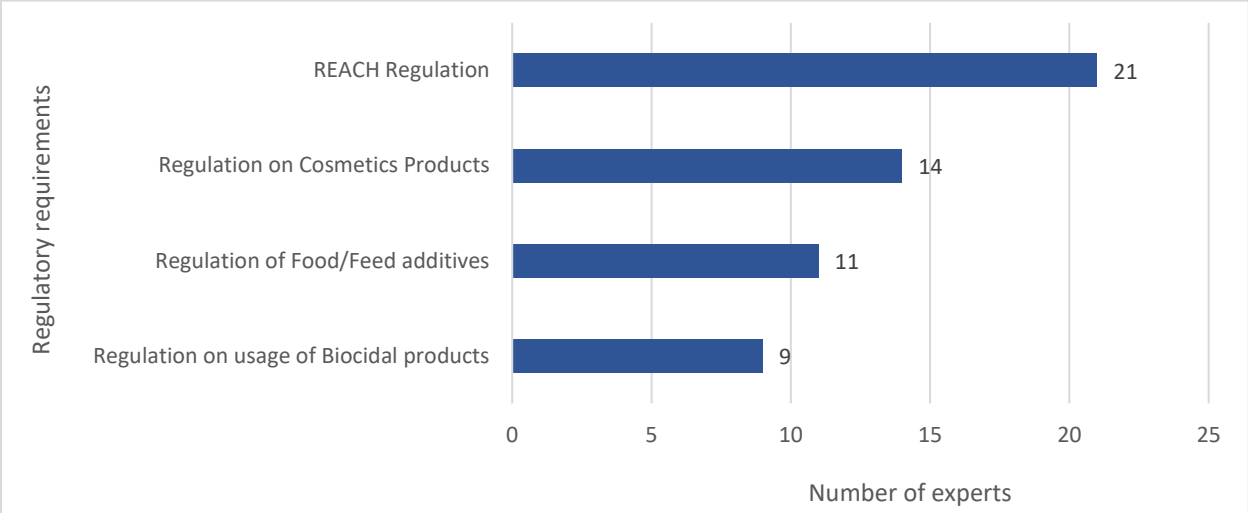


Figure 14. *European regulations that experts are familiar with*

Q2: Do you have any experience with NAMs?

As many as 21 respondents answered yes, and 4 that they had no experience with NAMs (Figure 15). Most respondents, 15, described their experience with NAMs as "I am or have been involved in the development of NAMs", 13 respondents indicated that "I have used/am using NAMs", and 9 respondents indicated that they have "assessed quality and/ or regulatory relevance of NAMs".

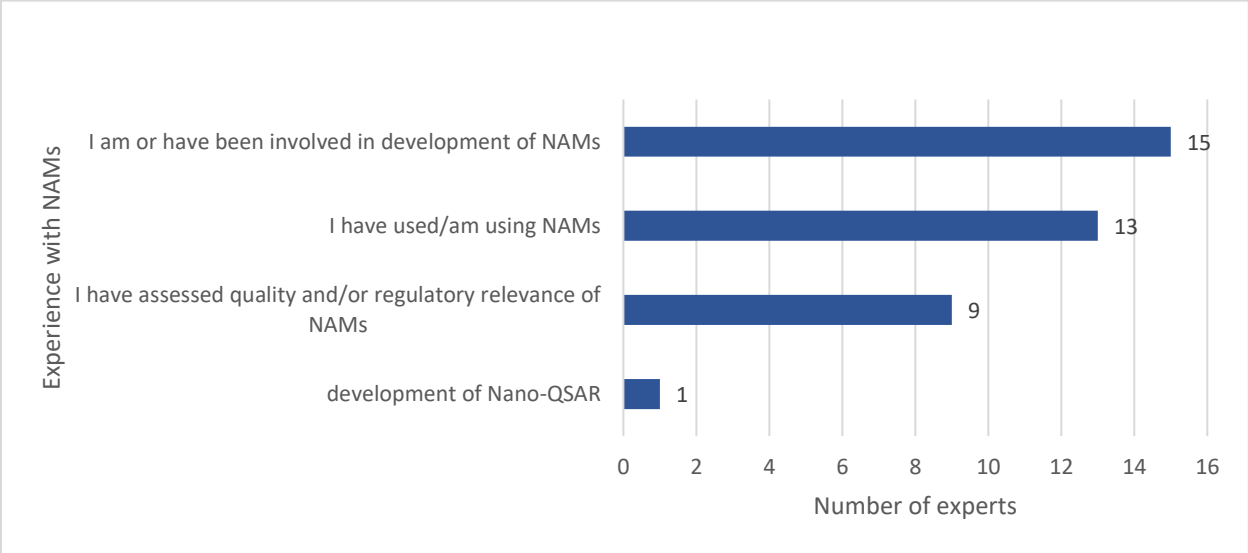


Figure 15. *Types of experience with NAMs*

Q3: Are you familiar with nano-specific NAMs in human safety assessment?

Third question was related to the expert knowledge in the topic of nano-specific NAMs in human safety assessment. Respondents answered similarly as for question 2: twenty one of them answered that they were familiar with nano-specific NAMs in human safety assessment. Four respondents replied that they had no such knowledge (Figure 16).
The majority of the experts had experience with nano-specific NAMs, having been involved in their development (11 experts). Eight experts indicated that they worked with nano-specific NAMs on a daily basis, and an equal number of experts stated that they were familiar with nano-specific NAMs because they have evaluated their quality and regulatory relevance. Some experts also reported that there were advancements in toxicology and exposure science, which contributes to their familiarity with developments related to NAMs (Figure 16).

