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Bioinformatics and machine learning to support nanomaterial grouping

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ABSTRACT

Nanomaterials (NMs) offer plenty of novel functionalities. Moreover, their physicochemical properties can be fine-tuned to meet the needs of specific applications, leading to virtually unlimited numbers of NM variants. Hence, efficient hazard and risk assessment strategies building on New Approach Methodologies (NAMs) become indispensable. Indeed, the design, the development and implementation of NAMs has been a major topic in a substantial number of research projects. One of the promising strategies that can help to deal with the high number of NMs variants is grouping and read-across. Based on demonstrated structural and physicochemical similarity, NMs can be grouped and assessed together. Within an established NM group, read-across may be performed to fill in data gaps for data-poor variants using existing data for NMs within the group. Establishing a group requires a sound justification, usually based on a grouping hypothesis that links specific physicochemical properties to well-defined hazard endpoints. However, for NMs these interrelationships are only beginning to be understood. The aim of this review is to demonstrate the power of bioinformatics with a specific focus on Machine Learning (ML) approaches to unravel the NM Modes-of-Action (MoA) and identify the properties that are relevant to specific hazards, in support of grouping strategies. This review emphasizes the following messages: 1) ML supports identification of the most relevant properties contributing to specific hazards; 2) ML supports analysis of large omics datasets and identification of MoA patterns in support of hypothesis formulation in grouping approaches; 3) omics approaches are useful for shifting away from consideration of single endpoints towards a more mechanistic understanding across multiple endpoints gained from one experiment; and 4) approaches from other fields of Artificial Intelligence (AI) like Natural Language Processing or image analysis may support automated extraction and interlinkage of information related to NM toxicity. Here, existing ML models for predicting NM toxicity and for analyzing omics data in support of NM grouping are reviewed. Various challenges related to building robust models in the field of nanotoxicology exist and are also discussed.

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1. Introduction

Engineering physicochemical properties of nanomaterials (NMs) such as size, morphology or surface chemistries has become common practice in order to meet the needs of specific applications. This has resulted in a large variety and a steadily increasing number of different NMs or nanoforms (NFs), as defined in specific regulatory frameworks (EC 2006). However, adjusting NM physicochemical properties does not only impact their desired functionalities but the fine-tuning also influences their original or expected behavior in a biological milieu including, their uptake by cells, biodistribution, dissolution rate and/or toxicity to humans or the environment, and more. To overcome the need to fully characterize each and every NM variant for all possible toxicological outcomes, the European chemicals legislation REACH (EC 2006) allows for grouping and read-across, to either justify waiving specific tests or to fill in data gaps.

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For chemicals, grouping is well established as a 'general approach for considering more than one chemical at the same time' (OECD 2014a; ECHA 2008). The idea behind grouping approaches is that chemicals which are similar enough with respect to certain criteria (e.g. structural, physicochemical properties, etc.) can be considered as a group. Chemicals within one group are then expected to show similar (eco-)toxicological and/or environmental fate behavior. Within this group, data gaps on toxicological behavior for a certain member of the group can therefore be filled by read-across using information from the other members in the group. In general, grouping may support risk assessment as well as Safe(r)-and-Sustainable-by-Design (SSbD) approaches. Groups are established initially on the basis of structural similarity, which can be based on various principles such as common functional groups, precursors, breakdown products or a constant incremental change of the properties of interest across the group (OECD 2014a; ECHA 2017). However, it then has to be demonstrated that these structural similarities result in a similar fate and/or (eco-)toxicity. Thus, knowledge of a common toxic mechanism or Mode-of-Action (MoA) can strongly facilitate grouping, since grouping always requires a proper scientific justification which is mainly supported by establishing a link between specific properties and the toxicological endpoint of interest. In addition, grouping is endpoint-specific meaning that group membership may vary depending on which toxicity endpoint is considered.

