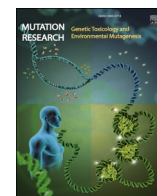




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# Mutation Research - Genetic Toxicology and Environmental Mutagenesis

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## New Approach Methods (NAMs) for genotoxicity assessment of nano- and advanced materials; Advantages and challenges

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### ABSTRACT

Genotoxicity assessment is essential for ensuring chemical safety and mitigating risks to human health and the environment. Traditional methods, reliant on animal models, are time-consuming, costly, and raise ethical concerns. New Approach Methods (NAMs) offer innovative, cost-effective, and ethical alternatives, playing a pivotal role in both traditional and next-generation risk assessment (NGRA) by minimizing the need for animal testing, particularly in genotoxicity evaluations. However, the development of NAMs often overlooks the particular physicochemical properties of nanomaterials (NMs), which significantly influence their toxicological behaviour and can interfere with genotoxicity evaluation. This underscores an urgent need for the standardization and adaptation of NAMs to address nano- and advanced material-specific genotoxicity challenges. In this review, we summarize the challenges associated with genotoxicity testing of NMs and highlight the suitability of existing *in vitro* and *in silico* NAMs for NMs and advanced materials, enabling genotoxicity testing across various exposure routes and organ systems. Despite considerable progress, regulatory validation remains constrained by the absence of approved test guidelines and standardized protocols. To achieve regulatory acceptance, it is crucial to adapt NAMs to NM-specific exposure scenarios, refine test systems to better mimic human biology, develop tailored *in vitro* protocols, and ensure thorough characterisation of NMs both in pristine form and dispersed in culture medium. Collaborative efforts among scientists, regulators, industry, and advocacy groups are vital to improving the reliability and regulatory acceptance of NAMs. By addressing these challenges, NAMs

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have the potential to revolutionize genotoxicity risk assessment, advancing it towards a more sustainable, efficient and ethical framework.

## 1. Introduction

The assessment of the genotoxic properties of chemicals, including nanomaterials (NMs), is a critical component of regulatory safety evaluations [19,125]. Genotoxicity refers to a substance's ability to damage genetic material in somatic and/or germ cells. Accumulated DNA damage is associated with adverse health effects, such as cancer or chronic and hereditary disorders [34,66]. The various kinds of DNA damage include gene mutations, structural chromosomal aberrations (clastogenicity), and numerical chromosomal aberrations (aneuploidy). Notably, genotoxic effects can occur even at low exposure levels. Understanding genotoxicity is crucial for the safety assessment of chemicals across fields, including NMs and advanced materials, nano- and microplastics, pharmaceuticals, consumer products, and agrochemicals.

Traditionally, genotoxicity assessments have relied heavily on animal testing, particularly in rodents. However, ethical concerns, regulatory pressures, and scientific advancements have driven interest in alternative approaches, collectively known as New Approach Methods (NAMs). NAMs encompass innovative techniques, or novel advances of existing tests, and models designed to reduce or replace animal use in hazard assessment, while providing reliable and relevant data for human risk assessment. These methods leverage advances in biological models and methods, including genomics and high-throughput screening, as well as computational biology, allowing more efficient and ethically acceptable genotoxicity assessment. Importantly, regulatory acceptance of NAMs could contribute to better protection of humans and the environment, and use of human-derived test systems could generate results that are more translatable to humans compared to animal studies [149, 206]. Further, use of NAMs supports tiered and targeted mechanism-based testing strategies, enabling robust safety evaluations, and advancing the Safe-by-Design (SbD) framework to get safer chemicals and advanced materials on the market in a time- and cost-efficient manner [94]. Most NAMs developed to date focus on chemical safety testing. As potential interferences with the test system are reported when performing hazard assessment of NMs, NAMs developed for chemical testing might require adaptations, standardization and inclusion of additional controls for reliable testing of NMs and advanced materials [71].

To address the challenges of applying NAMs for SbD and regulatory risk assessment of NMs and advanced materials, a framework for Next-Generation Risk Assessment (NGRA) is proposed. This framework is based on an Integrated Approach to Testing and Assessment (IATA) and follows a tiered, exposure-driven, and endpoint-specific strategy. A key aspect of NGRA is physicochemical characterization, which plays a crucial role in assessing the regulatory readiness of advanced materials. By providing essential data on both intrinsic and extrinsic properties, physicochemical characterization helps predict a material's environmental fate, biodistribution, and toxicological effects. This enables more targeted, accurate testing while ensuring compliance with regulatory requirements [109].

Despite the increasing availability of NAMs, a consensus has yet to be reached on their application and integration to assess the genotoxic potential of nano- and advanced materials. The European Union Observatory for Nanomaterials (EUON) has published an inventory of alternative methods for NM safety testing, where the need for genotoxicity tests tailored for specific organs, such as the lungs, liver, and gastrointestinal tract, is emphasized [115].

This review highlights the challenges in using NAMs for genotoxicity testing, provides an overview of existing organ-specific advanced models and methods in the scientific literature that could evaluate the genotoxicity of nano- and advanced materials across organs and organ

systems, and discusses their current status regarding standardization and validation.

## 2. Brief overview of nanomaterial-induced genotoxicity

There are different types of DNA damage related to distinct mechanisms of interaction with the DNA; (1) oxidation, (2) alkylation of bases, (3) base loss (4) bulky adduct formation, (5) DNA crosslinking, and (6) single and double stranded DNA breaks.

DNA damage induced by NMs can occur through various mechanisms, classified broadly as primary or secondary genotoxicity [69,145], (Fig. 1). Primary direct genotoxicity mechanisms involve the direct interaction of NMs with DNA, where the NMs themselves encounter the genetic material, leading to structural or base damage, such as strand breaks or other lesions. Thus, direct genotoxicity can only occur if the NM is taken up into the nucleus and interacts with DNA or chromosomes. The precise mechanism of NM penetration into the nucleus is not known. One possibility is that NMs enter through nuclear membrane pores (if the NM is small enough) or reach the DNA after dissolution of the nuclear membrane during mitosis. We can also speculate that the mechanism of NM uptake might be similar to endocytosis. Primary indirect genotoxicity can occur through oxidative stress, or via signalling molecules that can induce DNA lesions, interfere with DNA replication, or damage repair enzymes. However, oxidative stress is considered the main mechanism. Oxidative stress arises when the generation of reactive oxygen species (ROS) exceeds the cell's antioxidant defence capacity, leading to oxidation of DNA bases, strand breaks, and other lesions ([69, 78,125,145]; Evans et al., 2016).

Secondary genotoxicity mechanisms are driven by oxidative stress and ROS produced by inflammatory processes. Activation of oxidative bursts in phagocytic macrophages can produce excess ROS. During frustrated phagocytosis, a process where the phagocytes attempt, but fail, to engulf and clear larger or aggregation-prone NMs, prolonged activation of immune cells can lead to the release of pro-inflammatory cytokines and an exacerbated oxidative stress response. The resulting chronic inflammation and high levels of ROS can indirectly cause DNA damage, including base modifications, strand breaks, and the activation of genotoxic signalling pathways [69,78,125,145]. These secondary mechanisms underscore the complex interplay between the immune response, oxidative stress, and genotoxicity associated with NMs.

### 2.1. Challenges in testing genotoxicity of nano- and advanced materials with NAMs

NMs possess properties distinct from those of bulk materials, necessitating specialized methods for accurate behaviour and safety assessment. These include the characterization of NMs in their pristine forms and within biological systems or culture medium [58,125]. Genotoxicity of NMs is closely linked to their physicochemical properties, such as size, shape, stability, agglomeration state [155], mode of synthesis, and surface chemistry [52], as well as their cellular uptake [93]. Additionally, properties such as crystallinity and solubility significantly influence their toxicity [88].

A critical consideration in toxicity testing is whether to use properly dispersed or agglomerated NMs for cell exposure. Properly dispersed NMs represents the "idealized" form, enabling assessment of intrinsic physicochemical properties and mechanisms for toxicity and biological interactions. In contrast, agglomerated forms, with altered size and bioavailability, may better reflect physiological exposure conditions. The dispersion protocols significantly influence NM behaviour, bioavailability and interactions with biological systems, ultimately

impacting toxicity outcomes [53,117]. For mechanistic toxicity studies, achieving a stable and homogeneous dispersion, preventing agglomeration and sedimentation, is essential, as these factors can influence the toxicity of NMs [161]. Sonication requires calibrated settings (e.g. amplitude time, temperature) for reproducibility, while additives such as bovine serum albumin (BSA) can prevent agglomeration but may alter measured toxicity [117,226]. The timing for cell exposure after dispersion is also of importance, as agglomeration increases over time. Further, the dispersion method can modify structural features of the NMs, as demonstrated for multiwalled carbon nanotubes [29]. Dispersion medium could also influence the toxicity depending on the type of NM applied [29,98]. Thus, careful consideration of dispersion protocol and medium is important for the experimental design for testing of NMs, and harmonization and standardization of test procedures are critical for consistent and reproducible toxicity data [103].

The physicochemical properties of NMs have a large impact on uptake of NMs by cells and across different biological barriers [197]. Biotransformation of the NMs in biological fluids and biomolecular corona formation and change in surface chemistry all affect the potential genotoxicity of NMs. A biocorona can alter NM properties and influence function, biointeractions, fate, size, aggregation and toxicity [211]. Biocorona formation should therefore be taken into consideration for all exposure routes for NMs, as different routes can result in different coronas and thus different outcomes.

## 2.2. Nanomaterial-specific challenges associated with assay interferences

NMs pose challenges in *in vitro* testing due to their particular physical and chemical properties which can interfere with reagents, assay performance, and readouts, leading to potentially misleading results. These interferences can impact assay performance, for example by limiting exposure of the biological system to the NMs or by disrupting biological effects critical to endpoint analysis [71,95]. NMs may interfere with cellular assays by localizing within cellular compartments or inducing oxidative stress unrelated to the intended endpoint [227]. Interactions between NMs and culture medium components, such as serum proteins forming a biocorona, can alter NM behaviour and cellular interactions. Additionally, agglomeration and sedimentation in culture medium can cause uneven distribution and inconsistent exposure levels in cell-based

assays, leading to unreliable data [95,127].

NMs with strong optical properties, such as gold and silver nanoparticles, can absorb or scatter light, interfering with spectrophotometric assays relying on light transmission or fluorescence, while the autofluorescence of some NMs can overlap with assay signals and complicate data interpretation [5,71,95]. The high surface area of NMs enables adsorption of proteins, enzymes, or other assay components, depleting these from the solution and altering outcomes. Carbon nanotubes and graphene, for example, can adsorb significant amounts of protein or bind to dyes, affecting enzyme-linked immunosorbent assays (ELISAs) or other colorimetric assays [71]. Certain metal oxide NMs, such as cerium oxide, have catalytic properties that can alter assay chemistry, e.g. degrading hydrogen peroxide, while redox-active NMs, such as silver nanoparticles, can disrupt oxidation-reduction reactions in cell viability assays, causing unreliable or false results [71].

Addressing these issues requires well-designed and validated assays with proper controls to distinguish true biological effects from NM-induced artifacts. Developing standardized protocols and guidelines for NM testing is essential to mitigate these issues and enhance the reliability of NAMs for NMs and advanced materials.

## 2.3. Nanomaterial-specific challenges associated with regulatory requirements

From the regulatory perspective, genotoxicity testing requires a battery of tests addressing the key genotoxicity endpoints: gene mutations, chromosomal aberrations (clastogenicity) and aneugenicity [63]. All these endpoints are covered by OECD test guidelines (TGs) designed for standard cell culture models and provided in Table 1.