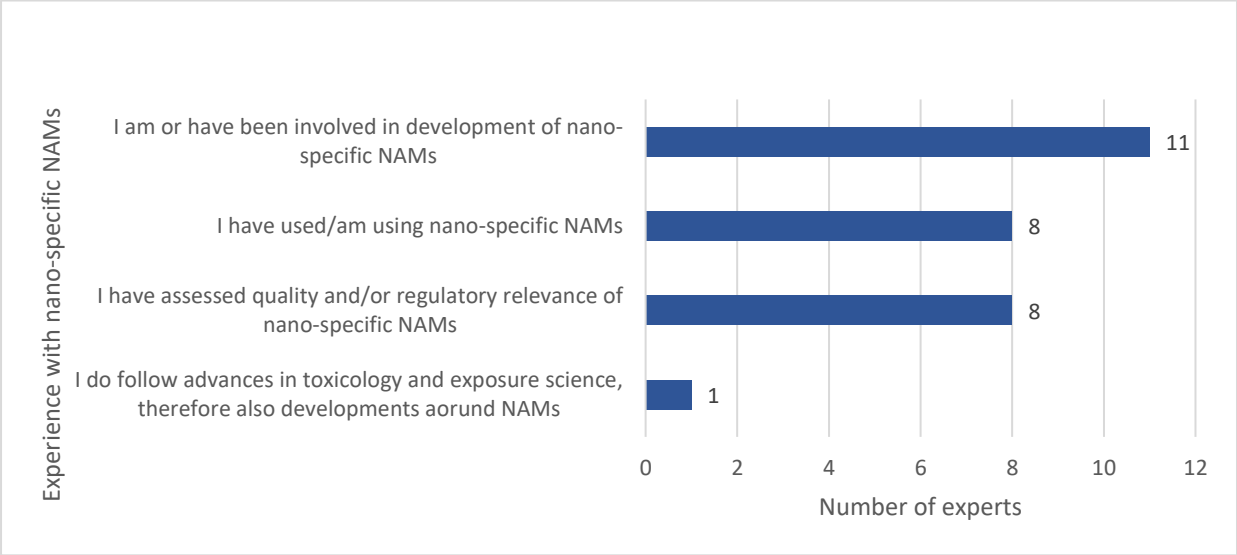


Figure 16. *Types of experience with nano-specific NAMs*

Summing up, the verification questions confirmed that the group was appropriately selected and that the experts had a thorough knowledge of the current regulations of the chemical industry, with 96% of respondents indicating so. In addition, 84% of the experts belonged to a group with experience working with NAMs and a similar percentage had experience operating in the field of nano-specific NAMs. It is worth noting that a significant number of respondents have experience in the development of nano-specific NAMs, as they are actively involved in this area.

Open-ended questions

The second part of the survey consisted of 9 open-ended questions, where the respondents were given the possibility to provide their answers in the form of handwritten opinion/text on specific topics. All open-ended questions are presented below, along with a brief summary of the respondents' answers.

Q4: Are you familiar with industry needs in relation to nano-specific alternative methods in human safety assessment? If yes, can you identify any NAMs that are currently used to assess the human health hazard of nanomaterials by the industry (manufacturers)?

Eighteen out of 25 respondents stated that they were familiar with the industry needs for nano-specific NAMs in human safety assessment. Most commonly mentioned NAMs were related to the skin sensitivity/irritation assays, reconstructed skin models and *in vitro* skin batteries, i.e., ISO 21699. Other answers included, in general, assays performed on cell cultures (cytotoxicity, morphological and metabolic alterations), measurement of biosolubility and dissolution in environmental media, AOP and transcriptomic approaches, *in vitro* assays to assess effects of nanomaterials on the respiratory system (i.e., short term inhalation toxicity, lung surfactant inhibition, biosolubility) and electrochemical screens on biomembranes. In addition, some commercially available 3D organotypic models that represent different routes of exposure, such as MatTek, Epithelix, AlveoliX, and ImmuOne were listed. Few respondents reported usage of *in silico* approaches, such as Quantitative structure–activity relationship (QSAR) and read-across or a mixture of several *in vitro* and *in silico* methods. Furthermore, answers suggested that currently, vast majority of NAMs used to assess hazard of nanomaterials in the industry are based on already existing methods used for classical chemicals, such as OECD Test Guidelines (OECD TG 487, OECD TG 476, OECD TG 473).

Can you point out any NAMs that should be prioritized and adapted to nanomaterials in relation to industry needs?

Experts prioritized industry needs mainly for tests related to specific types of endpoints and *in vitro* models for different types of organs (lung, liver, gut, organs). The mentioned endpoints included genotoxicity, carcinogenicity, dermal and oral absorption, inhalation toxicity, neurotoxicity, and toxicokinetics. Additionally, the responses indicated needs for: i) the adaptation of already existing regulatory guidelines (e.g. OECD TGs) for nanomaterials; ii) the development of complex *in vitro* models that would reflect specific organs; iii) the development of machine learning and *in silico* approaches using sufficient datasets; iv) the integration of -omics in safety assessments; v) the development of nano-specific AOPs; and vi) the prioritization of high-throughput screening to test cell toxicity endpoints.

Q5: Are there any existing, validated regulatory accepted NAMs (NAMs intentionally developed and validated for conventional chemicals) that can be adapted and applied to assess safety of nanomaterials? If yes, please indicate those NAMs.