In the last decade, several grouping frameworks have been developed for NMs, e.g. the MARINA grouping and read-across approach (Oomen et al. 2015) or the DF4nano Grouping Framework (Arts et al. 2015), which are comprehensively summarized in Oomen et al. (Oomen et al. 2018) and Giusti et al. (Giusti et al. 2019) The most recent and comprehensive framework is the GRACIOUS (Stone et al. 2020) grouping framework. Additional insights into NM grouping in the context of the EU chemicals legislation are detailed in Mech et al. (Mech et al. 2019) The most recent GRACIOUS framework is based on a hypothesis-driven approach. It proposes several grouping hypotheses, which link specific physicochemical properties with specific fates and/or toxicities, tested the hypotheses in case studies (Ag Seleci et al. 2022; Keller et al. 2021; Ruggiero et al. 2022; Jeliazkova et al. 2022a; Cross et al. 2022; Song et al. 2022) and lastly, offers guiding principles to support users to formulate their own grouping hypotheses (Murphy et al. 2023).

Nevertheless, grouping of NMs remains a challenge. In particular, unraveling relationships between specific physicochemical properties and toxicities is not trivial due to the large panel of partially interdependent physicochemical properties that are needed to describe a single NM (Lynch, Weiss, and Valsami-Jones 2014). The number-based particle size distribution, surface functionalization or treatment, shape or morphology as well as surface area are certainly the most central ones (Cosnier et al. 2021; Poulsen et al. 2016). Dissolution rate, state of agglomeration/aggregation and surface reactivity have also been shown to be of high relevance (EC 2018; Gutierrez et al. 2023). However, plenty of other NM properties exist that are not tested and, several of the NM properties are polydisperse already after production and additionally have the potential to change during the life cycle, depending on the environment/biological medium in which they are suspended or incorporated. This renders the physicochemical characterization of NMs both in their dry state and as applied, a complex task. Overall, identifying which physicochemical parameters are driving toxicity remains the key challenge in NM grouping (Ribeiro et al. 2017; Drasler et al. 2017).

Omics approaches are a very promising tool which have already frequently been applied for chemicals including NMs. Different omics layers can be investigated, e.g. levels of gene transcription (transcriptomics), protein abundances (proteomics) or levels of small molecule metabolites (metabolomics). Among them, transcriptomics is by far the most studied omics level for describing the molecular changes induced by NMs. This is mainly due to the fact that transcriptomics technologies are highly advanced, the evaluation is well standardized and interpretation of the results is relatively straightforward due to the well-studied and well-annotated state of this particular level of biological organization (Kinaret et al. 2020; Federico et al. 2020; Serra et al. 2020a) and well-established tools. On the other hand, while proteomics or metabolomics are closer to the actual phenotype, indicating the potential for more direct causal association with the adverse outcome (AO), i.e. endpoint of interest, they are more complex and do not at present have standardized protocols for the analysis and interpretation of data. The OECD reporting frameworks have been established for both transcriptomics (OECD 2021a) and metabolomics (OECD 2021b); however, standardized evaluation and interpretation for metabolomics and proteomics is still lagging behind.

While for now, it is possible to individually assess and report the single omics endpoints (transcriptomics or metabolomics or proteomics), their combined evaluation, which may be important to obtain a holistic view of the biological response to an exposure remains difficult. Thus, developing models that are based on omics data is also consistent with the major shift away from pure consideration of single endpoints toward a more mechanistic understanding which can be observed within the field of toxicology. In general, omics approaches yield the advantage that multiple endpoints are considered at the same time as a whole panel of cellular changes is measured in one single experiment, allowing for investigation of dependencies between events and endpoints.

Within NM experiments, usually omics data for a few materials are obtained and analyzed subsequently (Ma et al. 2023; Nguyen and Falagan-Lotsch 2023). While this provides useful insights into various biological changes in that specific experiment, it is difficult to derive general patterns and to extract which changes are most relevant in terms of grouping NMs with respect to a certain endpoint. Especially, the high-dimensional setting of omics experiments with the number of parameters being much larger than the number of samples is a major challenge. Here, meta-analyses may be very useful to counterbalance this situation. Due to data sharing rules in the community and corresponding journals, huge databases of publicly available datasets exist for different omics levels like the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) (Edgar, Domrachev, and Lash 2002) for transcriptomics or PROteomic IDEntification (PRIDE) database (Perez-Riverol et al. 2021) for proteomics. While these datasets cover a wide range of studied effects such as those cause by drugs, other types of chemicals or diseases, omics datasets should theoretically allow integration across those effects. Such meta-analyses may be beneficial for the detection of broad biological patterns as they are derived from a greater knowledge base and noise occurring in single studies may be canceled out.