While originally developed for chemical toxicity testing, the applicability of the standard assays to NMs must be demonstrated. Genotoxicity testing of NMs requires the adaptation and development of nano-specific OECD TGs and Guidance Documents (GDs) [58,72]. To address this, the OECD initiated a project in 2024 to adapt and validate the *in vitro* micronucleus assay [181]. Both the micronucleus assay as well as the chromosomal aberration test detect clastogenicity. However, the micronucleus assay is preferred as it can also be used to detect aneugenicity and is less laborious than the chromosomal aberration test. The micronucleus assay is also compatible with advanced *in vitro*

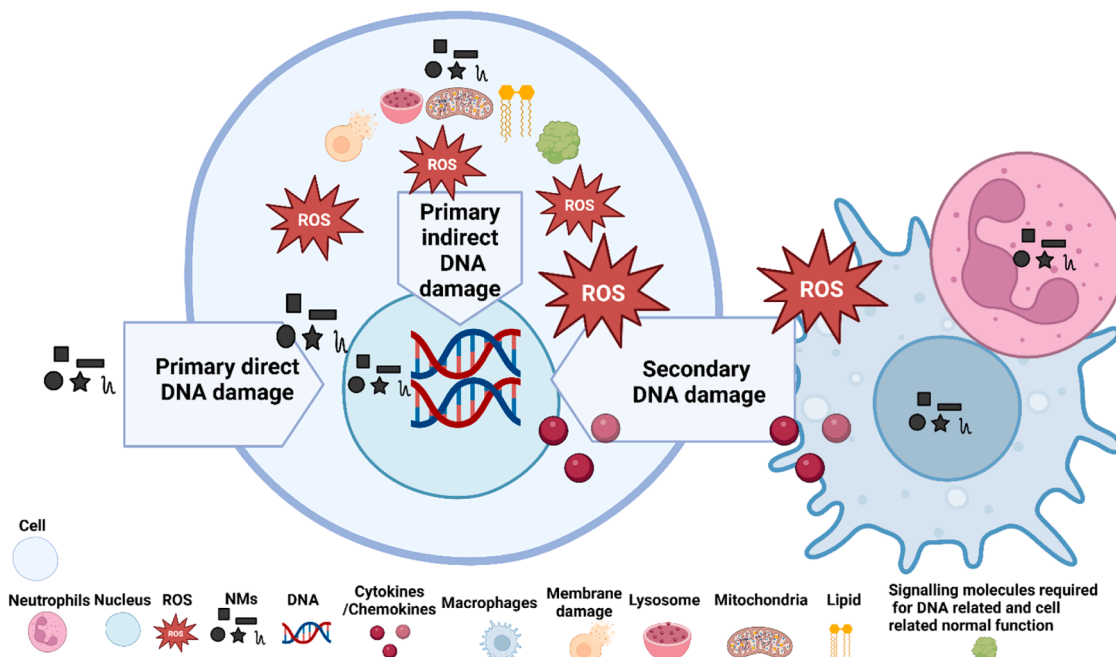


Fig. 1. Mechanisms for genotoxicity induced by nano-and advanced materials. NMs, nanomaterials; ROS, reactive oxygen species.

**Table 1**

Key genotoxicity endpoints covered by OECD test guidelines (TGs).

Genotoxicity endpoint	OECD Test guideline (TG)	OECD TG	Suitability for Nanomaterials	References
Gene mutations	Bacterial reverse gene mutation assay, or Ames test	TG 471	No*	OECD [172]
	Mammalian HPRT/XPRT gene mutation test	TG 476	Yes – adaptation may be needed	OECD [165]
	Mammalian TK gene mutation test	TG 490	Yes- adaptation may be needed	OECD [166]
Chromosomal aberrations (clastogenicity)	<i>In vitro</i> micronucleus assay	TG 487	Yes – adaptation for NMs is in progress at OECD	OECD [181]
Small deletions	Mammalian chromosomal aberration test	TG 473	Yes - adaptation may be needed	OECD [164]
	Mammalian TK gene mutation test	TG 490	Yes - adaptation may be needed	OECD [166]
Aneugenicity	<i>In vitro</i> micronucleus assay	TG 487	Yes - adaptation for NMs is in progress at OECD	OECD [181]

\* due to size of bacteria relative to NMs, and presence of bacterial cell wall [63,64].

models.

Induction of gene mutations can be tested using the bacterial OECD TG 471 [172] or mammalian OECD TG 490 [166] gene mutation tests. However, the bacterial gene mutation (Ames) test is not suitable for testing NMs due to the size of bacteria relative to NMs and the existence of the bacterial cell wall [63,64]. Therefore, the mammalian gene mutation test must be used instead. The OECD TG 476 [165] and TG 490 [166] are specific to certain mammalian cell types and require large number of cells and so can only be used with standard *in vitro* models and are less compatible with NAMs involving advanced three-dimensional (3D) models.

A promising genotoxicity test shown to be compatible with advanced 3D models, as well as with testing of NMs, is the *in vitro* comet assay (single cell gel electrophoresis assay) [32,74–76]. The comet assay is a sensitive and versatile method for detecting DNA strand breaks and can also identify oxidized and alkylated DNA bases when lesion-specific enzymes are included in the protocol [10,41,46,62,70,100,101,158,159,185]. As an indicator test for potential gene mutations, the comet assay can quantitatively measure DNA oxidation damage, such as 8-hydroxy-2'-deoxyguanosine (8-oxo-G), a biomarker for NM-induced genotoxicity induced via an oxidative stress mechanism [41]. A miniaturized version of the comet assay compatible with high-throughput systems has been developed for use with NMs within several European projects and pre-validated in the H2020 project RiskGONE [70] and shows promise for regulatory acceptance.

Both the micronucleus test and comet assay are suitable for testing NMs, and have been demonstrated as applicable in combination with advanced *in vitro* models [32,44].

#### 2.4. Nanomaterial-specific challenges associated with test system

Two-dimensional (2D) cell culture systems are widely employed for genotoxicity testing of NMs due to their simplicity, cost-effectiveness, and ease of use. These systems are effective for capturing primary genotoxicity mechanisms, such as direct and indirect DNA damage, and are commonly used in assays including the comet and micronucleus assays. However, secondary genotoxicity, which arises from complex biological processes, notably oxidative stress and inflammation, is often not represented in 2D models. 3D models, organoids, and co-culture systems offer improved physiological relevance, allowing for the study of NM-induced secondary genotoxicity, by including immune cells, within a context that better replicates human tissue and organ-level responses. These advanced models are crucial for accurately assessing the multifaceted genotoxic effects of NMs, particularly for regulatory risk assessment and safety evaluations.

### 3. NAM-based genotoxicity assessment of nano-and advanced materials using adverse outcome pathways (AOPs)

Before evaluating the suitability of NAMs, it is crucial to consider their integration into a broader framework for reliable genotoxic assessment, as no single method can serve as a complete replacement for *in vivo* animal studies. Although NAMs relevant to human genotoxicity assessment have been developed, challenges persist in integrating and

interpreting these data for regulatory purposes. The adverse outcome pathway (AOP) approach offers a promising solution to these challenges [12]. An AOP is a conceptual framework that organizes toxicological knowledge, linking molecular-level perturbations (molecular initiating events, MIEs) to adverse outcomes (AOs) through measurable biological events (key events, KEs) (Fig. 2). It also elucidates the relationships between KEs and AOs across biological levels [6]. AOPs assist in interpreting NAM data, evaluating KE-AO relationships, and bridging knowledge gaps in genotoxicity assessment.

To facilitate the use of NAMs in NM risk assessment, IATA have been developed to evaluate data from multiple sources, identify gaps, and incorporate NAM-generated data for robust regulatory decisions [12]. In NGRA, which emphasizes protection over prediction, bioactivity and *in silico* NAMs, combined with AOP-driven IATA assays, are applied in a tiered manner to establish safety margins [35]. AOPs form the basis for IATA, NGRA, and the SbD of NMs. Recognizing the critical role of AOPs, the OECD formalized their development through the Emerging Science in Chemical Assessment (ESCA) group, which has approved 35 AOPs, including only two related to genotoxicity, and 33 IATAs, with just one addressing genotoxicity.

The OECD-approved AOP 296 describes DNA oxidation damage as an MIE leading to mutations and chromosomal aberrations (AOP 296, <https://aopwiki.org/aops/296>), while AOP 15 highlights DNA alkylation in male pre-meiotic germ cells leading to heritable mutations (AOP 15, <https://aopwiki.org/aops/15>). Additional AOPs under development (e.g., AOP 303, AOP 330) also consider DNA damage as a KE leading to mortality or cancer. Efforts in the PARC project (Partnership for the Assessment of Risks from Chemicals) aim to establish an AOP network for genotoxicity, though current AOPs lack nano-specific considerations. While NMs may share some KEs with other chemicals, significant differences exist in their initial interactions with biological targets. Attempts to adapt chemical AOPs for NMs have been made using nanotoxicological data [27]. For instance, AOPs describe TiO<sub>2</sub> NMs inducing lung and intestinal cancer through KEs such as oxidative stress, inflammation, and DNA damage [27,199].

A nano-specific AOP for lung carcinogenicity was developed, showing how pulmonary NM deposition triggers secondary genotoxicity and cancer, with assays linked to each KE [162]. Recent work demonstrated the integration of nano-specific MIEs and KEs into existing chemical AOPs [156,157]. Developing NAMs to measure nano-specific MIEs is critical for genotoxic evaluation of NMs, especially where such MIEs are lacking. As shown above, secondary genotoxic mechanisms play a crucial role in the progression of several AOs. Therefore, focus should be given to the development of NAMs that can capture secondary genotoxicity mechanisms related to NMs and advanced materials. Collaboration between nano-genotoxicity experts and initiatives such as PARC and the Health and Environmental Sciences Institute (HESI) are essential to integrate nano-specific requirements into AOPs, enabling the development and standardization of NAMs for regulatory applications.



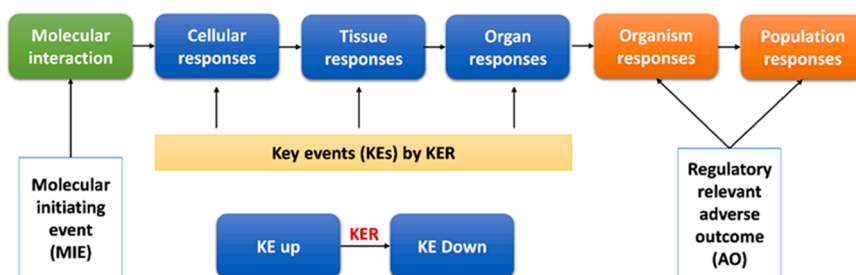


Fig. 2. Schematic representation of the adverse outcome pathway (AOP) framework [156]. KER, key event relationship.

#### 4. New approach methods in genotoxicity testing of nanomaterials

As NMs can affect the body through various exposure routes, such as inhalation, ingestion, dermal contact, or injection, there is a need for the diverse NAMs across different organs to simulate realistic exposure scenarios. A recent systematic review of more than 200 alternatives to animal studies for safety testing of NMs [115], identified only 8 NAMs as nano-specific and accepted for regulatory testing. This emphasizes the need to speed up the validation processes for the approximately 120 nano-specific NAMs under development. For complex endpoints such as genotoxicity, carcinogenicity, neurotoxicity and reproductive toxicity, or germ cell mutagenicity there are no or only a few NAMs under

development [115]. Here we describe advanced *in vitro* assays and models for genotoxicity assessment that utilize cells from different organs and tissues in order to replicate complex tissue architectures (Fig. 3).

NAMs based on advanced human *in vitro* models offer a more physiologically relevant test than lower tier *in vitro* assays (e.g. 2D monocultures) or animal models, where it is often difficult to extrapolate results to humans. Intensive efforts are being made to develop advanced human 3D *in vitro* models to be used for hazard assessment of NMs as well as other chemicals. Due to the particular properties of NMs, standardization and validation of the models for compliance with NM testing need to be performed. Models are needed for all organs in the body, especially for lung, liver and gastrointestinal tract as well as testis. For

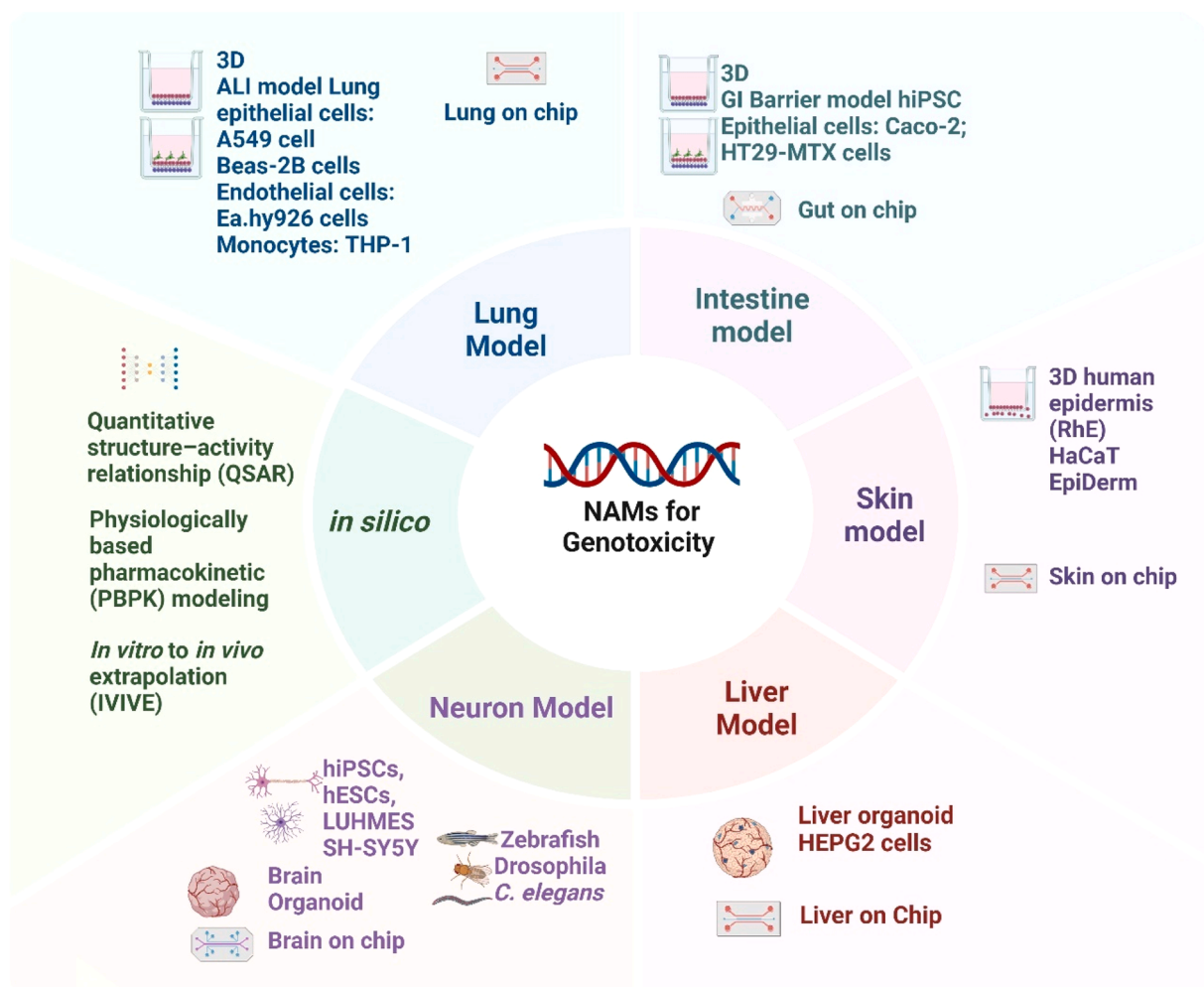


Fig. 3. Overview of New Approach Methods (NAMs) and most advanced *in vitro* models for different organs for genotoxicity assessment of chemicals, including nano- and advanced materials. ALI, air-liquid interface; hiPSC, human induced pluripotent stem cells; hESCs, human embryonic stem cells.