Thirteen out of 25 respondents answered yes to above question, two answered no, and ten that they did not know. Responses included in general (no specific indication) NAMs for skin sensitization, skin and eye irritation/corrosion, phototoxicity, genotoxicity. In addition, several OECD Test Guidelines were listed, such as: TG442D, TG455, TG456, TG458, TG471, TG487, TG490, and TG476. The respondents indicated that most of the validated local toxicity tests can be adapted for nanomaterials with proper consideration of the nano-specific properties, their dispersion, and cell uptake.

If no, please indicate what, in your opinion, needs to be done to make those NAMs relevant for nanomaterials testing.

The experts raised several main issues regarding the use of NAMs in nanomaterials safety assessment. Firstly, experts indicated that there were several evaluated nano-specific NAMs and their alternatives, and the priority should be to validate them. However, the often very limited funding to support the validation process, which can be time-consuming and resource-intensive was considered as the biggest barrier. Another issue that has been indicated is the need for adaptation of regulatory relevant NAMs/endpoints to be considered for nanomaterials. Additionally, the characterization of nanomaterials, both in their pristine form and in culture media should be considered as an integral part of every NAM. Lastly, experts suggested that some alternative methods, such as high-throughput screening and omics approaches, may not be feasible for regulatory acceptance due to the lengthy and extensive nature of the process of implementing. As these methods tend to evolve quickly, it will be difficult to keep up with the necessary regulatory standards.

Q6: Are there any recently developed/under development (not yet regulatory accepted) NAMs (intentionally developed and validated for conventional chemicals) that can be adapted and applied to assess safety of nanomaterials? If yes, please list known recently developed/under development NAMs that in your opinion can be adopted for nanomaterials testing? Please indicate, if possible, the reasonable timeframe for regulatory acceptance (up to 1 year, 1-3 year, 3 -5 years, more than 5 years)

Twelve out of 25 respondents have indicated to be familiar with recently developed or under development NAMs that can be adapted to assess safety of nanomaterials. According to the experts' answers, there are several recently developed or under development NAMs that can potentially be adapted and applied to assess the safety of nanomaterials. These include NAMs for genotoxicity testing using 3D models such as liver spheroids and lung co-culture models, which are not yet validated and may take more than 5 years to become regulatory accepted. There are also NAMs for genotoxicity testing on 3D reconstructed skin that have been adapted for nano and an OECD TG is currently under preparation, which could take 1-3 years to become regulatory accepted. The EU H2020 Project PATROLS (https://www.patrols-h2020.eu/) developed liver and lung models, which may take 3-5 years to become regulatory accepted. Additionally, the outcomes of NanoHarmony project funded through Horizon 2020, (https://nanoharmony.eu/) were mentioned, as the project goal is to deliver Test Guidelines and Guidance documents for eight nanomaterials-adapted test methods. Other NAMs that have been mentioned include several *in silico* approaches such as: read-across, quasi-SMILES, IVIVE (*In Vitro* to *In Vivo* Extrapolation), PBTK (Physiologically Based Toxicokinetic) modelling. Additional NAMs that have been mentioned included the ALI (Air-Liquid-Interface) respiratory model, 3D liver model, gut model, co-culture models, ToxTracker (https://toxys.com/toxtracker/), Cell Transformation Assay for morphological transformation, 3D skin model for genotoxicity, reverse dosimetry, organoids, organ on a chip, NAMs for developmental neurotoxicity, *in vitro* carcinogenicity, and OECD TG249.

Q7: Do you know any recently developed/under development (not yet regulatory accepted) nano-specific NAMs? If yes, please list known recently developed/under development nano-specific NAMs that in your opinion can be adopted for nanomaterials? Please indicate, if possible, the reasonable timeframe for regulatory acceptance (up to 1 year, 1-3 year, 3 -5 years, more than 5 years)

Fourteen out of 25 respondents have indicated to be familiar with any recently developed/under development (not yet regulatory accepted) nano-specific NAMs. Respondents answered that several nano-specific NAMs have recently been developed or are under development for the evaluation of nanomaterials, including *in vitro* alveolar macrophage assay, quasi-SMILES technique, transcriptomics-driven predictive modeling, genotoxicity assays, ALI respiratory model, 3D liver model, GUT model, co-culture models, ToxTracker, Cell Transformation Assay for morphological transformation, and 3D skin model for genotoxicity. Some of these methods may be suitable for regulatory acceptance within the next 1-3 years, while others may take longer (up to 5 years). PATROLS project has also developed experimental tools to predict potential human and environmental hazards from engineered nanomaterial exposure. Likewise, the NanoHarmony project, has the mission to support the development of Test Guidelines and Guidance Documents for eight endpoints where nanomaterial-adapted test methods have been identified as a regulatory priority.
Moreover, experts mentioned *in vitro* methods suitable for investigating the mechanism of gastrointestinal absorption. The OECD is developing an integrated *in vitro* approach for the intestinal fate of orally ingested nanomaterials based on a coculture of three different cell lines. The EFSA has also launched a grant for covering the *in vitro* uptake of nanofibres. Microphysiological systems, conventionally named as gut-on-a-chip, reproduce peristaltic movements, and can include the interaction of the nanoparticles with the gut microbiome; these systems could become a suitable alternative to *in vivo* systems in the future.

Q8: Are there any NAMs that, while identifying the potential hazards, incorporate exposure and dose into the final determination on a nanomaterial's safety to a particular population? If yes, please list those NAMs and comment on their suitability for the risk assessment for nanomaterials.