Machine learning (ML), a subfield of Artificial Intelligence (AI), may be highly valuable for addressing this task. The greatest advantage of ML is that such models are able to automatically learn patterns in large datasets and derive associated predictions. Generally, data to be modeled is described in a feature vector or matrix. This comprises values for measured input features like physicochemical properties or others. Additionally, a respective outcome variable, describing for instance a certain toxicity outcome can be linked to the input data. ML algorithms range from classical linear or logistic regression models to more complex ones such as Random Forests (RFs) or Support Vector Machines (SVMs) and Deep Learning models like Neural Networks (NNs). The choice of ML algorithm used in a study should be based on the amount and complexity of the available data as well as the specific goal in order to obtain robust trustworthy results. In an optimal dataset for the development of ML models, the number of samples should be much larger than the number of features describing these samples. However, in biological settings this is rarely ever the case. Therefore, the main goal for implementing useful ML models is to find the right balance between model complexity and generalizability – thus, avoiding overfitting. Model complexity means that a model is complex enough to be able to describe a set of so-called training data, but at the same time not too complex in order to be able to predict outcomes for previously unseen test data as well. Also, there is usually a tradeoff between flexibility/power and interpretability/inference capacity of a ML method (Gareth James, Hastie, and Tibshirani 2021; Gareth James et al. 2023). This means that simplest methods are also the most easily interpretable ones, such as linear regression (and variants such as elastic net regression) or decision trees. More complex methods such as RF and SVMs are significantly less interpretable, and the least interpretable models are NNs and the multilayered deep neural networks (deep learning models). Explainable AI (XAI) is a subset of AI and ML techniques focused on making AI systems more understandable, transparent, and interpretable for humans. This has implications also for regulatory toxicology, as models should be maximally interpretable for reasons of transparency and governance.

Again, ML is optimally suited to aid the analysis of omics data for several reasons. Omics datasets are high-dimensional and rich in describing many cellular alterations within each single measurement, in which patterns and relationships can be detected and that may not be visible using traditional statistical methods. However, the high dimensionality of the data can also be challenging. Here, moving from datasets limited to single experiment or materials toward integration of various datasets in a meta-analysis setting may be beneficial. In such cases, ML is well suited to handle the complexity of the datasets, unraveling the hidden interactions

or dependencies between molecular components. ML can also be used to automatically select the most relevant features such as transcripts, proteins or metabolites related to specific molecular events or key event (KE), thereby reducing the dimensionality and improving the interpretability, resulting in identification of potential biomarkers. ML can also directly be used for omics-driven predictive modeling. Additionally, meta-analyses are great tools for handling noise in the data and distinguishing true from random signals (Cervantes-Gracia, Chahwan, and Husi 2022). ML can support integration of multiple omics layers. Finally, a great advantage of ML methods is that they can easily be adapted by re-training once new data become available. Thus, ML models are well suited for reducing the complexity of analysis of omics datasets, revealing the important biological traits perturbed after exposure. Moving forward, once NMs with similar MoAs have been identified, one may investigate corresponding similarities and differences in physicochemical properties and elucidate the most relevant properties for formulating a grouping hypothesis, and also to provide design principles for NMs with reduced hazards. Advances in high-throughput transcriptomics facilitate the creation of large and uniform datasets that are ideally suited for ML and grouping applications as the available technologies reduce the cost of transcriptome profiling by up to 10- to 20-fold (Ye et al. 2018). Coupling together omics and high-throughput screening technologies in a tiered approach increases the granularity and informativeness of the data even further (Grafström et al. 2015). XAI may also facilitates MoA discovery as it helps to interpret the decision made by ML models. In the context of transcriptomics-derived MoAs, tools need to be developed, in part, to conform to XAI principles while maximizing predictive ability (Boyadzhiev et al. 2021).

Some recent reviews have summarized ML models in the NM field (Stone et al. 2020; Lamon et al. 2019; Basei et al. 2019; Furxhi et al. 2020). However, they have not or only scarcely considered the potential of omics data in the context of NM grouping. The aim of this review is to provide an overview of existing ML models that enable complex data integration and analysis in support of NM grouping and to shed light on how omics data may be used in conjunction with ML models in the development of reliable grouping frameworks. The review focuses only on hazard endpoints which are considered relevant under REACH (see Table 1) for human toxicity. ML models for other endpoints like cytotoxicity or ecotoxicity as well as those predicting NM uptake or protein corona formation are excluded from this review.