skin models, there are several non-nano-specific regulatory accepted NAMs that would need nano-specific validation [115]. The complexity of the models is constantly increasing, making them more realistic and better reflecting organ and tissue structures, both at a dimensional aspect (2D to 3D to organoids) and by including multiple cell types to mimic the intercellular interactions and signalling occurring *in situ*. Here we summarize the most important advanced 3D *in vitro* models for genotoxicity of NMs, representing the major exposure routes.

To evaluate potential challenges, limitations, and advantages of NAMs for each route of exposure and organ/system, we performed a literature search restricted to the following key words: **specific NAM (lung, liver, skin, gut)** and **genotoxicity** and **NMs/nanoparticles**. Results of this search and references are included in Table 2.

#### 4.1. Advanced respiratory system models

Inhalation is the most important human exposure route for airborne NMs and particulate matter (PM), making the respiratory system a primary target organ [23]. It is well demonstrated that pulmonary exposure to NMs, PM or other chemicals may lead to adverse health effects that can also be studied *in vitro* [97,193]. Interaction with and deposition of inhaled NMs is likely to occur in the bronchial and alveolar regions, dependent upon the physicochemical properties of NMs, such as size and solubility but also on aerodynamics in the respiratory system [163].

With regard to the human respiratory system, it is important that the correct cell model is selected, considering the mode of action (MoA) of the chemical under investigation, since the bronchi and alveoli differ in their physiological functions and responses to external stimuli. Thus, a relevant biological endpoint for the bronchial region is the beating frequency of the cilia, which favour clearance, while for the alveolar region, a relevant endpoint is represented by the barrier function (expressed as Trans Epithelial Electrical Resistance (TEER) or permeability to fluorescent molecules). Various *in vitro* models are generated by selecting different epithelial cells derived from the respective tissue [83,84]. Considering the human respiratory system structure, the human alveolar lung epithelial type II cell line A549 and the human bronchial epithelial cells BEAS-2B or Calu-3 have been widely used as representative models to study potential effects of inhaled NMs, representing lower and upper inhalation models, respectively [27,229,235]. These cell lines were initially cultured in conventional 2D submerged systems and primarily utilised to investigate the primary genotoxic mechanisms under submerged conditions.

To mimic *in vitro* the exposure of the airways, the cultivation of the cells has advanced into an air-liquid-interface (ALI) [131]. Exposing respiratory cell models to NMs at the ALI provides a controlled environment for studying genotoxic effects from inhalation [91]. This method also enables NMs and PM to interact directly with the cells, without the interference of a biocorona that forms on NM surfaces in submerged cultures. The biocorona can significantly influence the toxicity of the NMs [230].

Different ALI devices exist that allow exposure to liquids, gases or powders [131]. *In vitro* ALI models have been applied with genotoxicity assays such as the comet assay, micronucleus assay, and  $\gamma$ -H2AX immunostaining to detect DNA strand breaks, chromosomal damage, and DNA repair kinetics, providing insights into the genotoxic potential of tested substances [30,32,75]. Cells exposed to NMs in ALI models can also be coupled to omics technologies, to comprehensively analyse molecular responses associated with genotoxicity, such as gene expression, signalling pathways, and protein profiles, enhancing our understanding of underlying mechanisms and contributing to development of AOPs for genotoxicity. Advanced imaging techniques, including confocal microscopy, live-cell imaging, genome-scale imaging, and high-content screening, enable detailed visualization and quantification of genotoxic effects in ALI models. High-content analysis allows researchers to assess multiple cellular endpoints simultaneously, providing a comprehensive view of genotoxicity induced by NMs and its

spatial and temporal dynamics within lung tissue [42].

To further advance the complexity of the model and increase its physiological relevance, co-culturing of several cell types has been established. Epithelial cell lines are commonly co-cultured with endothelial cells, such as the human EA.hy926 cells, and immune cells, such as human THP-1 differentiated macrophages, as well as with fibroblasts [14,32,75,124,186]. Elje et al. [75] utilized a combined model of BEAS-2B and A549 cells in mono- and co-cultures with EA.hy926 to measure DNA strand breaks and chromosomal damage after exposure to silver nanoparticles NM-300K. BEAS-2B mono-cultures were the most sensitive, while co-cultures showed reduced DNA damage but increased pro-inflammatory responses, highlighting the interplay between cell types in mitigating or exacerbating genotoxicity induced by NMs.

Evans et al. [79] utilized 16HBE14o<sup>-</sup> mono- and co-culture of 16HBE14o<sup>-</sup> epithelial cells and macrophages exposed to  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>2</sub>O<sub>3</sub> nanoparticles. In 16HBE14o<sup>-</sup> monocultures only  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles induced chromosomal damage. In contrast co-culture treatments with immune cells showed both  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>2</sub>O<sub>3</sub> to be genotoxic to 16HBE14o<sup>-</sup> cells due to secondary genotoxicity promoted by immune cell interaction. Burgum et al. [30] used TT1 epithelial cells co-cultured with dTHP-1 cells to study functionalized few-layer graphene using micronucleus assay and oxidative stress markers.

Camassa et al. [32] employed a 3D ALI lung model with A549 epithelial cells, EA.hy926 endothelial cells, and dTHP-1 monocytes exposed to NM-300K using the VITROCELL® cloud system. DNA strand breaks and DNA oxidation damage measured by the enzyme-modified comet assay, showed dose-dependent effects, underscoring the sensitivity of the model for detecting primary and secondary genotoxic responses. Elje et al. [73] used a similar triple-culture ALI model and demonstrated significant DNA damage in basolateral endothelial cells, measured via an enzyme-modified comet assay, as well as oxidative stress markers such as HMOX1 expression, showcasing the suitability of ALI systems for capturing multi-layer interactions. Pantzke et al. [186] employed a triple-culture model combining epithelial cells, macrophages, and fibroblasts to evaluate multi-walled carbon nanotubes (MWCNTs). DNA strand breaks and oxidative stress markers such as MDA release were used to assess primary and secondary genotoxicity. The fibroblast-mediated interactions significantly amplified oxidative stress and DNA damage. Friesen et al. [86] tested mechanically treated (mCF) and thermo-mechanically treated carbon fibres (tmCF) in mono-cultures, co-cultures, and triple-cultures using ALI systems, measuring DNA strand breaks by the alkaline unwinding method. The study revealed moderate genotoxicity only in triple-cultures, emphasizing the role of immune-stromal interactions in exacerbating genotoxic responses under realistic exposure conditions.

In addition, complex 3D models based on lung cell lines have become increasingly available [32,36,75,124] and have been used to study effects of particle exposures ranging from diesel exhaust [24,83,84,104,124], silver NMs and silver nanowires [75,76,82], and nano-silica [208].

The added value of the increased complexity of ALI models has been shown by the increased similarity of active gene networks of co-culture models such as ALIsens® when compared with the *in vivo* human counterpart [148]. Several commercially available complex models, such as the ALIsens® model from Invitrolize™ or the EpiAlveolar™ model from Epithelix, include immune cells such as macrophages and/or dendritic cells, which can reflect the interplay between different cell types and secondary genotoxic mechanisms induced by NMs [14,43].

#### 4.2. Challenges, limitations and advantages of respiratory models for testing nano- and advanced materials

Both ALI and co-culture models exhibit significant strengths in assessing genotoxicity induced by NMs and advanced materials. ALI models are ideal for studying genotoxicity due to their realistic exposure conditions and ability to replicate inhalation scenarios. However, they

**Table 2**

Summary of genotoxicity studies with nanomaterials (NMs) using Advanced in vitro models.

Organ/ System	In vitro model	NMs Tested	Protocol for Exposure	Genotoxicity Endpoints	Outcome of the Test	Usefulness for Genotoxicity Assessment of NMs	Reference
Respiratory	Advanced 3D Lung Model at ALI (human alveolar epithelial (A549), endothelial (EA.hy926) cells and differentiated monocytes (dTHP-1)	NM-300K (Ag NMs)	Aerosol exposure in VITROCELL® cloud system	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified Comet assay)r	Positive Genotoxicity observed with dose-dependent responses	Effective in understanding genotoxicity of NMs	[32] doi.org/10.3390/nano12152609
Respiratory	Triple-culture model (epithelial cells, macrophages and fibroblasts)	Multi-walled carbon nanotubes (MWCNTs)	In vitro exposure on Transwell inserts for 24 and 48 h	DNA strand breaks (Comet assay) and oxidative stress (MDA release)	Positive Significant increase in DNA damage (secondary genotoxicity) and oxidative stress markers in complex cultures	Effective in revealing secondary genotoxicity and pro-fibrotic changes through cell-cell interactions	[186] doi. org/10.1080/15376516.2022.2156008
Respiratory	BEAS-2B Mono-cultures, Co-cultures (BEAS-2B/dTHP-1), and Triple-cultures (BEAS-2B/dTHP-1/CCD-33Lu)	Mechanically treated (mCF) and thermo-mechanically treated carbon fibers (tmCF)	Air-liquid interface exposure in a Vitrocell® system	DNA strand breaks (Alkaline Unwinding method)	Positive Slight genotoxicity in mono- and co-cultures; moderate genotoxicity in triple-cultures	Effective for highlighting secondary genotoxicity and interaction-driven responses	[86] doi.org/10.3390/ijms24031927
Respiratory	BEAS-2B and A549 Mono-cultures, Co-cultures with EA.hy926	NM-300K (silver nanomaterials)	Air-liquid interface exposure in VITROCELL® Cloud system	DNA damage (Comet assay), Chromosomal damage (Micronucleus assay)	Positive BEAS-2B mono-culture showed highest sensitivity for DNA strand breaks; Negative for co-culture for DNA damage but increased pro-inflammatory response	Effective in capturing primary and secondary genotoxic effects, highlighting model-specific differences	[75] doi.org/10.3390/nano13030407
Respiratory	TT1 Mono-culture and TT1/d.THP-1 Co-culture	Neutral-FLG, Amine-FLG, Carboxyl-FLG, CB particles	24-hour exposure; adapted in vitro CBMN assay	Chromosomal damage (Micronucleus assay), Oxidative stress (NAC inhibition study)	Positive Genotoxicity in mono-cultures (primary-indirect mechanisms) Positive in co-cultures; secondary genotoxicity driven by oxidative stress	Highly effective in distinguishing primary and secondary genotoxic mechanisms	[30] doi. org/10.1186/s12951-021-00769-9
Respiratory	Mono-culture (A549) and Co-culture (A549 + differentiated THP-1 macrophages)	Crystalline Quartz (DQ12)	Submerged and Air-Liquid Interface (ALI) conditions; 24-hour exposure	Gene expression changes associated with oxidative stress and inflammation	Positive Significant upregulation of inflammatory and oxidative stress-related genes under ALI conditions compared to submerged conditions	Effective in identifying molecular pathways involved in nanomaterial-induced respiratory tract responses	Freisen et al., 2022 Doi.org/10.3390/ijms23147773
Respiratory	Mono-culture (16HBE14o-),	γ-Fe2O3 and Fe3O4 (dSPIONs)	Conditioned media and co-	Chromosomal damage	Positive γ-Fe2O3	Highly effective in identifying	[79] doi.org/10.1186/s12989-019-0291-7