Eight out of 25 respondents answered “yes” for question #8. Experts mentioned several NAMs that incorporate exposure and dose into the final determination of a nanomaterial's safety. Among listed, there were: quasi-SMILES technology, air-liquid-interface model, *in vitro* alveolar macrophage assay, biosolubility and an inhalation-ingestion bioaccessibility assays. Respondents additionally mentioned useful risk assessment tools that consider exposure and dose, such as: NanoSafer (http://www.nanosafer.org/), ECETOC NanoApp (https://nanoapp.ecetoc.org/), Stoffenmanager (https://nano.stoffenmanager.com/).

Q9: What is an expected timescale, in your opinion, needed for potential transfer of the nano-specific NAMs into EU regulations considering current gaps and development needs in particular regulations (i.e., REACH, Cosmetics Directives)?

Fourteen experts out of 25 asked, gave an answer with estimated timescale needed for potential transfer of the nano-specific NAMs into EU regulations considering current gaps and development needs in particular regulations. Eight of the experts responded that they believe this process could take up to 5 years, while 6 experts estimated a timescale of 5 to 10 years. One expert believed that the process could be completed within a shorter period of 2 to 3 years. Other experts raised issues related to sufficient funding as well as intensified/well-coordinated efforts to support validation of the methods.

Q10: What are, in your opinion, the development gaps and needs of the NAMs to fulfil nanomaterials specific safety testing requirements?

Several development gaps and needs aimed towards NAMs in order to fulfil safety testing requirements for nanomaterials have been mentioned. Respondents pointed out that extensive physicochemical characterization of nanomaterials should be incorporated in those methods, and performed under appropriate conditions, in which human exposure is likely to occur. Experts also listed a need for increased accessibility, usability, and validation of NAMs followed by systematized databases that are easily accessible, and for the implementation of NAMs in the area of safe-and-sustainable-by-design (SSbD) through case studies. The availability and reusability ("FAIRness" - Findability, Accesibility, Interoperability and Reusability) of data is also crucial in supporting the development and application of NAMs, as well as increasing trust in these methods. The precautionary principle is often applied in the absence of sufficient data on the toxicological effects of nanomaterials, but it is important for NAMs to be exposure-driven in order to accurately assess potential risks.

It is important to focus on the biological relevance of NAMs and their alignment with human biology, as well as their ability to provide information that leads to health protective decisions. Nano-specific NAMs should consider different exposure routes, such as topical, oral, and inhalation, and there is a significant gap in understanding *in vitro* dosimetry for nanomaterials compared to conventional chemicals. It should be noted that the occurrence and probability of the appearance of nanoforms in the tested materials and their ability to reach the tested biological system should be adapted to the real-life use scenarios. Experts imply, that there are a lot of data about potential hazard properties of pure nanoforms, however in real life situations they constitute only a fraction of used raw materials, especially from the industry perspective, where pristine material properties are negligible in industrial use case scenarios due to materials being specifically stabilized and coated to ensure lack of these effects. Hence, for example, in the context of cosmetic formulations, the experts point out, that there is a need for detailed recommendations on the use of transport enhancement techniques in NAMs, i.e., which techniques are allowed, and which are non-appropriate for hazard assessment purposes (because of too excessive modification of nanomaterial or biological system behaviour). There is also a significant lack of understanding

among the general public about the nature of nanomaterials and the representativeness of test results for these materials, which has led to stigmatization of these materials.

Q11: Which of the nano-specific NAMs, being currently developed, are the most promising ones from the regulatory point of view (preferably, list them in a descending order of importance)?

Experts listed several nano-specific NAMs currently in development that are considered promising from a regulatory perspective. These include mainly methods being developed by OECD and ISO (i.e. OECD TG 442D, OECD TG 201, OECD TG 202, OECD TG 203), and those being developed within the Malta initiative (specifically the outcomes of the Gov4nano, PATROLS and NanoHarmony projects). Very often, the experts mentioned co-culture models, 3D liver and skin models, gastro-intestinal and inhalation models for toxicity, inflammatory and genotoxicity testing as the most promising ones from the regulatory point of view. Few mentioned *in silico* methods, including read-across techniques and, in general, reuse of the existing data and databases. Other mentioned NAMs included *in vitro* alveolar macrophage assay, high-throughput screening of basic cell toxicity endpoints, electrochemical screens using fabricated membrane microelectrodes.

Q12: (For industry only): In the context of industry needs, which of the nano-specific NAMs under development are the most promising ones (or should be prioritized) to gain the regulatory acceptance?

Experts mentioned several nano-specific NAMs, that are the most promising ones (or should be prioritized) to gain the regulatory acceptance in the context of industry needs. These include mainly NAMs developed by the OECD and ISO (such as OECD TG 442D, OECD TG 201, OECD TG 202, and OECD TG 203). Genotoxicity testing and inhalation toxicity are seen as the most urgent areas of focus, with other methods such as the Cell Transformation Assays, 3D skin model, ALI respiratory models, 3D liver models, gut models, and co-culture models being suggested as promising ones. Some answers suggest prioritization of *in silico* methods using machine learning. Furthermore, it is recommended to prioritize methods developed in the Malta initiative, in particular outcomes of the Gov4nano, NanoHarmony and PATROLS projects

4. Summary and main conclusions

4.1 Main findings of literature review

In order to identify alternative methods for evaluating toxicological endpoints relevant to human safety, including ENMs, the available documents were reviewed (i.e., OECD TG/GD, ISO standards, ECVAM repositories, SOPs, scientific publications, nano-relevant AOPs, EU project deliverables and the OECD Working Plans). In this Report we did not review all available scientific publications on NAMs, thus, did not consider methods that are currently under development for conventional chemicals (in the case of conventional chemicals only methods that are already validated and regulatory accepted were considered). Hence, this Report should not be considered as complete or comprehensive review of research efforts in the area of NAMs, but

rather the summary of NAMs that may be useful for taking practical actions to increase the use and acceptability of NAMs for ENMs testing.