2. Relevant hazard endpoints

Grouping allows waiving of tests or filling the data gaps related to a target substance by using data from a previously tested source substance. In the European Union, the most important overarching regulation for chemicals is REACH. The Annexes VI to XI of the REACH regulation (EC 2006) specify which information the manufacturers need to

Table 1. Relevant endpoints for human health across multiple legislations (according to Jagiello et al. 2021, with modifications).

Endpoint	Description
Acute toxicity	Adverse effects after single dose, multiple doses given within 24 hours or inhalation exposure of 4 hours
Skin corrosion/ irritation	Irreversible (corrosion) or reversible (irritation) damage to the skin
Serious eye damage/ irritation	Serious eye damage: tissue damage in the eye, or serious physical decay of vision, which is not fully reversible within 21 days Eye irritation: changes in the eye, which are fully reversible within 21 days
Skin sensitization	Allergic response following skin contact
Mutagenicity	Alteration of the structure, information content or segregation of DNA
Carcinogenicity	Induction of cancer or increase of its incidence
Reproductive toxicity	Adverse effects on sexual function and fertility in adult males and females or developmental toxicity in the offspring (before or after birth)
Specific target organ toxicity (STOT) – single exposure	Specific, non-lethal target organ toxicity after single exposure
STOT – repeated exposure	Specific, non-lethal target organ toxicity after repeated exposure
Neurotoxicity	Disruption of the nervous system
Endocrine disruption	Adverse effects connected to endocrine system like developmental malformations, disorders of immune and nervous systems functions or increased cancer risk
Hypersensitivity / food intolerance	Adverse reaction to food including or excluding the immune system
Immunotoxicity	Adverse effects on the structure and function of the immune system
Phototoxicity	Toxic response after exposure to environmental light
Hypersensitivity/ Food intolerance	Adverse reaction of the digestive system to a substance
Toxicokinetics	Behavior of a substance within the body according to ADME (absorption, distribution, metabolism and excretion)
Water solubility or dissolution rate in relevant biological media	Amount of substance that dissolves in water and relevant media like cell culture media or body fluids in a certain time and at a specific temperature

provide when registering a substance. These requirements are dependent on the tonnage of production per year. Thus, higher tonnages lead to more extensive toxicological testing requirements. In order to tackle specific information requirements for NMs, the REACH annexes VI to X have been updated to specifically consider nano-specific information needs (EC 2018). In addition, work is underway to modify or adapt test guidelines in consideration of specific properties and property specific effects of NMs (Rasmussen et al. 2019).

Apart from REACH, other specific legislations exist for chemicals with specific applications, e.g. cosmetics (EC, 2009a), food contact materials (EC 2004) or pesticides (EC, 2009b). All these regulations consider (to a large extent) similar toxicological endpoints. The relevant endpoints for several legislations for NMs have also been collected in the European Union Observatory for NMs (EUON) report on New Approach Methodologies (NAMs, referring to a set of innovative techniques or testing strategies used in toxicology and risk assessment to evaluate the safety of chemicals) for NMs (Jagiello et al. 2022) as well as in a recent publication from Bleeker et al. (Bleeker 2023) which intends to support harmonization of the testing requirements for NMs across EU legislations. An overview on relevant hazard classes for human health under REACH or in at least two other legislations as described in these documents is given in Table 1.

While for some of the above-mentioned endpoints such as skin corrosion/irritation or serious eye damage/irritation, NAMs are well established and NAM data is accepted for regulatory decision making, for other more complex endpoints, NAM development is still on-going. Although a push for replacing animal tests with validated NAMs is in full swing internationally (Schmeisser et al. 2023), to date, toxicological testing for complex endpoints still largely relies on animal studies.