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Table 2 (continued)

Organ/ System	In vitro model	NMs Tested	Protocol for Exposure	Genotoxicity Endpoints	Outcome of the Test	Usefulness for Genotoxicity Assessment of NMs	Reference
	Co-culture (16HBE14o- + dTHP-1 macrophages)		culture models; 22–26 h exposure	(micronucleus assay)	induced micronucleus frequency in mono- and co- culture; Fe3O4 induced damage only in co-culture (secondary genotoxicity)	secondary genotoxicity via immune interactions	
Respiratory	Triculture model (A549, dTHP-1 macrophages, EA.hy926 endothelial cells)	NM-300K (silver nanoparticles)	Air-liquid interface exposure using VITROCELL® Cloud system for 24 h	DNA strand breaks and DNA oxidation lesions (Alkaline and enzyme- modified comet assay), Oxidative stress (HMOX1 expression)	Positive Significant DNA damage in basolateral endothelial cells; oxidative stress marker upregulated in both apical and basolateral compartments	Effective in assessing genotoxicity, oxidative stress, and inflammation in complex models; highlights inter-laboratory variability issues	[73] doi.org/10.3390/nano14231888
Respiratory	Mono-culture (A549), Co- culture (A549 + THP-1a macrophages)	ZnO (NM-110), CuO (NRCWE-072), TiO2 (Rutile, Anatase, Cube), DQ12 (quartz)	Submerged and air-liquid interface (ALI) exposure; 24- hour exposure in Vitrocell Cloud system	DNA strand breaks (Comet assay), IL-8 expression (inflammation)	Positive Significant genotoxicity observed in CuO and ZnO in both systems; TiO2 showed varied responses based on crystal structure	Effective in capturing primary genotoxicity and inflammatory responses with good in vitro-in vivo correlation	[56] doi.org/10.1016/j.tiv.2024.105897.
Skin	2D HaCaT keratinocytes	polyethylene terephthalate (PET) NM 781 nm average size and negative surface charge	Exposure in suspension at 37 °C for 24 h 1, 10, and 100 µg mL-1	Chromosomal damage (OECD TG 487-based cytokinesis- block micronucleus assay, evaluation by high content analysis)	Positive Significant genotoxicity (100 µg mL-1)	Effective to detect primary genotoxicity	[48] doi.org/10.1016/j. chemosphere.2024.141813
Skin	2D human keratinocytes Ker-CT and 3D skin explants	TiO2 P25 Aeroxide nanoparticles and 5 NPs extracted from Sunscreens: sphere (ExS), pristine sphere (PrS), extracted rod (ExR), and pristine rod (PrR).	cells exposed to the NPs for 24 h 0.01–5 µg/cm2 (	DNA damage (phosphorylated γH2AX) only with Ker-CT cells)	Negative in 2D No increase in phosphorylated γH2AX	Skin explant cultures provide a more realistic alternative to monolayer cell cultures. However, genotoxicity study was performed only on Ker-CT cells	De [130] doi: 10.1186/s12989-024-00610-9
Skin	EpiDerm™	AgNP (25 mg/L) Graphen oxide GO flakes dispersed in the aqueous solution at the 4 mg/mL Complex AgNP-GO	1 h treatment topical with AgNP-, GO-, AgNP-GO- treated, and NC (DPBS), post- incubation 24 h,	8-hydroxy-2'- deoxyguanosine (8-OHdG) by an enzyme-linked immunosorbent assay (ELISA) kit	Negative No increase in 8-OHdG in any group	3D skin model maybe useful to understand genotoxicity of NMs	[240] doi: 10.3390/pharmaceutics14071398.
Skin	EpiDerm™ MatTek versus 2D	BASF Levasil® silica NP (16 and 85 nm)	Topical administration (ALI) 24 and 48 h ≤ 300 µg/mL	the 3D reconstructed skin micronucleus (RSMN) assay	Negative In 3D model no significant DNA damage Positive In 2D model	Importance of tissue microarchitecture in defining nanomaterial exposure closer to in vivo Consideration of top dose – possible air-liquid interface suffocation	Will et al., 2016 doi: 10.1186/s12989-016-0161-5
Skin	LC-like dendritic cell (DC) line XS52	CMS (DendroSol®, product number: 10-18-350)	CMS: 50, 100, 150 µg/mL Silver NP 5µg/	DNA strand breaks (alkaline comet assay)	Negative CMS and CMS- ICC	Only 2D model was used	[67] DOI: 10.1016/j. biomaterials.2018.01.058

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Table 2 (continued)

Organ/System	In vitro model	NMs Tested	Protocol for Exposure	Genotoxicity Endpoints	Outcome of the Test	Usefulness for Genotoxicity Assessment of NMs	Reference
Skin	2D HaCaT cells	CMS-ICC (labeled with fluorescent dye indocarbocyanine) Slover NPs as positive control ZnO NPs 5 and 10 µg/mL	mL24h in medium	DNA strand breaks (Alkaline comet assay), Chromosomal damage (Micronucleus assay) DNA fragmentation and cell cycle (G2/M arrest)	Positive silver NPs  Positive in both comet and micronucleus assays	Only 2D model was used	[221] DOI: 10.1093/mutage/gez017
Intestine	Caco-2/HT29-MTX, differentiated	12 nm and 24 nm (NP1 and 2), 118 nm (E171)	1–100 µg/mL (10 and 50 µg/mL, 24 h) E171 or TiO <sub>2</sub> -NMs, from day 1 to day 21 days post-seeding.	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay)	Positive in Caco-2/HT29-MTX (	Relevant for studying mucus-mediated protective effects	[60] doi.org/10.1080/17435390.2017.1349203
Intestine	Caco-2/HT29, differentiated	30 –160 nm (NP-sphere), D = 20 –180 nm and L = 250 nm (NP-rod), D = 4 –26 nm and L = 100 nm (NP-wire)	All NPs - 12.5, 50, 150, 350 µg/mL for 24 h	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay)	Alkaline comet assay Positive for the three NPs at all tested concentrations (except 350 µg/mL NP-wire) Fpg-modified comet assay Positive outcome only with NP-rods (all tested concentrations) and NP-spheres (350µg/mL only).	Relevant for studying genotoxicity induced by barrier translocated NMs	Alba [87] doi.org/10.1186/s12989-018-0269-x
Intestine	Caco-2/HT29-MTX, non differentiated	12 nm and 24 nm (NP1 and 2), 118 nm (E171)	1–100 µg/mL E171 or TiO <sub>2</sub> -NPs, From three days of seeding for 24 h	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay) and 8-oxo-dGuo (by HPLC-MS/MS)	Negative outcome in comet assay, alkaline and Fpg-modified (tested at 50 µg/mL for 24 h), in 53BP1 foci count assay (similar to gamma-H2AX). No significant increase of 8-oxodGuo level (HPLC-MS/MS).	Relevant for studying impact differentiated and non-differentiated intestinal cell types	[61] doi.org/10.1016/j.mrgentox.2018.11.004
Intestine	HT29-MTX-E12 (mucus-secreting)	Cellulose microfibrils (CMF-ENZ and CMF-TEMPO)	3–50 µg/mL for 3–24 h	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay), Micronucleus assay	DNA damage observed at higher doses for undigested CMFs; digested samples showed negligible effects due to mucus-mediated protection	Relevant for assessing effects in mucus-secreting environments	[224] https://doi.org/10.1007/s00204-024-03911-2
Intestine	triculture small intestinal epithelium (SIE) model	carboxylated polystyrene spheres (PS25C and PS1KC) Aqueous suspensions of 25 and 1000 nm and	1, 5, and 20 µg/mL for 24 or 48 h subjected to simulated GIT digestion	CometChip	Positive DNA damage increased in a time- and concentration-	Relevant for assessing effects of NM, nano and microplastics in an integrated in vitro ingestion platform	Zhenning et al., 2024 DOI: 10.3390/nano14090807

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Table 2 (continued)

Organ/ System	In vitro model	NMs Tested	Protocol for Exposure	Genotoxicity Endpoints	Outcome of the Test	Usefulness for Genotoxicity Assessment of NMs	Reference
Liver	2D and 3D HepaRG-based models. 3D spheroids formation by use of ultra-low attachment (ULA) plates.	incinerated polyethylene (PEI PM <sub>0.1</sub> )  TiO <sub>2</sub> (Aeroxide P25), and two ZnO NPs (ZnO S. NMs and NM110)	Various concentrations up to 100 µg/mL. Time: 48 h for TiO <sub>2</sub> , 24 h for ZnO	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay) (standard and high throughput formats)	dependent manner  TiO <sub>2</sub> Negative for 2D No significant cytotoxicity or genotoxicity, 3D: slight increase in % tail DNA at high concentrations (100 µg/mL). ZnO S. NMs Negative (Genotoxic only at cytotoxic concentrations) NM110: Positive DNA damage increase in both models, with oxidative DNA damage observed in some cases.	including simulated gastrointestinal digestion and a triculture SIE model. Promising approach with increased sensitivity observed in 3D models and high throughput comet assays (CometChip®)	[222]. <a href="https://doi.org/10.1016/j.chemosphere.2023.140975">https://doi.org/10.1016/j.chemosphere.2023.140975</a>
Liver	3D HepG2-based spheroid model. Spheroids formed by hanging drop technique, then transferred to ULA plates coated with 1.5 % agarose gel to prevent adhesion	TiO <sub>2</sub> and Ag	Acute (24 h) and long-term (120 h) exposure to 5 µg/mL	Micronucleus assay	TiO <sub>2</sub> and Ag: Positive after acute 24 h exposure Negative a after long-term 120-hour exposure	High potential for assessing chronic ENM exposure with realistic dosimetry. Highlights the importance of using 3D models over traditional 2D methods for improved physiological relevance	[139], DOI: 10.3791/61141
Liver	HepG2-based 3D liver spheroids formed by hanging drop technique, then transferred to agarose-coated wells to support longer-term exposure experiments	ZnO	Long-term exposure (5 days) with concentrations of ZnO from 0.2–2.0 µg/mL	Micronucleus assay	Positive Dose-dependent increase in MN frequency in mononucleated cells (MN/MN %). For binucleated cells (MN/BN %), significant genotoxicity was only observed at the lowest ZnO concentration (0.2 µg/mL).	Need to adapt assay for longer-term exposures to accurately assess genotoxicity of NMs. Utility of the 3D HepG2 model for both acute and prolonged exposure regimes, emphasizing its physiological relevance over 2D systems.	Conway et al., Mutagenesis (2020), DOI: 10.1093/mutage/geaa018
Liver	3D HepG2-based spheroid model. Spheroids formed by hanging drop technique, then transferred to agarose-	TiO <sub>2</sub> NM–105, ZnO NM–111, BaSO <sub>4</sub> NM–220, CeO <sub>2</sub> NM–212, Ag 576832, Sigma Aldrich	Acute (24 h) and long-term (120 h) exposure at 0.2–10 µg/mL	Micronucleus assay	Acute exposure: All NMs caused significant genotoxicity, with ZnO showing the strongest effect. Long-term exposure: Only ZnO induced genotoxicity.	3D liver spheroid models are realistic and effective for NM hazard assessment.	[141], Journal of Nanobiotechnology, DOI: 10.1186/s12951-021-00938-w

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Table 2 (continued)

Organ/System	In vitro model	NMs Tested	Protocol for Exposure	Genotoxicity Endpoints	Outcome of the Test	Usefulness for Genotoxicity Assessment of NMs	Reference
Liver	coated ULA plates 3D HepG2-based spheroid model. Spheroids formed by hanging drop technique, then transferred to ULA plates	TiO <sub>2</sub> anatase, ZnO NM110, Ag NM300K	Acute 24-hour exposure at concentrations ranging from 3–212 µg/mL (1–75 µg/cm <sup>2</sup> )	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay)	TiO <sub>2</sub> : No significant cytotoxicity or genotoxicity observed. ZnO: Induced cytotoxicity and DNA damage at high concentrations; significant effects only in 2D cultures. Ag: Induced cytotoxicity and concentration-dependent DNA damage; significant effects observed only at cytotoxic concentrations in 2D cultures.	Demonstrates the utility of HepG2 3D liver spheroids for nanotoxicity assessment while highlighting the higher sensitivity of 2D models for detecting genotoxicity.	Elje et al., Nanomaterials (2020), DOI: 10.3390/nano10030545
Liver	3D human liver microtissue model (InSphero system) co-cultured using primary human hepatocytes and non-parenchymal cells	ZnO (NM110), Ag (NM300), MWCNT (NM400), TiO <sub>2</sub> (NRCWE 002)	Single and multiple exposures over 15 days at concentrations: ZnO/Ag: 0.5–8 µg/mL TiO <sub>2</sub> /MWCNT: 16–250 µg/mL	DNA strand breaks and DNA oxidation lesions (Alkaline and Fpg-modified comet assay)	Single exposure: DNA damage induced at higher concentrations for all NMs, with ZnO and Ag showing the most significant effects. Multiple exposure: Increased genotoxicity for ZnO and Ag compared to single exposures; MWCNT and TiO <sub>2</sub> showed limited effects.	Demonstrates the relevance of liver microtissues for nanotoxicology, particularly for repeated low-dose exposure studies, over conventional 2D models.	Kermanizadeh et al., Particle and Fibre Toxicology (2014), DOI: 10.1186/s12989-014-0056-2

ALI: Air-Liquid-Interface;

require specialized equipment and present challenges in reproducibility due to inter-laboratory variability, as highlighted by Elje et al. [73]. Such challenges could be mainly attributed to the differences in size in different laboratories despite an identical dispersion protocol being used [73].