There are different limitations of NAMs, not only nano-specific ones, when used for regulatory purposes. Despite that the potential of several NAMs was demonstrated, their regulatory acceptance has mostly not been established yet, mainly due to the difficulty of addressing complex endpoints (e.g., repeated dose toxicity), the issue of translating the concept of adversity to NAMs, doubts of stakeholders about the level of chemical safety ensured by NAMs, and lack of internationally harmonised guidance on the interpretation of NAM-derived data. [337] For practical discussion on the use of NAMs in regulatory decisions for chemical safety and how they can be integrated into the Next Generation Risk Assessment (NGRA) methodologies for decision-making purposes, please see a report from the EPAA Workshop.[338]

In the conclusions section we tried to provide a simple and general prioritisation of the research needs, which should be directed towards developing validated NAMs for ENMs testing for different toxicity endpoints. The prioritization was based on a general relevance of all the NAMs (as a group) addressing a particular endpoint, rather than on an analysis of regulatory applicability of individual NAMs in the group. Our proposal was based solely on the experience of the QSAR Labs' researchers; however, it was consulted with an external expert in the field of NAMs for ENMs testing. The Report indicates gaps, needs and future directions in the context of NAMs, therefore can serve as a starting point in the further development of nano-specific NAMs.

An inventory of all NAMs covered - Table 7 presented in "Results" section of "Alternative methods for assessing the human safety of nanomaterials" in this Report is reproduced below for convenience.

Table 7. *The number of identified NAMs for each endpoint and classification of the NAMs according to their stage of regulatory acceptance and development.*

Regulatory relevant endpoint	*Nano-specific regulatory accepted NAMs*	*Nano-specific under development NAMs*	*Nano-specific under validation NAMs*	*Non-nano-specific regulatory accepted NAMs*	*Non-nano-specific under validation NAMs*
Acute toxicity oral	-	1	-	1	-
Acute toxicity inhalation	-	26	-	-	-
Acute toxicity dermal	-	-	-	-	-
Carcinogenicity	-	1	-	3	-
Dermal absorption	-	-	-	1	-
Developmental neurotoxicity	-	2	-	-	-
Effects on gut microbione	-	-	-	-	-
Endocrine disruption	-	-	-	5	14
Eye damage/eye irritation in vitro	-	-	-	22	1
Gastrointestinal digestion	-	12	1	-	-
Hypersensitivity/Food intolerance	-	-	-	-	-
Immunotoxicity/developmental immunotoxicity/allergenicity	-	8	-	-	-
In vitro toxicity testing* *cytotoxicity/cell viability* *oxidative stress* *(pro-)inflammation* *gastrointestinal barrier integrity*	5	26	-	-	-
Mutagenicity/genotoxicity	-	11	2	6	-
Neurotoxicity	-	2	-	-	-
Phototoxicity	1	-	1	3	-
Repeated dose toxicity	-	17	-	-	-
Reproductive toxicity/Endocrine disruption/Developmental toxicity	-	3	-	3	3
Respiratory sensitization	-	-	-	-	-
Skin corrosion in vitro	-	-	-	6	-
Skin irritation in vitro	-	-	-	6	-
Skin sensitisation in vitro/in chemico	-	-	1	11	-
Toxicokinetics	-	11		-	1
Water solubility and dissolution in biological media (including stability in lysosomal fluid)	2	-	-	1	-
Total number of NAMs:	**8**	**120**	**5**	**68**	**19**

* In the case of '*in vitro* toxicity testing', this endpoint is recommended by EFSA (2021: Guidance on risk assessment of nanomaterials) and relevant for the food regulations (Regulation (EU) No 2015/2283 amending Regulation (EU) No 1169/2011 and repealing Regulation (EC) No 258/97, Regulation (EC) No 1852/2001), and refers to testing cytotoxicity/cell viability, oxidative stress, (pro-)inflammation, gastrointestinal barrier integrity after exposure to different food components. However, many of the NAMs included in the table for 'Toxicity *in vitro* testing' can be useful in assessment of other endpoints (e.g., acute toxicity inhalation or oral).

Main conclusions based on the shortened and adapted Table 27:

Table 27. *Nano-specific under development NAMs sorted according to their prevalence. A corresponding number of non-nano-specific regulatory accepted NAMs are provided as well.*