3. NAMs and NAM frameworks

For many chemicals, and especially for NMs, the data for complex endpoints is very scarce and an increasing number of new materials is entering the market every year. Filling the data gaps and testing new materials using animal studies raises not only ethical concerns but is also limited with respect to time and cost efficiency. In addition, human relevance of animal models is frequently questioned and the underlying toxicity mechanisms are often less obvious in animal models. Therefore, NAMs are becoming increasingly important for safety assessment in the light of the 3R principles for reducing, refining and replacing animal studies. Several NAMs exist and are based on *in vitro*, *in chemico* and *in silico* methods. This also includes high-throughput screening, allowing for testing of multiple chemicals at a time and high-content methods like omics approaches that enable comprehensive understanding of the underlying mechanisms (ECHA 2016).

While *in vitro* and *in chemico* methods are targeting the retrospective observation of NM toxicity, *in silico* models are able to predict potential hazards prior to development and testing and thus are well suited for SSbD approaches. Among the most frequently used *in silico* methods are Quantitative Structure-Activity Relationship (QSAR) models. QSAR models establish quantitative relationships between relevant physicochemical properties of NMs and their biological activity. These relationships are represented by mathematical functions in terms of regression models. Various QSAR models have been introduced for NMs and have been reviewed in multiple publications (Ciura et al. 2024; Li et al. 2022; Burello 2017). However, QSARs usually show certain limitations. The mathematical relationship usually holds true for few materials or material classes only. This is called the applicability domain (Roy, Kar, and Ambure 2015). In addition, most QSAR models are developed for simple toxicity endpoints like cytotoxicity due to the fact that modeling complex endpoints sufficiently well is usually not feasible. However, such endpoints are typically not of regulatory relevance. Also, mechanistic explanations on how the descriptors used for QSARs development are related to the outcome are usually missing and thus explainability of such approaches is limited which again leads to the fact that QSARs do not generalize well across different material classes on which they have not been developed. More complex ML models and omics approaches are well suited to overcome these limitations.

One major advantage of some NAMs is that they allow unraveling toxicity mechanisms which may greatly improve hazard and risk assessment in the future. However, single NAMs may not be sufficient to describe an AO. Instead, a battery of NAMs may be required to sufficiently assess an AO. Therefore, NAM frameworks combining multiple individual NAMs are needed.

To date, several NAM frameworks have been developed. Within REACH, one of the most important alternatives relying on NAM frameworks is the concept of grouping and read-across. In grouping,

chemical similarity is assessed in terms of similarity of their physicochemical properties, their toxicity with respect to a certain endpoint as well as other properties which could be relevant in terms of their associated hazard. In case chemicals are similar with respect to all of these properties, they form a group. The main aim is to determine which physicochemical properties are most relevant with respect to the studied endpoint such that for new chemicals, for which no toxicity assessment has been performed so far, one may conclude whether this new chemical is likely to belong to the established group and thus induce similar toxicity or not. For chemicals in general, grouping is already well established and frequently used in regulatory decision making, with structural similarities or common functional groups being some of the key parameters defining similarity. In contrast, for NMs, the situation is much more complex and establishing reliable grouping approaches is not trivial. This stems from the facts that 1) the number of physicochemical properties needed to sufficiently describe a NM is much large and to date, no simple relationship between any single property and the toxicological outcomes has been consistently observed. This may also be due to the polydispersity of NM (or any other particle) in their properties, such that not all particles in one test preparation exhibit the exactly identical properties; 2) the NM physicochemical properties change during the lifecycle and/or in different environments; 3) other tasks such as exposure characterization and dose estimation are an issue; and 4) NMs interfere with some assay components, requiring optimization of existing methods or development of novel methods. All of this has resulted in inconsistencies in results and reporting, leading to an inability to generalize the observed results across NMs of similar properties.

For NM grouping approaches, one of the most critical questions is whether it is sufficient to establish them on the level of intrinsic physicochemical properties like chemical composition, primary particle size or morphology in combination with the toxicity data. Predictive models and grouping approaches based on intrinsic physicochemical properties describing the chemical and physical structures have two major advantages: 1) they can mostly be controlled directly during the production process and 2) many of the intrinsic properties can be measured more easily compared to extrinsic properties, which often require more complex methodologies, which are currently not well standardized. However, intrinsic physicochemical properties alone often seem to be insufficient for determining whether or not NMs behave similar with respect to a certain toxicity endpoint in a reliable manner. Instead, extrinsic properties like hydrodynamic diameter in relevant media, zeta potential or the formation of a protein corona, may be better suited for reflecting the actual biological activity of NMs. Thus, approaches based on such extrinsic properties may be superior compared to approaches based on simple physicochemical properties (Wohlleben, Mehling, and Landsiedel 2023) and they need to be derived to separate distinct NM hazard groups sufficiently well. However, existing grouping approaches of NMs are still not perfect and intrinsic as well as extrinsic physicochemical properties suffer from the fact that the applicability domain usually restricts models to very specific subsets of NMs. Due to all these reasons, NM grouping may be viewed as a complex endeavor and further efforts are needed to develop effective grouping strategies.