Co-culture models are particularly suited for studying secondary genotoxic mechanisms, such as those mediated by immune and stromal cells. They effectively reveal the role of oxidative stress and inflammation in genotoxicity. However, these models require careful optimization of cell ratios and culture conditions to ensure reliability.

Careful consideration is required to address potential interferences when evaluating genotoxicity induced by NMs. For instance, metal oxide NMs such as ZnO and CuO release soluble ions, which can induce cytotoxicity and complicate the interpretation of genotoxicity data. Di Ianni et al. [56] stressed the need to optimize NM concentrations to avoid excessive cytotoxicity (>20 %) that may obscure assay results [56]. Similarly, Burgum et al. [30] noted that oxidative stress markers must be analysed carefully to distinguish true genotoxicity from assay artefacts.

## 5. Advanced gastro-intestinal models

*In vitro* gastro-intestinal models assess intestinal toxicity and absorption after oral exposure. Cells are cultured on the apical side of membrane inserts, typically using Caco-2 enterocytes alone or co-cultured with mucus-secreting HT29-MTX cells [228]. Enterocytes form the barrier and mediate NM uptake [90], while mucus influences NM interactions with the epithelium [47].

Microfold cells (M cells) are specialized cells, found in the Peyer's patches of the gut-associated lymphoid tissue, that facilitate transcytosis [45,57] by internalizing NMs for transport via the lymphatic system, bypassing hepatic metabolism [31,225]. Though rare and hard to isolate, M cells can be induced *in vitro* by co-culturing Raji B cells with Caco-2 cells [3,16,55,96,122]. Triple-cultures of Caco-2/HT29-MTX/Raji B were established a decade ago [7,9,203] and are now part of the OECD Test Guidelines Programme [184,223], offering a valuable tool for nanosafety research [80].

Including immune cells such as human primary macrophages or differentiated THP-1 cells on the basolateral side of intestinal *in vitro*

models enables toxicity studies on immune-modulating NMs. Le et al., [132]. Alternatively, THP-1 and MUTZ-3 cells can be embedded in a collagen scaffold under epithelial cells [213]. However, these models mimic inflammatory conditions but lack M cells, limiting their use in NM research.

The commercially available 3D EpiIntestinal™ model (MatTek) consists of primary intestinal cells grown on a fibroblast-derived lamina propria, replicating human small intestine responses. The tissues can be maintained *in vitro* for > 10 days while remaining proliferative, differentiating and shedding from the apical surface. The reconstructed Epi-Intestinal™ model (MatTek) was designed for genotoxicity testing using micronucleus and comet assays [112]. A micronucleus assay protocol was developed to detect clastogens and aneugens, including those requiring metabolic activation [138]. However, it lacks mucus secretion.

García-Rodríguez and colleagues employed a Caco-2/HT29 co-culture to investigate the effects of differently shaped TiO<sub>2</sub> NMs (nanospheres, nanorods, and nanowires) [87]. The study assessed endpoints such as barrier integrity (TEER), paracellular transport, cellular uptake, and DNA damage using the comet assay. The results revealed shape-dependent toxicity, with nanowires showing the greatest adverse effects, significantly reducing TEER and increasing permeability, indicative of barrier compromise. Furthermore, nanowires induced higher levels of DNA damage compared to nanospheres and nanorods, highlighting the critical role of NM shape in modulating intestinal toxicity.

Dorier et al. [60] used a mucus-secreting Caco-2/HT29-MTX co-culture model and exposed them acutely (6–48 h) or repeatedly (twice a week for 3 weeks) to evaluate the genotoxic effects of three different types of TiO<sub>2</sub> including E171, a food-grade TiO<sub>2</sub>, by the comet assay. The study showed that repeated exposure to E171 led to significant oxidative stress, evidenced by ROS accumulation and oxidized DNA bases, but no evidence of DNA strand breaks or endoplasmic reticulum (ER) stress was found. Dorier et al. [61] expanded on previous studies by exposing a Caco-2/HT29-MTX model to A12 and NM105 TiO<sub>2</sub> NMs. The findings showed ROS accumulation and slight DNA damage without significant cytotoxicity, as measured by comet assays and cell viability tests.

Vital et al. [224] investigated the cyto- and genotoxicity of cellulose NMs (CNMs), specifically CNF-TEMPO and CMF-ENZ, using intestinal co-culture models comprising Caco-2 and HT29-MTX cells. The study incorporated a standardized *in vitro* digestion protocol to simulate gastrointestinal processing and evaluated endpoints including reactive oxygen species (ROS), and DNA damage by the micronucleus and comet assays. Results demonstrated limited cytotoxicity, with cell viability maintained even at higher concentrations (200 µg/mL for undigested CNMs). Moreover, the study revealed no mutagenic effects across tested conditions. Interestingly, digestion appeared to mitigate the genotoxic potential of CNMs, as observed by reduced DNA damage markers in digested samples compared to undigested ones. However, the persistence of DNA damage, particularly chromosomal aberrations induced by CNF-TEMPO at low concentrations, raises concerns and highlights the need for further investigation into the long-term safety of CNMs under real-world exposure scenarios.

A triple-culture (Caco-2, HT29-MTX, and Raji B cells) small intestinal epithelium (SIE) model and the CometChip assay were used to investigate primary and secondary genotoxicity of carboxylated polystyrene spheres (PS25C and PS1KC), and incinerated polyethylene (PEI PM<sub>0.1</sub>) [59,233]. Simulated gastrointestinal tract digestion created physiologically relevant exposures, applied to the SIE model, showing that carboxylated polystyrene spheres caused DNA damage in a time- and concentration-dependent manner.

Investigating co-ingestion, cellular uptake, and translocation of NMs in a multicell layer setting is vital for assessing potential synergistic adverse effects, particularly genotoxicity. Kharaghani et al. [123] demonstrated co-ingestion of 25 nm sized nanoplastics significantly increased arsenic uptake and translocation in a triple-culture small

intestinal epithelium model (Caco-2, HT29-MTX, and Raji B cells), underscoring potential synergistic effects from combined NM and pollutant exposure.

### 5.1. Challenges, limitations and advantages of gastro-intestinal models for testing nano- and advanced materials

The suitability of co-culture intestinal models for hazard assessment of NMs is well-established due to their ability to replicate key physiological features of the intestinal barrier. These models have been effectively utilized to assess the toxicity of various NMs, including TiO<sub>2</sub> nanoparticles and cellulose NMs, by evaluating endpoints such as barrier integrity, oxidative stress, and genotoxicity. Studies highlight the critical role of NM shape and digestion in modulating intestinal toxicity [87,224].

It has been demonstrated that oxidative stress, rather than direct DNA damage, may be a key driver of genotoxicity in TiO<sub>2</sub>-exposed intestinal cells, suggesting that even in the absence of overt cytotoxic effects, a genotoxic risk could be present [60]. These models have proven valuable in assessing both acute and chronic exposure scenarios, and elucidating both primary and secondary genotoxicity mechanisms [61]. Their use in these studies highlights their relevance in providing a more realistic and comprehensive assessment of NM safety. However, it remains essential to address the potential long-term effects of exposure, which is a critical consideration for the broader application of these models in regulatory and safety assessments.

The physiochemical properties of NMs have a large impact on phagocytosis and uptake of NMs across the intestinal barrier [197]. Biotransformations, such as biocorona formation and surface chemistry changes during digestion process, impact biointeractions and NM toxicity. Such biotransformations should be taken into consideration, and *in vitro* gastrointestinal digestion could be performed in test tube assays using static or dynamic protocols, such as the INFOGEST 2.0 consensus protocol [25]. Further, the low pH of the gastric acid alters NM bioidentity, affecting uptake and toxicity [211].

Co-culture intestinal models are valuable for studying NM interactions with the intestinal barrier, offering high physiological relevance through mucus-secreting cells and tight junctions. They effectively assess NM shape- and exposure-dependent effects but lack immune components and microbiota, limiting their ability to study secondary genotoxicity. Enhancing these models by incorporating immune cells and microbiota, developing long-term exposure protocols, and standardizing testing guidelines are essential steps to improve their complexity, reproducibility, and broader applicability in nanotoxicology research. However, the limited availability of normal human tissues and the high cost of commercially available models may pose challenges for some research facilities, potentially limiting the widespread adoption and use of these advanced models.

## 6. Advanced liver models

Numerous studies have demonstrated the ability of NMs to accumulate in the liver and induce hepatotoxicity and genotoxicity [107, 121,141]. *In vitro* liver models, using cell lines or primary hepatocytes, serve as a valuable model to assess NM metabolism and bioactivation of pro-genotoxic compounds including NMs [212]. Biotransformation of NMs or chemicals occurs via phase I (cytochrome P450) and phase II (glutathione S-transferase) enzymes, providing insight into genotoxic potential and reactive intermediates.

Traditional 2D hepatic cell cultures have low metabolic activity and altered phenotype [212]. Liver spheroids, formed using techniques such as the hanging drop method or ultra-low attachment plates, provide a more physiologically relevant model for toxicology and drug discovery [74,76,141,212]. Commonly used cells to form spheroids include HepG2, C3A, HepaRG, iPSC-derived hepatocytes, and primary hepatocytes [15,141,212]. Hepatocytes can be co-cultured with Kupffer,



stellate, and endothelial cells enhancing physiological relevance, enabling better insights into cell interactions, immune responses, and genotoxicity [141].

3D spheroids lack vasculature, leading to uneven NM distribution due to restricted transport from the outer layer [141]. However, they improve *in vitro* relevance by mimicking physiological conditions such as fluid flow and cell interactions [234].

Advanced liver models are employed with various genotoxicity assays to evaluate the genotoxic potential of chemicals and NMs. Common genotoxicity assays applied to NMs include the comet assay for DNA strand breaks [76,222] and the micronucleus assay for chromosomal aberrations [139–141,44,76]. In these studies, different types of TiO<sub>2</sub>, Ag, ZnO, BaSO<sub>4</sub> and CeO<sub>2</sub> NMs have been tested on liver spheroids. The genotoxic potential of several NMs has been investigated in 2D and 3D hepatic HepaRG models. Interestingly, comet assay sensitivity was increased when combining the high-throughput format of the assay with 3D spheroids, compared to the 2D model [222]. Differences in genotoxic sensitivity, measured by the comet assay, were also seen in 3D HepG2 spheroids after exposure to chemicals [74,76].

HepaRG and HepG2 spheroids have also been applied for low-dose and longer-term exposure to NMs and genotoxicity assessment by the micronucleus assay, showing their potential as a relevant model for longer-term toxicity testing [44].

A 3D human liver microtissue model (InSphero system) co-cultured using primary human hepatocytes and non-parenchymal cells has also been used to assess the DNA damage induced by ZnO, Ag and TiO<sub>2</sub> NMs after repeated exposure [121]. The relevance of liver microtissues for nanotoxicology was noted, in particular with respect to repeated low-dose exposure studies.

Liver models combined with ‘omics’ technologies allow investigation of molecular mechanisms behind genotoxicity. Omics enable analysis of gene expression, metabolic profiles, and DNA damage response pathways in liver cells. They also assess hepatotoxicity, such as, cytotoxicity and mitochondrial dysfunction, often linked to genotoxic effects due to the liver’s role in metabolism, offering a comprehensive view of chemical-induced liver injury and DNA integrity.

### 6.1. Challenges, limitations and advantages of liver models for testing nano- and advanced materials

The ongoing research indicates that 3D hepatic cell models provide a more physiologically relevant model compared to 2D cell cultures, thus representing a valuable option to enhance the precision of *in vitro* toxicity assessments, and improving safety evaluations of chemicals and NMs. The different techniques available allow for relatively large-scale and cost-effective production of spheroids, which are easy to handle and with minimal need for specialized materials and equipment. A challenge or limitation of these models is the formation of a necrotic core due to insufficient oxygen and nutrient diffusion, leading to cell death and compromising the model’s functionality [141]. Besides, a specific challenge in the use of these models with NMs is the capability of the particles to penetrate the spheroid. The translocation of the particles through the 3D structure has been reported to vary considerably depending on the model, and also on the particles themselves (based on properties such as NM size) [141]. Thus, a case-based characterization is needed to ensure an accurate representation of NM distribution in the spheroid. Despite these challenges, advanced *in vitro* liver models have been proven to serve as valuable platforms for studying genotoxicity, offering insights into mechanisms underlying genotoxicity, the metabolic activation of pro-genotoxic compounds, the mechanisms of DNA damage, and the cellular responses involved in DNA repair and toxicity. These models play a crucial role in safety assessment of NMs and advanced materials, drug development, and environmental risk evaluation by providing relevant data on genotoxic hazards and informing decision-making processes aimed at protecting human health.