Regulatory relevant endpoint	*Nano-specific under development NAMs*	Regulatory relevance of the NAMs as a group for ENMs testing (High, limited, low)	Development needs of the NAMs to fulfil nanomaterials specific safety testing requirements (High, limited, low)	*Non-nano-specific regulatory accepted NAMs*
Acute toxicity inhalation	26	limited	high	-
*Toxicity in vitro testing**	26	too wide specificity to be assessed	high	-
Repeated dose toxicity	17	high	high	-
Gastrointestinal digestion	12	limited	high	-
Mutagenicity/genotoxicity	11	high	high	6
Toxicokinetics	11	too diversified levels of development to be assessed	high	-
Immunotoxicity/developmental immunotoxicity/allergenicity	8	low	high	-
Reproductive toxicity/developmental toxicity/endocrine disruption	3	limited	high	3
Developmental neurotoxicity	2	too diversified levels of development to be assessed	high	-
Neurotoxicity	2	too diversified levels of development to be assessed	high	-
Acute toxicity oral	1	limited	high	1
Carcinogenicity	1	limited	high	3
Serious eye damage/eye irritation in vitro	-			22
Skin sensitisation in vitro/in chemico	-			11
Skin corrosion in vitro	-			6
Skin irritation in vitro	-			6
Endocrine disruption	-			5
Phototoxicity	-			3
Dermal absorption	-			1
Water solubility and dissolution in biological media (including stability in lysosomal fluid)	-			1
Acute toxicity dermal	-			-
Effects on gut microbione	-			-
Hypersensitivity/Food intolerance	-			-
Respiratory sensitization	-			-
	120			68

* In the case of 'Toxicity *in vitro* testing', this endpoint is recommended by EFSA (2021: Guidance on risk assessment of nanomaterials) and relevant for the food regulations (Regulation (EU) No 2015/2283 amending Regulation (EU) No 1169/2011 and repealing Regulation (EC) No 258/97, Regulation (EC) No 1852/2001), and refers to testing cytotoxicity/cell viability, oxidative stress, (pro-)inflammation, gastrointestinal barrier integrity after exposure to different food components. However, many of the NAMs included in the table for 'Toxicity *in vitro* testing' can be useful in assessment of other endpoints (e.g., acute toxicity inhalation or oral).

C1. There is a severe shortage of **nano-specific regulatory accepted NAMs** (according to our search, there are only 8 NAMs available so far). Most of the few accepted NAMs are available only for 3 endpoints, mainly for "Toxicity *in vitro* testing" (N=5). This number strongly indicates urgent needs for expediting validation processes for nano-specific NAMs which are currently under development for different endpoints.

C2. Definitely the highest number of NAMs belongs to the category **nano-specific under development NAMs** (N=120). As this group of NAMs seems to be the most interesting from the future perspective of regulatory implementation, we attempted to sort them according to their prevalence and simultaneously classify them in relation to their relevance for different endpoints and development needs to fulfil ENMs specific safety testing requirements. The classification was based on the subjective opinion of the QSAR Labs' researchers, however after taking into consideration the framework developed by Parish et al. (2020) for analysis of information on the various core principles and criteria available for a given NAM.

C3. For acute toxicity by inhalation, repeated dose toxicity, or toxicokinetics, which are among the most complex endpoints, a high number of **NAMs are under development**, with no (or only a single) corresponding **non-nano-specific regulatory accepted NAMs**.

This indicates rather high potential of the NAMs, but also a need to accelerate their validation and entering the phase of formal regulatory acceptance.

C4. Endocrine disruption, which is another extremely complex endpoint, is covered by 14 **non-nano-specific under validation NAMs**. However, for other complex endpoints like carcinogenicity, neurotoxicity or reproductive toxicity, very little **nano-specific under development** or **non-nano-specific regulatory accepted NAMs** have been found.

C5. For the majority of endpoints for which currently there are no **nano-specific under development NAMs**, e.g. phototoxicity, eye damage, or skin corrosion/irritation, there are at least several **nano-specific regulatory accepted NAMs**. This may indicate that the speed of current efforts towards application and validation trials of the latter NAMs for ENMs testing is too slow and the scientific community needs rethinking on how to improve the situation.

C6. The most complicated situation is for the endpoints for which currently there are neither **nano-specific under development NAMs** nor **nano-specific regulatory accepted NAMs** available, i.e., for: acute toxicity by dermal exposure, effects on gut microbiome, hypersensitivity/food intolerance, respiratory sensitisation. This situation may result and be understandable considering the complexity of the endpoints, however the population size of workers and consumers who are potentially exposed is high, implying the urgent need for developing **nano-specific** NAMs for these endpoints.

C7. In summary, the problem of development of nano-specific NAMs is complex. In the opinion of the authors of this Report, the unequal speed of development of the NAMs depends on the specific endpoint, which in turn depends on its complexity. Additionally, further efforts in the area should be based on a more constructive and effective dialogue between all interested stakeholders, especially involving the regulatory bodies.

4.2 Main findings of experts' surveys

The state of research on alternative methods is definitely improving year by year, and regulatory acceptance of nano-specific NAMs has become a very high priority. Experts pointed to the usefulness and suitability of several existing alternative methods for assessing the risk of nanomaterials. Currently, NAMs most commonly used by the industry to assess the hazard associated with nanomaterials are mainly *in vitro* skin sensitization/irritation assays, reconstructed skin models, *in vitro* skin batteries and ISO/TS 21633:2021 (Label-free impedance technology to assess the toxicity of nanomaterials in vitro). Experts very clearly indicated the usefulness of commercially available 3D organ models that represent different exposure routes (i.e., MatTek, Epithelix, AlveoliX, ImmuOne). Several respondents indicated use of *in silico* methods in general i.e., read-across, QSAR. Furthermore, the answers suggest that currently, the vast majority of NAMs used to assess hazard of nanomaterials in the industry are based on already existing methods used for conventional chemicals, such as OECD Test Guidelines (OECD 487, OECD 476, OECD 473).