Instead of purely relying on overall potency read-outs from *in vivo* or *in vitro* testing, information on common MoAs or toxicity mechanisms may greatly advance NM grouping approaches and can help to justify an existing grouping hypothesis (OECD 2014a). Knowledge on MoAs may also aid some of the aforementioned challenges with implementing reliable NM grouping approaches. Especially, MoAs can provide qualitative information on whether two NMs induce the same kind of changes even if the actual quantitative values are not directly comparable due to challenges in dosimetry and dispersion. In addition, they drastically reduce the characterization efforts as instead of measuring a huge number of physicochemical properties, one only needs to perform very few omics measurements. These measurements are also directly related to induced changes while for the physicochemical properties if and how they are related to toxicity is not clear. Omics measurements may also be helpful to separate different MoAs and thus solve the difficulties for NM grouping that result from the fact that some AOs *in vivo* or *in vitro* may feature different MoAs and in other cases, different MOAs may lead to the same AO. The knowledge of underlying MoAs can then in turn greatly advance two important concepts: Adverse Outcome Pathways (AOPs) and Integrated Approaches to Testing and Assessment (IATAs).

AOPs (Ankley et al. 2010) are conceptual frameworks which aim to causally link certain biological events in a sequential manner, starting from a

molecular initiating event (MIE), inducing multiple KEs and finally leading to an AO. The MIE thereby represents the interaction of a NM with a biomolecule or an event after its first interaction. That is followed by KEs at multiple levels of biological organization that are essential to the disease progression and can be measured, e.g. at the cellular, tissue or organ level. The AO may then be one of the endpoints mentioned in Table 1. Several AOPs have been proposed for chemical induced toxicity and may also be applicable or adaptable to NMs (Halappanavar et al. 2020; Gerloff et al. 2017; Gromelski et al. 2022; Nymark et al., 2021). One such example is AOP173 from the AOPwiki (https://aopwiki.org/aops/173) which describes the development of pulmonary fibrosis after substance interaction with pulmonary resident cell membrane components, which is relevant to NMs (Halappanavar 2023).

Often AOPs are used as a basis to establish IATAs, which are frameworks for evaluating complex hazard endpoints by integrating multiple sources of information for studying various aspects of the toxicity endpoint under consideration (Nymark et al., 2021; Halappanavar et al. 2021b). A proper understanding of the underlying MoA and related AOPs is important for developing reliable IATAs, as they enable identification of the right assays reflecting KEs to be tested. IATAs allow integration of the different assay outcomes and provide information on a potential hazard in a weight-of-evidence manner. Within an IATA, different kinds of NAMs can be combined.

In the following sections, an overview of existing approaches based on ML and omics supporting NM grouping is given.

4. ML And existing ML models for NMs

ML models are well suited for predicting outcomes with respect to NM toxicity and for selecting the most relevant descriptors influencing toxicity. In general, supervised and unsupervised ML models, as well as some mixed types exist. In supervised models, the goal is to map labels assigned to each sample to variances observed in the input data. In the case of NM grouping, these labels are usually representing a toxicity endpoint, e.g. the outcome of an *in vivo* study or an *in vitro* assay. Depending on the nature of this response variable, ML models can be divided into regression models in which the outcome variable is continuous and classification models with discrete outcome variables. Instead, unsupervised models use unlabeled data and thus, only rely on variances in the input data and seek to find patterns therein. Additionally, semi-supervised methods and reinforcement learning exist but are less frequently used. Semi-supervised ML methods combine labeled and unlabeled data thereby increasing the dataset size which can be used for training without the need for further labeling which might be time- or cost-intensive.