### 6.2. Advanced neurotoxicity and neurodevelopmental toxicity models

NMs can cross the blood-brain barrier (BBB) and affect the central nervous system (CNS) through transporters, adsorptive-mediated transcytosis, or the intranasal route [89]. Once inside, they can cause neurotoxicity [205], DNA damage, and contribute to neurodegenerative diseases, notably Parkinson’s and Alzheimer’s, owing to the brain’s high metabolic activity and limited repair ability. New developments in NAMs for neurotoxicity, particularly genotoxicity, are crucial for understanding these diseases and assessing risks of nanoscale materials. NAMs for neurotoxicity are divided into developmental (DNT) and adult neurotoxicity (ANT) [205]. The OECD is working on a battery of *in vitro* DNT tests for regulatory acceptance, as current methods rely on rat studies (OECD TG 426). The OECD’s initial recommendation for DNT-IVB [177] provides a foundational framework for the adoption of *in vitro* testing methods. Additionally, a recent review by Tal et al. [215] evaluates NAMs and their potential for regulatory use.

Neurotoxicity can be assessed using various *in vitro* models, including human iPSCs, hESCs, 3D brain organoids, spheroids, primary neurons, or immortal cell lines. The human multineurotransmitter assay (hMNR) using hiPSC-derived BrainSpheres can record spontaneous electrical activity [22,102]. BBB models based on hiPSCs [8] replicate neurovascular units [37]. Additionally, a 3D neuroimmune co-culture system modelling the Parkinson’s disease microenvironment is a promising NAM for genotoxicity assessment of NMs and advanced materials [201].

*Caenorhabditis elegans* is a promising model system for studying neurodegenerative mechanisms, owing to its simplicity, short life span, well-defined genetics and well-characterized nervous system. Transgenic *C. elegans* strains expressing human disease-associated proteins, e. g. for Parkinson and Alzheimer diseases, are engineered to help to understand neurodegeneration, neuron-coordinated behavioural changes, and cognition defects [204,205].

Research on the genotoxicity of NMs in mammalian cells is extensive [128], but studies on their neurotoxicity and effects on neuronal models are more limited. TiO<sub>2</sub> NMs have been shown to cause DNA damage in neuroblastoma cells using the comet assay [219]. *In vitro* neuronal models, including primary cell lines, astrocytes, rodents, zebrafish, and *C. elegans*, are valuable tools for assessing the neurotoxicity of NMs, particularly TiO<sub>2</sub>, by measuring mitochondrial dysfunction, neurodegeneration, and oxidative stress [238]. Additionally, CeO<sub>2</sub> NMs have been found to induce DNA damage in neurons and glial cells, detectable using  $\gamma$ -H2AX assays [81]. These findings underscore the potential of neuronal NAMs in detecting the neurotoxic effects of NMs.

Advanced neuronal NAMs – co-culture and 3D spheroids – have started to be used to study neurotoxicity after NM exposure. Cytotoxicity has been performed in neuronal co-cultures and 3D brain spheroid NAMs from human D384 astrocytes and SH-SY5Y neuroblastoma cells exposed to iron oxide nanoparticles [49–51]. The uptake of gold nanoparticles has been successfully demonstrated in 3D brain spheroids generated from human dopaminergic neurons differentiated from the LUHMES cell line and in human iPSC-derived brain spheroids (BrainSpheres) [133]. NMs such as silver nanoparticles reduce the expression and co-localization of cytoskeleton proteins in hiPSC cerebral organoids, eventually influencing neurite growth [111]. Moreover, the potential usefulness of BBB NAMs to study the effect of NMs has been demonstrated in a few studies [1,135,136,188].

Despite increasing attempts to apply the NAMs developed for neurotoxicity, genotoxicity testing of NMs using the neuronal NAMs is lacking. This highlights a significant gap in the field, indicating substantial opportunities for further research.

A systematic review on the effect of NMs on embryonic stem cell neural differentiation has been published [195], indicating some potential for NMs (mainly polymer-based composites) in neural tissue engineering.

### 6.3. Challenges, limitations and advantages of neurotoxicity and neurodevelopmental NAMs for testing nano- and advanced materials

3D brain NAMs more accurately mimic the structural and functional complexity of the human brain physiology, including cell-cell and cell-matrix interactions, as compared to the simple 2D neuronal cell culture NAMs. Furthermore, these models represent the brain's microenvironment more accurately, generating *in vitro* findings more relevant to *in vivo* conditions. Understanding how NMs can impair neurodevelopment and cause neurotoxicity in a human-relevant context is one of the many advantages of neuronal NAMs. A high throughput platform can be used for cost-effective assessment of the risk to human health of different NMs, while adhering to ethical standards. Little has been done to study genotoxicity of NAMs exposed to different NMs, but well-established neuronal NAMs can be successfully used to understand the DNA damage and response to NMs.

Among the major challenges are the need to establish these models, data reproducibility, and the quantification of NM uptake. The penetration and distribution of nanoparticles is challenging due to differences in diffusion dynamics compared to *in vivo* conditions. The lack of BBB and inadequately replicating complex human brain is one of the major limitations of these NAMs.

## 7. Advanced skin models

Traditional monolayer skin cell cultures, particularly keratinocytes grown in submerged conditions, have been widely used for studying skin responses to chemical exposure. However, these 2D cultures have significant limitations, as they fail to replicate the complex architecture and functional properties of human skin. The lack of multiple interacting cell types and stratified layers does not accurately reflect the physiological environment, limiting their applicability for safety assessments of cosmetics, pharmaceuticals, and other chemicals that come into contact with the skin.

To address these limitations, significant efforts have been made to develop advanced 3D skin models that better mimic the structural and biological complexity of native human skin. These models incorporate multiple layers of differentiated keratinocytes, often supported by fibroblasts in a dermal-like matrix, creating a more physiologically relevant system for studying chemical penetration, metabolism, and cellular responses [108,200].

One of the earliest and most successful applications of 3D skin models was in the assessment of skin irritation and sensitization. These endpoints are critical in dermatotoxicology and have been well-characterized using reconstructed human epidermis (RHE) models. As a result, several OECD TGs have been established, including Skin irritation (OECD TG 439), Skin corrosion (OECD TG 431), Skin sensitization (OECD TG 442E), Skin absorption (OECD TG 428). These standardized tests provide reliable *in vitro* alternatives to traditional animal testing, aligning with regulatory frameworks and ethical considerations.

Advanced skin models have undergone significant advances in recent years, particularly in their application to the genotoxicity testing of chemicals [192,232]. Advanced skin models, such as reconstructed human epidermis and full-thickness skin equivalents, consist of multiple cell types, including the epidermis, dermis, and appendages, in 3D arrangement, to better replicate the architecture and functionality of human skin and its responses to genotoxic agents, and they offer a more physiologically relevant platform for genotoxicity testing [4,13,112,190,192].

The immune system plays a critical role in skin homeostasis and the response to genotoxic insults. Immune cells, such as Langerhans cells, dendritic cells, and T cells are incorporated into skin models, to simulate the immune response to genotoxic agents. This integration enhances the model's ability to assess inflammatory responses, immune cell activation, and cytokine release following chemical exposure, providing a more comprehensive evaluation of genotoxicity [200]. The authors

developed skin Immuno-CometChip in 3D versus 2D cultures to screen topical toxins and skin-specific cytochrome inducers.

Protocols for both micronucleus [4,38,39,190] and comet assay [191,198] have been developed in combination with reconstructed 3D skin models to enhance the *in vitro* prediction of genotoxicity for dermally applied chemicals. The reconstructed skin micronucleus test (RSMN) and RS comet assay have gone through extensive validation with several coded chemicals showing high predictivity. The metabolic competency of commercially available 3D human RS skin models, specifically EpiDerm™ and Phenion® Full-Thickness Skin Model, has also been investigated; it was found to be similar to native human skin [106,231], confirming that RS skin models have *in vivo*-like metabolic properties.

The genotoxicity of various NMs—including silica, silver, ZnO, TiO<sub>2</sub>, polyethylene terephthalate (PET), graphene oxide (GO), and core multishell nanocarriers—has been investigated in both 2D and 3D skin models (Table 2) [48,67,130,221,232,240]. These studies assessed DNA damage using the comet assay, phosphorylated γH2AX detection, ELISA for 8-hydroxy-2'-deoxyguanosine (8-OHdG), DNA fragmentation, cell cycle arrest (G2/M phase), and clastogenicity via the micronucleus assay.

Wills et al. [232] adapted a 3D reconstructed RSMN model to test silica NMs (16 and 85 nm) following topical application. They compared 3D dose-response results to a 2D micronucleus assay using monocultured human B cells (TK6) exposed in growth medium. The 3D models, at doses up to 300 µg/mL, did not show significant DNA damage or impact on cell viability. Higher doses likely caused air-liquid interface suffocation.

Zielińska-Górska et al. [240] also observed negative results in DNA oxidation in 3D EpiDerm models after exposure to silver nanoparticles, graphene oxide, and complexes of silver and GO NMs. These findings contrast with studies using 2D models [48,67,130], which reported both positive and negative results.

3D skin models for genotoxicity can be crucial in following up positive results from standard 2D testing, especially in cosmetic regulation which bans *in vivo* genotoxicity testing (7th Amendment to the EU Cosmetics Directive effective since 2009; Notes of guidance for the testing of cosmetic ingredients and their safety evaluation SCCS/1647/22) [19,20]. Advantageously, the use of comet and micronucleus RS assays allows the investigation of all key modes of genotoxic activity mandatory for regulatory testing (even though the comet assay detects transient DNA lesions and is considered as an indicator assay). The RSMN and RS comet assay have already supported safety assessments of hair dye ingredients in the EU [154] and are internationally validated at the OECD level to be adopted into an OECD TG for global use in chemical safety assessment, such as within the REACH framework.

The development of OECD TGs for genotoxicity testing in reconstructed skin, including RSMN and comet assays, represents a significant step forward in this field, providing crucial data for evaluating potential carcinogenic and mutagenic risks. However, for NMs they would need further validation.

### 7.1. Challenges, limitations and advantages of skin models for testing nano- and advanced materials

Considering the resemblance of the 3D skin models to real exposure conditions, they are highly relevant for risk assessment of dermally applied chemicals when compared with standard monocultures. A comparison study with OECD TG439 skin irritation was performed with 2D HaCaT keratinocytes, primary human keratinocytes and RhE with TiO<sub>2</sub>, ZnO, CuO, Ag, SiO<sub>2</sub> and NM300K NMs, with measurement of cytotoxicity and pro-inflammatory markers. The 2D HaCaT model was found to be more sensitive than the RhE model, and more aligned with the RhE model than with the primary keratinocytes [152]. However, for NMs they would need a further validation step as the situation is more complex considering the generally poor dermal absorption of NMs and

very poor translocation through intact stratum corneum of the skin, and limited data are available. Additionally, the top concentration might be challenging as increasing dose by topical exposures may lead to air-liquid interface suffocation.

Nevertheless, the outcome of the recent studies on NM skin exposure shows the importance of tissue microarchitecture in defining NM exposure and suggests that 3D *in vitro* models could play a role in bridging the gap between *in vitro* and *in vivo* outcomes in nanotoxicology. Robust exposure characterisation and uptake assessment methods are essential to interpret nanogenotoxicity studies successfully.

## 8. NAMs for phototoxicity and photogenotoxicity

Phototoxicity and photogenotoxicity refer to the adverse effects caused by the interaction of chemicals with sunlight or artificial light, leading to skin irritation, inflammation, and in some cases, DNA damage and mutations. NAMs are increasingly being utilized to assess phototoxic and photogenotoxic potential for chemicals. However, for NMs they would need further validation.

NAMs applied for evaluation of phototoxicity and photogenotoxicity of chemicals include various *in vitro* assays designed for use with human-derived skin cells or 3D skin models. These assays typically involve exposing cultured cells or tissues to test substances in the presence or absence of light irradiation, followed by the evaluation of cell viability, inflammatory responses, and oxidative stress markers. High-throughput screening platforms enable the rapid screening of large numbers of chemicals for phototoxic effects, allowing for the prioritization of compounds for further evaluation.