On the other hand, there are several regulatory relevant endpoints and specific NAMs that, according to experts, should be prioritized in terms of both industrial needs and regulatory acceptance. These include tests related to specific types of organs such as lung, liver, gastrointestinal tract. The endpoints of highest priority include genotoxicity, carcinogenicity,

dermal and oral absorption, inhalation toxicity, neurotoxicity and toxicokinetics. Respondents indicate very high priority of adaptation of already existing validated OECD Test Guidelines for safety testing of nanomaterials.

Regarding the current progress in the development of nano-specific NAMs, the responses indicated several alternative methods that could potentially be adapted for use with nanomaterials within specific timeframes. Only a few of these methods were estimated to be ready for regulatory acceptance within the next 1-3 years, while most responses suggested that a timeframe for readiness may be up to 5 years or longer.

Several issues were raised concerning the fulfillment of nano-specific testing requirements that influence the time for regulatory acceptance of NAMs and their potential transfer into EU regulations. These included insufficient funding support and the need for intensified efforts from regulators to fully validate the methods. Additionally, consideration of exposure-driven scenarios/methods that take into account different routes of exposure of nanomaterials, as well as adjusting the test systems to mimic human biology are of high importance, i.e., development of appropriate *in vitro* exposure protocols that take into account the behavior of nanomaterials. Moreover, experts indicate the need for proper nanomaterial characterization, both in pristine forms and in culture media, which is often neglected. Other answers suggested needs for reusable data and accessible databases to support the development and validation of *in silico* methods.

5. Conclusions

The conclusions derived from both the literature review and the expert interviews indicate that the challenge of advancing nano-specific NAMs is exceedingly complex. In this context, it appears reasonable and scientifically justified to consider the possibility of adapting existing methods that have already been validated and regulatory accepted for conventional chemicals for use in ENMs testing. Respondents have identified the adjustment of validated OECD TGs for safety testing of nanomaterials as a matter of great importance. Among the NAMs that are predominantly employed by the industry for assessing the hazard linked with nanomaterials are primarily *in vitro* skin sensitization/irritation assays, reconstructed skin models, *in vitro* skin batteries, and ISO/TS 21633:2021 (Label-free impedance technology to assess the toxicity of nanomaterials in vitro). With regards to the most promising techniques, the usefulness of 3D organ models portraying different exposure routes was highlighted.

The most pressing needs regarding nano-specific NAMs, as identified by the literature review, align with the views expressed by experts who emphasize the need for prioritizing tests concerning specific organ types such as the lung, liver, and gastrointestinal tract. The endpoints that demand the highest priority encompass genotoxicity, carcinogenicity, dermal and oral absorption, inhalation toxicity, neurotoxicity, and toxicokinetics. The scarcity of techniques pertaining to these specific endpoints is comprehensible given their intricacy. Consequently, the uneven progress of NAMs development is a result of the complexity of the endpoint, which dictates the pace of its advancement. Therefore, it is essential that further endeavors in this domain are founded on a more productive and efficacious discourse involving all pertinent parties, with regulatory entities being accorded particular importance.

In order to expedite the regulatory acceptance of nano-specific NAMs and their potential implementation into EU regulations, it is imperative to consider the following measures: (i) adapting exposure-driven scenarios to account for diverse routes of ENM exposure; (ii) adjusting test systems to emulate human biology; (iii) developing appropriate in vitro exposure protocols that consider nanomaterial behaviour; (iv) developing effective methods for characterizing nanomaterials in their pure forms and within culture media; and (v) utilizing existing data and accessible databases to endorse the creation and validation of in silico methods.

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ANNEX 1: Expert survey - List of questions

Please confirm that you are aware of the goal of the survey and accept that your answer will be used by QSAR Lab to prepare the report commissioned by EUON.

Confirm and Accept/Do not accept
In case of selection 'Do not accept', the survey will be closed!

Please confirm that you hereby consent to your personal data being processed by QSAR Lab for the purpose of preparing the report commissioned by EUON.

Confirm/Prefer to be anonymous

Introductory – bringing some information about the respondent:

1. Full name:
2. Institution:
3. E-mail address:
4. Working area:
   Select from (you can choose more than one):
   - industry (please specify what kind?)
   - academia
   - regulatory affairs (please specify what kind?)
5. Field of expertise (the endpoints below have been selected based on relevance in different EU regulations):
   Select from (you can choose more than one):
   - Gastrointestinal digestion
     - *In vitro*
     - *In chemico*
     - *In silico*
   - Stability in lysosomal fluid
     - *In vitro*
     - *In silico*
     - *In chemico*
   - *In vitro* toxicity testing
     - *In vitro*
     - *In silico*
   - Skin corrosion
     - *In vitro*
     - *In silico*
     - *In chemico*
   - Skin irritation
     - *In vitro*
     - *In silico*
     - *In chemico*

- Serious eye damage/eye irritation
  - *In vitro*
  - *In silico*
  - *In chemico*
- Skin sensitisation
  - *In vitro*
  - *In silico*
  - *In chemico*
- Phototoxicity
  - *In vitro*
  - *In silico*
  - *In chemico*
- Dermal absorption
  - *In vitro*
  - *In silico*
  - *In chemico*
- Respiratory sensitisation
  - *In vitro*
  - *In silico*
  - *In chemico*
- Acute toxicity
  - *In vitro*
  - *In silico*
- Repeated dose toxicity
  - *In vitro*
  - *In silico*
- Neurotoxicity
  - *In vitro*
  - *In silico*
- Reproductive toxicity
  - *In vitro*
  - *In silico*
- Developmental toxicity (including neurotoxicity)
  - *In vitro*
  - *In silico*
  - *In chemico*
- Endocrine disruption
  - *In vitro*
  - *In silico*
  - *In chemico*
- Immunotoxicity
  - *In vitro*
  - *In silico*
  - *In chemico*
- Hypersensitivity
  - *In vitro*
  - *In silico*
  - *In chemico*

- Toxicokinetics
  - *In vitro*
  - *In silico*
  - *In chemico*
- Carcinogenicity
  - *In vitro*
  - *In silico*
  - *In chemico*
- Mutagenicity
  - *In vitro*
  - *In silico*
- Other (please specify what kind?)