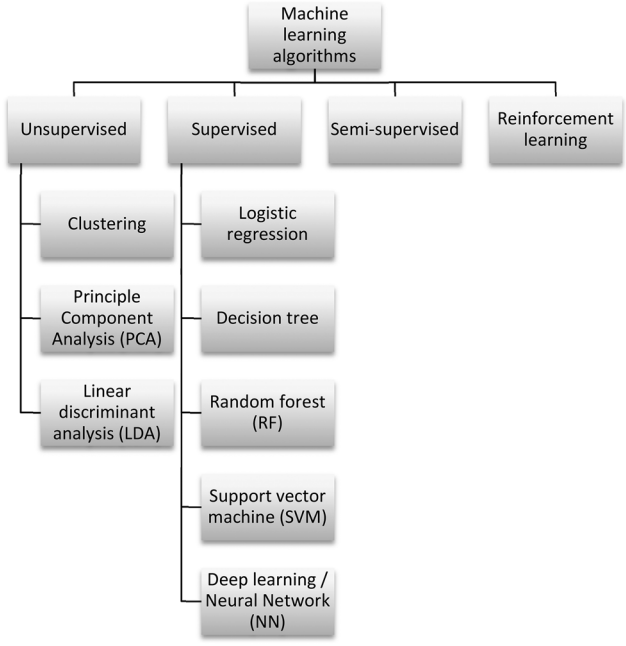

Figure 1. Overview on common ML algorithms.

In reinforcement learning an agent learns an optimal strategy for choosing actions in a way that the reward obtained for a sequence of actions is maximized. An overview of frequently used ML algorithms (Gareth James, Hastie, and Tibshirani 2021; Gareth James et al. 2023) is given in Figure 1.

To identify relevant studies, a search in Scopus using the following search query: TITLE-ABS-KEY ((*nanoparticle* OR *nanomaterial OR nanoparticles* OR *nanomaterials*) AND (*'in silico'* OR *computational* OR *'machine learning'* OR *'case study'*) AND *toxicity*), was conducted. This search matched 988 publications of which 657 publications were tagged as primary publications (Stand: 11/2023). From these publications, those using a ML model to predict one of the relevant endpoints in Table 1 were identified. In addition, relevant studies from previous reviews on ML models in the NM field (Stone et al. 2020; Lamon et al. 2019; Basei et al. 2019; Furxhi et al. 2020) were also added. An overview of the relevant approaches is provided in Table 2.

Except for one study, all identified approaches concentrate either on mutagenicity and genotoxicity or on STOT as the modeled endpoint. In addition, almost all models include supervised approaches. Most frequently, tree-based approaches, namely decision trees or RFs, are used to predict toxicity. Often, these are preceded by an unsupervised analysis using hierarchical clustering or Principle Component Analysis (PCA). Overall, the predictive performance of the models was found quite good (0.7 to 1.0). However, it is also easily visible from Table 2 that the number of available datasets is very small in most cases and usually not sufficient to build robust ML models. In addition to predicting toxicity outcomes, many studies also perform feature selection to reduce the model to only the most relevant descriptors. From Table 2, it becomes obvious that the selected descriptors vary largely across studies. Thus, even though most models show relatively high predictive performance, it may be expected that their applicability domain is rather limited and that they may be expected to be overfitted for the training data and therefore not generalize well to new datasets. With respect to selected materials, there is a strong focus on metal oxide NMs and multi-walled carbon nanotubes (MWCNTs). Other materials are not sufficiently covered so far and thus, cannot easily be assessed with the available models. As properties by which different material classes and materials of different shapes can be described may differ, integrating various types of NMs in a common model is not straightforward.

5. Omics approaches revealing NM MoA

As described before, omics data have the potential to support NM grouping approaches by informing about underlying MoAs and induced AOPs. Thus, this review also focuses on predictive models for NM toxicity which include omics data as descriptors as well as other omics approaches which may potentially support NM grouping. Therefore, all omics-based tools and approaches were searched and below, important primary NM omics studies are described.