The 3T3 Neutral Red Uptake Phototoxicity Test (OECD TG 432) [169] and the RhE model (OECD TG 498) [182] are validated assays for assessing photoirritation and phototoxicity by measuring cell viability, membrane integrity, and cytokine release after chemical exposure and light irradiation. These assays can predict phototoxicity and be used alone or in a tiered strategy (Krakowian et al., 2024). The Assay for Photoreactivity (OECD TG 495) measures ROS production [170], and an ICH and ISO standard for NM phototoxicity testing is available [113, 114].

Assessments of TiO<sub>2</sub> nanoparticle phototoxicity in the 3D skin model were performed by [187] and [216], applying non-toxic doses of UV-A and variable exposure times [187,216]. The results on reduction of MTT indicated that TiO<sub>2</sub> nanoparticles did not exhibit phototoxicity in the 3D skin model in the presence of UV radiation.

To assess the photomutagenic effect of irradiated TiO<sub>2</sub> particles, Nakagawa et al. [160] used the chromosome aberration test in Chinese hamster ovary (CHO) cells. Although the result was positive, it could not be reproduced by Theogaraj et al. [217] who found no induction of chromosome aberrations in the same cell type when TiO<sub>2</sub> was irradiated with UV-A and UV-B light. In the study by Petkovic et al. [189], using the comet assay, anatase TiO<sub>2</sub> NPs after UV irradiation reduced the viability of HepG2 cells and induced significant increases in DNA strand breaks and DNA oxidation damage compared with non-irradiated TiO<sub>2</sub>.

### 8.1. Challenges, limitations and advantages of the phototoxicity and photogenotoxicity NAMs for testing nano- and advanced materials

Photogenotoxicity assays evaluate the ability of chemicals to induce DNA damage under light exposure using *in vitro* genotoxicity testing methods. The common Ames test with photomodification (Ames MPF), which assesses the mutagenic potential of chemicals in the presence of light [153] can be used for photogenotoxicity testing of chemicals; however, this test is not suitable for testing of NMs owing to the size of bacteria and their cellular walls which hamper internalisation of NMs.

The most promising photogenotoxicity test is the alkaline comet assay modified with lesion-specific enzymes which detects DNA strand breaks and oxidized bases in cells exposed to both chemicals (including NMs) and ultraviolet (UV) light. In combination with the reconstructed

human epidermis, which better reflects real skin, this assay can provide information on the photogenotoxic potential of substances and can help to identify compounds that may pose a risk of DNA damage upon exposure to sunlight or artificial light sources.

Overall, NAMs offer valuable tools for assessing the phototoxic and photogenotoxic/photomutagenic potential of conventional chemicals and nano- and advanced materials. The OECD has launched a project for developing GD with an IATA for phototoxicity [183].

## 9. Organ-on-a-chip models

Organ-on-a-chip (OoC) models are advanced microfluidic systems that replicate human organ functions, offering more accurate genotoxicity testing than traditional cell cultures [119,143]. They provide precise control over flow rates, nutrient delivery, and waste removal, improving toxicity assessments. OoC models also support automation, high-throughput screening, and real-time monitoring of cellular responses, enabling efficient and cost-effective evaluation of NMs and their genotoxic effects [194].

Although numerous OoC models have been developed to assess chemical and drug toxicity in tissues such as skin, lung, liver, gut, heart, kidney, spleen, and bone marrow, their application in nanotoxicity research remains limited [11]. A lung-on-a-chip system employing human alveolar epithelial and endothelial cells highlighted the distinct effects of ZnO and TiO<sub>2</sub> NMs, revealing oxidative stress, apoptosis, and disruptions to the alveolar-capillary barrier, consistent with previous findings [237]. Similarly, a placenta-on-a-chip model, featuring co-cultured BeWo trophoblast cells and endothelial cells, demonstrated that high concentrations of TiO<sub>2</sub> NMs impaired barrier integrity and induced significant cell death [236]. Esch et al. [77] further developed a body-on-a-chip device integrating intestinal (co-cultured Caco-2/TH29-MTX) and liver models (HepG2/C3A) via fluidic connections. When carboxylated polystyrene NMs were introduced to the intestinal model, the liver model exhibited a dose-dependent increase in tissue-specific enzyme production, indicative of liver damage. This multi-organ system underscored the exacerbated liver injury caused by interactions between the liver and intestinal epithelium. However, none of these studies included assessments of genotoxicity, highlighting a critical gap in current research.

### 9.1. Challenges, limitations and advantages for organ on a chip for testing nano-advanced materials

OoC models provide promising platforms for assessing the genotoxicity of NMs due to their ability to replicate complex human organ microenvironments, precise control of cellular conditions, and integration of real-time monitoring technologies. However, current studies have primarily focused on toxicity endpoints such as oxidative stress and apoptosis, with no further investigation of genotoxicity endpoints. Challenges include the need for models capable of long-term exposure simulations, integration of immune system components, and the development of standardized protocols for genotoxicity testing. Additionally, materials used in OoC construction, such as PDMS, can interfere with NM behaviour, further complicating assessments [17]. Future advancements should focus on multi-organ integration, personalized models using induced pluripotent stem cells (iPSCs), and incorporating advanced sensing technologies for real-time monitoring of genotoxic responses, paving the way for more comprehensive and predictive toxicological evaluations of NMs.

## 10. Molecular biology methods and endpoints

Omics approaches, including genomics, transcriptomics, proteomics, and metabolomics, provide insights into the molecular mechanisms of NMs and chemical toxicity [118]. Integrated into advanced models, they analyze gene expression, signalling pathways, and metabolic changes,



deepening our understanding of genotoxicity and aiding risk assessment. Omics techniques excel in NM toxicity studies where conventional methods fall short, offering high-throughput, precise data to explore DNA damage, repair, and genotoxic mechanisms.

Recent biomarker discovery leverages multi-omics approaches to detect molecular alterations (e.g., DNA methylation, mRNA, metabolites, proteins), linking exposures to phenotypic outcomes. High-throughput omics techniques screen DNA, RNA, proteins, lipids, and metabolites *in vitro* and *in vivo*, including 3D models such as triple-culture lung models and brain organoids. Omics also apply to non-animal models like *C. elegans*, *D. rerio* (zebrafish), and *D. melanogaster*. RNA sequencing (RNA-seq) and DNA sequencing (DNA-seq), and metabolic profiling, identify gene expression changes linked to genotoxic stress, while proteomics via mass spectrometry detects post-translational modifications and protein expression shifts [205].

An assay that can be used for mechanistic genotoxicity assessment of NMs is the stem cell-based ToxTracker reporter assay, specifically focusing on detecting DNA damage and related cellular responses. It combines the use of genetically modified cells with reporter genes that fluoresce in response to specific molecular events associated with genotoxicity, such as DNA damage, oxidative stress, and DNA repair. The ToxTracker assay has been used to test various NMs, including TiO<sub>2</sub>, silica, and carbon-based NMs [26,33,105,120,150,151,242].

Another sensitive approach is to measure transcriptional profiling to predict genotoxicity [218]; for example the TGx-DDI (Transcriptomics-Based Genotoxicity Direct Drug Interaction) test uses expression of a particular set of mRNAs for the prediction [40,134]. This assay provides mechanistic insight including potential identification of the types of damage induced and holds promise as a sensitive genotoxicity assay for testing NMs. Mutations induced by environmental stressors are rare low-frequency genetic errors, and recent developments in next generation sequencing have opened up an arena for mutagenicity assessments that is suitable for regulatory purposes of chemicals including NMs. These technologies (in particular error-corrected DNA sequencing) predict which stressor is responsible for the mutations induced (molecular fingerprinting) and give valuable mechanistic information regarding their mode of action [202,209,220]. These methods hold promise to advance genotoxicity and carcinogenicity testing and have already been used to understand the genetic toxicity of chemicals [147]. Moreover, epigenetics also plays a role in predicting chemical toxicity, with observed changes linked to various substances, including endocrine-disrupting chemicals and NMs [64,210].

Mapping of toxicogenomics data to AOPs facilitates the inclusion of this evidence in regulatory safety assessments. A computational method developed by Saarimäki et al. [214] systematically allocates toxicogenomics data to key events and AOPs, enabling better comparisons between *in vitro* and *in vivo* data. Even more, the sensitivity of modern omics techniques allows the detection of PoD events, which in turn can allow the prediction of long-term outcomes even after short exposure of the *in vitro* biological systems.

Omics technology can also be used with neural models (e.g., human 2D and 3D *in vitro* models, brain organoids, *C. elegans*, Zebrafish, and *Drosophila*) to identify gene expression changes linked to genotoxic stress, shedding light on neurodegeneration pathways, post-translational modifications and protein expression changes or metabolic profiling to identify pathway changes related to genotoxicity and neurodegeneration [205].

#### 10.1. Challenges, limitations and advantages of novel molecular biology methods for testing nano- and advanced materials

Novel molecular biology methods including omics techniques have been used to assess biological responses to NMs and are crucial for future risk assessment strategies [137,144]. They can detect subtle genotoxic effects that might not be evident with traditional methods.

In the context of using omics techniques for genotoxicity testing of

NMs, gene expression and epigenetic changes play key roles as important endpoints. Several reviews provide valuable information on the deregulation of genes involved in DNA damage response and repair, as well as epigenetic responses, including DNA methylation, non-coding small single-stranded RNAs (miRNAs), and histone modifications [125]. Recently, reviews focusing on the epigenetic changes induced by silica [239], and TiO<sub>2</sub> nanoparticles (Wells et al., 2023) have also been published.

In conclusion, toxicogenomics and epigenetics techniques have proven valuable in assessing the genotoxicity of NMs. However, before these techniques can be incorporated into regulatory testing frameworks, it is essential to harmonize and validate the protocols. Inconsistent or even contradictory results for NMs have been observed, likely due to differences in particle properties, exposure conditions, and cell types. Therefore, it is crucial to develop standardized protocols and guidelines for evaluation. Given the complexity of the endpoints and the potential for multiple abnormal epigenetic changes to occur simultaneously, it is important to investigate a range of changes to draw definitive conclusions.

#### 11. *In silico* methods for genotoxicity assessment of NMs

Assessing NM risks is challenging owing to the impracticality of testing all nanoforms *in vivo* or *in vitro*, given their diversity and rapid development [146]. Small variations in nano-scale properties can significantly impact toxicity [129]. Computational approaches, traditionally used for chemical hazard assessment, leverage intrinsic properties to predict toxicity [196]. *In silico* modelling has also proven useful for safety assessment of NMs and for identifying descriptors for genotoxicity [2,70,142,241]. Quantitative structure-activity relationship (QSAR) models are particularly valuable in this context, predicting biological activity and aiding in identifying potential biomarkers by assessing NM interactions with biological targets [18,28,65,70,110,142].

While QSAR models offer a cost-effective, rapid alternative for NM genotoxicity assessment, they are not stand-alone solutions and should complement other methods [18]. The Weight-of-Evidence (WoE) approach, integrating *in vitro*, *in vivo*, and *in silico* data, is crucial for comprehensive NM genotoxicity assessment, ensuring more accurate risk evaluations. The REACH advocates combining *in vitro*, *in chemico* and *in silico* models [65] to predict toxicity based on stressor features. Grouping and read-across, supported by hypotheses and scientific justification, enable virtual screening by assuming that similar structures will likely exhibit comparable hazards, aiding predictions for untested substances [18,70,110,142].

Advanced computational models, such as physiologically based PBPK, combined with *in vitro* to *in vivo* extrapolation (IVIVE), further enhance the ability to predict NM distribution in a biological system [116]. The properties of NMs depend not only on their chemical composition and structure, but also on the surrounding environment. Consequently, the attainment of reliable and reproducible outcomes from *in vivo* and *in vitro* dosimetry studies is crucial for the creation of a reliable PBPK model. DeLoid et al. [53] have developed an approach to standardize NM testing, which involves three key elements: 1) reaching stable NM suspensions in the cell culture medium; 2) a comprehensive colloidal analysis of the suspended NMs with particular emphasis on the properties that affect particle dynamics in *in vitro* environment, including the size distribution and effective density of agglomerates; and 3) accurate measurement of the concentration of NMs transported into cells over the course of the *in vitro* exposure, using robust numerical fate-and-transport modelling. The outcome of research conducted using this protocol is a one-dimensional distorted grid (DG) model that estimates the delivered dose of metal oxide NMs suspended in culture medium [54]. Halder et al. [99] developed a perturbation approach based on a quantitative structure-toxicity relationship (QSTR) model for predicting genotoxic effects using diverse biological targets under various



experimental assay conditions of NM-NM pair cases. Another comparative approach using a self-organizing map to predict genotoxicity based on comet assay for silicon and metal oxide nanoparticles was presented by Sizochenko et al., [207]. There are numerous studies in the present literature correlating NM structure and genotoxicity. For instance, Golbamaki et al. [92] developed a nano-QSAR model for metal oxide NMs based on quantum chemical properties, using the comet assay for validation, but noted challenges in data consistency. Similarly, Kotzabasaki et al. [126] described a QSAR model for MWCNTs, integrating *in vitro* and *in vivo* data to predict genotoxicity. In a similar study, a QSAR model was used for the assessment of several reference NMs from the EU Joint Research Centre (JRC) using data from the *in vitro* comet assay in two cell lines [70]. The aforementioned models employ different descriptors to predict genotoxicity, reflecting the distinct mechanisms of action of various groups of NMs.