Background information:

Q1: Are you familiar with European regulatory requirements in assessing human safety of chemicals?

Yes/No

If yes, please provide the information which regulatory requirements are you familiar with (you can choose more than one):

- REACH Regulation
- Regulation on Cosmetics Products
- Regulation of Food/Feed additives
- Regulation on usage of Biocidal products
- Other (please specify)

Q2: Do you have any experience with NAMs?

Yes/No

If yes, please provide the information what kind of experience do you have (you can choose more than one):

- I am or have been involved in development of NAMs
- I have used/am using NAMs
- I have assessed quality and/or regulatory relevance of NAMs
- Other (please specify)

Q3: Are you familiar with **nano-specific NAMs** in human safety assessment?

Yes/No

If yes, please provide the information on what kind of experience do you have?

- I am or have been involved in development of nano-specific NAMs

- I have used/am using nano-specific NAMs
- I have assessed quality and/or regulatory relevance of nano-specific NAMs
- Other (please specify)

Q4: Are you familiar with industry needs in relation to **nano-specific alternative methods** in human safety assessment?

Yes/No

If yes, can you identify any NAMs that are currently used to assess the human health hazard of nanomaterials by the industry (manufacturers)?
Also, can you point out any NAMs that should be prioritized and adapted to nanomaterials in relation to industry needs?

Closed-ended questions about NAMs and their adaptation for nanomaterials:

Q5: Are there any **existing, validated regulatory accepted NAMs** (NAMs intentionally developed and validated for conventional chemicals) that can be adapted and applied to assess **safety of nanomaterials**?

Yes/no/do not know

If yes, please indicate those NAMs.
If no, please indicate what, in your opinion, needs to be done to make those NAMs relevant for nanomaterials testing.

Q6: Are there any **recently developed/under development** (not yet regulatory accepted) **NAMs** (intentionally developed and validated for conventional chemicals) that can be adapted and applied to **assess safety of nanomaterials**?

Yes/no/do not know

If yes, please list known recently developed/under development NAMs that in your opinion can be adopted for nanomaterials testing? Please indicate, if possible, the reasonable timeframe for regulatory acceptance (up to 1 year, 1-3 year, 3 -5 years, more than 5 years)

Q7: Do you know any recently developed/under development (not yet regulatory accepted) **nano-specific NAMs**?

Yes/no/do not know

If yes, please list known recently developed/under development nano-specific NAMs that in your opinion can be adopted for nanomaterials? Please indicate, if possible, the reasonable timeframe for regulatory acceptance (up to 1 year, 1-3 year, 3 -5 years, more than 5 years)

Q8: Are there any **NAMs**, that while identifying the potential hazards, **incorporate exposure and dose** into the final determination on a nanomaterial's safety to a particular population?

Yes/no/do not know

If yes, please list those NAMs and comment on their suitability for the risk assessment for nanomaterials

Open-ended questions about NAMs and their adaptation for nanomaterials:

Q9: What is an expected timescale, in your opinion, needed for potential transfer of the **nano-specific NAMs** into EU regulations considering current gaps and development needs in particular regulations (i.e., REACH, Cosmetics Directives)?

Q10: What are, in your opinion, the development gaps and needs of the NAMs to fulfil nanomaterials specific safety testing requirements?

Q11: Which of the **nano-specific NAMs**, being currently developed, are the most promising ones from the regulatory point of view (preferably, list them in a descending order of importance)?

Q12 (For industry only): In the context of industry needs, which of the **nano-specific NAMs** under development are the most promising ones (or should be prioritized) to gain the regulatory acceptance?

ANNEX 2: List of NAMs assigned to toxicological endpoints.xlsx

Delivered in separate file. Click here to access it.

ANNEX 3: Complete list of experts that took part in the survey

Only details of the experts who agreed to be listed in the annexes of this study are provided in the table below.

ACADEMIA	
Clift Martin	Swansea University
Nelson Laurence Andrew	University of Leeds
Nymark Penny	Karolinska Institute
Marcos Ricard	Universitat Autònoma de Barcelona
Rogiers Vera	Vrije Universiteit Brussel
Toropova Alla P.	Istituto di Ricerche Farmacologiche Mario Negri IRCCS Via Mario Negri 2, 20156 Milano, Italy
Toropov Andrey A.	Istituto di Ricerche Farmacologiche Mario Negri IRCCS Via Mario Negri 2, 20156 Milano, Italy
INDUSTRY	
Bialas Iwona	CosmetoSAFE Consulting
Jantunen Paula	Sweco Finland
Pikal Petr	Precheza a.s.
Sergent Jacques-Aurélien	Solvay
Sharma Monita	PETA Science Consortium International
Stucki Andreas	PETA Science Consortium International e.V.
Suarez Blanca	TEMAS Solutions GmbH
REGULATORY AGENCY	
Dusinska Maria	NILU-Norwegian Institute for Air Research
Kass Georges	EFSA