### 11.1. Challenges, limitations and advantages of *in silico* methods for genotoxicity testing of nano- and advanced materials

For regulatory applicability, rigorous validation of QSAR-like models against experimental data is essential but often challenging due to data limitations [178]. It is also of great importance to choose relevant biological endpoints to predict NM toxicity with computational approaches for human health risk assessment [85] as the use of prokaryotic cells (e. g. bacteria) is questionable in terms of their relevance to human health risk assessment.

Also, it is not feasible to create a universal model that could serve as a standalone NAM, as different descriptors to identify genotoxicity of NMs have been revealed. However, *in silico* models, given their predictive capabilities, can be utilized for screening and prioritizing NMs for further research. The advancement of these methods is contingent upon the accessibility and quality of genotoxic data, already identified as a challenge in several studies [92,126].

Promising *in silico* NAMs are integrated into the nQTB tool (<http://nqtb.app>) to predict results of *in vitro* micronucleus and comet assays for nanoforms of titanium and silicon dioxides. The data used for calibration of QSAR models in these tools have been rigorously verified according to European Food Safety Authority (EFSA) criteria [68], allowing comprehensive genotoxicity screening across various cell lines. These models require basic nanostructure information and adhere to the principles of OECD (Q)SAR Assessment Framework for validation and uncertainty assessment [175,176,178].

## 12. Standardisation and validation

Standard testing approaches for NMs have notable deficiencies, including inadequate physicochemical characterization in both pristine forms and complex matrices, insufficient exposure times, limited demonstration of cellular uptake, and incomplete coverage of genotoxic modes of action [58,125].

A critical step in the development of NAMs for regulatory applications is the formal regulatory validation of the proposed methods, with the aim of assessing the performance, reproducibility and transferability of the methods, as well as applicability domains and technical boundaries. NAMs for testing NMs are evolving and new methods are being developed, validated, and adopted to address emerging scientific advancements and regulatory needs. Several NAMs have been approved as OECD TGs, internationally recognized standards for conducting chemical testing and risk assessment.

In the absence of formal regulatory validation, use of the methods will be mostly limited to research and development activities, with regulatory use limited to expert evaluation e.g. following the OECD GD211 [167] for describing non-guideline *in vitro* methods, the Scientific Committee for Consumer Safety (SCCS) guidance on the safety assessment of nanomaterials in cosmetics [19–21], or expert evaluations such as those conducted by the ECHA Risk assessment committee.

This restriction hinders the broader acceptance of newly developed NAMs within a larger regulatory framework. The primary reason for developing regulatory-validated OECD methods is the Mutual Acceptance of Data (MAD) principle, under which data generated in a Member State using validated models are accepted by other countries, ultimately reducing time and costs associated with safety assessments.

Currently, the OECD is undertaking several specific activities to develop new TGs and GDs for NMs or to adapt existing ones. These ongoing projects, part of the Program on Safety of Manufactured NMs, focus on validating and adopting NAMs for NMs, assessing the reliability, relevance, and their regulatory acceptance for safety evaluation. In addition to toxicity testing, OECD projects also address the standardization of methods for characterizing the physicochemical properties of NMs, including size, shape, surface chemistry, and aggregation/agglomeration behaviour (Table 3). Appropriate characterization is crucial for toxicity testing of NMs. Harmonized characterization methods are essential for ensuring reproducibility and comparability of data across different studies and laboratories.

While some existing *in vitro* genotoxicity TGs for conventional chemicals can be used for NMs, not all are suitable (e.g., the Ames test) and others such as the micronucleus assay may need adaptation (an ongoing process). OECD projects also address the validation of *in vitro* genotoxicity tests for NMs, including assays for DNA damage,

**Table 3**

Validation status of methods to characterise nanomaterials for physicochemical properties, fate and dosimetry related to human hazard assessment.

Validation status	Methods	References
OECD approved Test Guidelines (TGs)	Test No. 124: Determination of the Volume Specific Surface Area of Manufactured Nanomaterials	OECD [174]
	Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials	OECD [179]
	Test No. 126: Determination of the Hydrophobicity Index of Nanomaterials Through an Affinity Measurement	OECD [180]
	Test No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media	OECD [168]
OECD approved Guidance Documents (GD)s	GD No 328 Guidance Document for the testing of dissolution and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies	OECD [171]
Under validation by OECD	No. 382 Study Report on Applicability of the key event-based TG 442D for <i>in vitro</i> skin sensitization testing of nanomaterials	
	Project 1.6: GD Identification and quantification of the surface chemistry and coatings on nano- and microscale materials	
	Project 1.8: TG on Determination of the Dustiness of Manufactured Nanomaterials	
	Project 1.10: GD on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies	
	Project 3.10: TG on dissolution rate of nanomaterials in aquatic environment	
	Project 3.12: New GD on assessing the apparent accumulation potential for nanomaterials	
	Project 3.16: GD Environmental abiotic transformation of nanomaterials	
	Project 4.146: New GD on toxicokinetics to accommodate testing of nanoparticles	
	Project 4.158: New Guidance Document on IATA for intestinal fate of orally ingested nanomaterials	
	Update of the Guidance Document on Sample Preparation and Dosimetry (GSPD) Grouping of Nanomaterials	

Adapted from OECD Test Guidelines, work program and ECHA report [115].

mutagenicity, and chromosomal aberrations. Also, several advanced *in vitro* models with genotoxicity endpoints are currently undergoing validation (Table 4).

OECD TGs for *in silico* models, such as QSAR models, are also currently being developed and validated for predicting chemical properties, toxicity endpoints, and environmental fate, providing alternatives to experimental testing.

The OECD project Development of Integrated Testing Strategies (ITS) for Nanomaterial Safety Assessment focuses on the development and validation of ITS that combine multiple NAMs to assess comprehensively the safety of NMs. These ITS may include assays, models, and physico-chemical characterization techniques to evaluate various toxicological endpoints.

Several European projects such as NanoHARMONY, PATROL, Risk-GONE, NANOMET and others have contributed to the development, standardisation and validation of NAMs for testing NMs. Extensive interlaboratory pre-validation studies were conducted to demonstrate the reliability, relevance, and reproducibility of the developed NAMs. Through these efforts, a large amount of data has been generated and included in the open database supporting the development of standardized, reliable, and accepted NAMs for the testing and regulation of NMs.

### 13. Conclusion and outlook

Despite their many advantages, NAMs also present challenges, including the need for standardization, validation, and acceptance by regulatory authorities. The ECHA, the U.S. Environmental Protection Agency (EPA), SCCS, EFSA and other regulatory agencies and risk assessment committees recognize the importance of using NAMs and alternative testing approaches to predict and mitigate the risks associated with NM-induced adverse effects, promoting the development and adoption of innovative methods in regulatory frameworks.

Advancing nano-specific NAMs is a highly complex process, necessitating the adaptation of validated methods designed for standard chemicals for use with NMs. The most advanced NAMs include RSMN and RS comet assay.

Prioritization is needed for specific organs such as the lung, liver, and gastrointestinal tract, with a focus on genotoxicity as an endpoint to be measured with these advanced models. As highlighted in a recent survey on NAMs for NMs [115] for regulatory acceptance and EU implementation, key measures include:

- Adapting exposure scenarios for diverse NM exposure routes,
- Adjusting test systems to mimic human biology,
- Developing *in vitro* exposure protocols considering NM behaviour,
- Creating methods for characterizing NMs in pure form and culture medium,
- Using existing data and databases to support *in silico* method validation.

Addressing these challenges requires collaboration among scientists, regulators, industry stakeholders, and advocacy groups to ensure the reliability and robustness of NAMs in risk assessment. Continued research towards development of NAMs for NMs and advanced materials, their validation, and adoption by OECD will further enhance their effectiveness and pave the way for a more sustainable and ethical approach to risk assessment.

NAMs can play a crucial role in future IATA by providing alternative, mechanistic insights into genotoxicity while reducing reliance on animal studies. In NGR, advanced *in vitro* and *in silico* models, and AOP-driven IATA strategies applied in a tiered manner can establish reliable safety margins for NMs and advanced materials. However, to ensure regulatory acceptance, further efforts are needed to refine nano-specific NAMs that capture primary and secondary genotoxicity mechanisms and align with international initiatives such as OECD programs and the PARC project.

**Table 4**

Validation status of New approach methods (NAMs) and their suitability for the assessment of NM-induced genotoxicity.

Validation status	Assays/Methods	References
OECD approved test guidelines (TGs)	*OECD TG 476 - <i>In vitro</i> Mammalian Cell Gene Mutation Tests using the <i>Hprt</i> and <i>xprt</i> genes *OECD 490 - <i>In vitro</i> Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene *OECD TG 487 - <i>In vitro</i> Mammalian Cell Micronucleus Test *OECD TG 473 - <i>In vitro</i> mammalian chromosomal aberration test	OECD [165] OECD [166] OECD [181] OECD [164]
OECD Guidance documents (GDs)	OECD GD No 359: <i>In vitro</i> micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials	OECD [173]
Under validation by OECD	*Project 4.125: New TG: ToxTracker assay: a stem cell-based reporter assay for mechanistic carcinogenicity hazard assessment *Project 4.139: <i>In vitro</i> genotoxicity testing for dermal exposure using 3D skin models: reconstructed skin micronucleus test and reconstructed skin Comet assay *High content imaging of DNA double strand breaks DSBs (phosphorylated H2AX staining) *Project 4.139: Update of TG 489 Comet Assay for gonadal cells to study germ cell specific genotoxic effects	
NAMs under development for chemicals and nanomaterials	Alkaline single cell gel electrophoresis in A549 cells Alkaline single cell gel electrophoresis in THP-1 cells <i>In vitro</i> Comet assay modified with lesion specific enzymes for chemicals including nanomaterials CD59 gene loci mutation assay <i>in vitro</i> FE1 Muta™ Mouse Lung Epithelial Cell Line gene mutation assay Secondary genotoxicity testing by using <i>in vitro</i> methods based on conditioned media or co-cultures Comet assay on 3D HepG2 spheroids for chemicals including nanomaterials Micronucleus and comet assay on air liquid interface lung model for chemicals including nanomaterials Micronucleus assay on 3D HepG2 spheroids for chemicals including nanomaterials <i>In silico</i> predictions of responses in <i>in vitro</i> micronucleus tests for TiO <sub>2</sub> NMs (separate simulations for 16 cellular models) <i>In silico</i> predictions of responses in <i>in vitro</i> comet assay for TiO <sub>2</sub> NMs (separate simulations for 23 cellular models) <i>In silico</i> predictions of responses in <i>in vitro</i> micronucleus tests for SiO <sub>2</sub> NMs (separate simulations for 6 cellular models) <i>In silico</i> predictions of responses in <i>in vitro</i> comet assay for SiO <sub>2</sub> NMs (separate simulations for 13 cellular models)	

and ECHA report [115]. \* Tests that might need adaptation for testing nanomaterials.

Adapted from OECD Test Guideline work program [184], H2020 projects (PATROL, RiskGONE, etc.

## CRediT authorship contribution statement

**Gutleb Arno C.:** Writing – review & editing, Writing – original draft, Conceptualization. **Weber Pamina:** Writing – review & editing. **Longhin Eleonora Marta:** Writing – review & editing, Writing – original draft. **Arnesdotter Emma:** Writing – review & editing. **Hardie Olsen Ann-Karin Hardie:** Writing – review & editing. **Puzyn Tomasz:** Writing – review & editing. **SenGupta Tanima:** Writing – review & editing. **Serchi Tommaso:** Writing – review & editing. **El Yamani Noaule:** Writing – review & editing, Writing – original draft. **Grudzinski Ireneusz P.:** Writing – review & editing. **Judzinska Beata:** Writing – review & editing. **Cambier Sebastien:** Writing – review & editing. **Skakalova Viera:** Writing – review & editing, Conceptualization. **Wyrzykowska Ewelina:** Writing – review & editing. **Jirsova Katerina:** Writing – review & editing. **Jagiello Karolina:** Writing – review & editing. **Dusinska Maria:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **McFadden Erin:** Methodology. **Murugadoss Sivakumar:** Writing – review & editing, Writing – original draft. **Shaposhnikov Sergey:** Writing – review & editing, Conceptualization. **Stepnik Maciej:** Writing – review & editing. **Collins Andrew:** Writing – review & editing. **Rundén-Pran Elise:** Writing – review & editing. **Honza Tatiana:** Methodology.

## Declaration of Competing Interest

On behalf of all co-authors I declare that there are no conflicts of interest. All authors reviewed and approved the manuscript.

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## Data availability

No data were used for the research described in the article.

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