Several studies have been performed to describe the changes induced by NMs on the level of transcriptomics. Various *in vitro* (Bajak et al. 2015; Boyadzhiev et al. 2021; Snyder-Talkington et al. 2015) as well as *in vivo* (Halappanavar et al. 2015; Chézeau et al. 2018; Poulsen et al. 2021; Decan et al. 2016; Gosens et al. 2021; Solorio-Rodriguez et al. 2023) approaches have been described. Although scarcer, literature on the effects of NM treatment on other omics layers also exists. For proteomics, mass spectrometry gives the most comprehensive results and is therefore frequently used nowadays (Gallud et al. 2020; Torres et al. 2022; Billing et al. 2020; Miranda et al. 2022). Also, metabolomics changes are addressed in multiple studies (Bannuscher et al. 2020a; Cui et al. 2019; Chen et al. 2019). In addition, some approaches considered multiple omics layers at the same time (Bannuscher et al. 2020a; Karkossa et al. 2019; Nymark et al. 2015; Seidel et al. 2021; Aragoneses-Cazorla et al. 2022; Dekkers 2018). While this is not a comprehensive list of available studies, it is already clear, that these several omics datasets shed light on molecular changes induced by various NMs *in vitro* and *in vivo* from different perspectives, using different omics layers, techniques and methods, cell models, species and so on. The main question that remains is, how to use this existing information to support NM grouping. An overview of predictive models as well as other useful approaches will be given in the next section. In addition, the different obstacles rendering the development of omics-based models non-trivial are discussed.

In general, the biological model, the exposure pattern as well as the detection method for the respective omics layer may largely impact the result of a study. *In vivo* studies naturally represent the gold standard as they reflect the biological complexity present in animals and can inform about long-term effects. Depending on the use of the NM under study different exposure scenarios may be

Table 2. Predictive ML models for NM toxicity and grouping in mammals or mammalian cell models.

Modeled endpoint	References	Study type	Model type	Feature selection performed	Computational validation performed	Study design / dataset	Selected descriptors for best model	Predictive performance of best model
Germ cell mutagenicity	(Lamon et al. 2018)	*In vitro*	Hierarchical clustering, PCA, RF	Gini index	/	6 TiO_2, testing Read-Across Assessment Framework (RAAF) workflow, read-across for Comet assay	Content of organic matter, total non-TiO_2, biodurability in different media	/
	(Aschberger et al. 2019)	*In vitro / in vivo* (different rodents)	Hierarchical clustering, PCA	PCA loadings	/	19 MWCNTs, testing RAAF workflow, different genotoxicity assays (Comet, micronucleus) *in vitro* and *in vivo*	Length, specific surface area, combustion elemental analysis of hydrogen and nitrogen	/
	(Murugadoss et al. 2021)	*In vitro*	PCA, regression and RF	/	Trainings and test set	TiO_2 case study	Agglomerate size	$R^2 = 0.658$
	(Kotzabasaki et al. 2021)	*In vitro / in vivo* (different rodents)	PCA, SVM, RF, Logistic regression, Naïve Bayes	Recursive Feature Elimination	Trainings and test dataset (Kennard-Stone algorithm)	15 MWCNTs from literature	Length, zeta potential, purity, polydispersity index	Accuracy = 0.8
	(Sizochenko et al. 2019)	*In vitro / in vivo* (Wistar rats)	SVM, Naïve Bayes, k-nearest neighbors (kNN), and Decision Trees (DT), Self-organizing maps (SOM)	/	/	21 metal oxide NMs, genotoxicity in Comet assay and Ames test	Electronegativity of the metal and the charge of its ions	Balanced accuracy = 0.75 for Comet assay and 0.83 for Ames test
	(Ambure et al. 2020)	*In vitro / in vivo* (different rodents)	LDA, RF	Genetic algorithm (GA) and best subset selection (BSS)	Internal (training set) and external (test set) validation, Y-randomization test and 10-fold cross-validation for RF	7 metal oxides nanoparticles (SiO_2, ZnO, TiO_2, CuO, Fe_2O_3, Fe_3O_4, Al_2O_3,) classification-based multi-tasking QSAR model predicting genotoxicity	Concentration exposed, core size of nanoparticle, exposure time, experimental protocols, and cell lines + 5 calculated descriptors	Accuracy = 0.94
	(El Yamani et al. 2022)	*In vitro*	PLS			Seventeen NMs derived from TiO_2, ZnO, Ag and SiO_2, Comet assay	Difference between highest occupied and lowest unoccupied molecular orbital energy energy, ionization potential, highest occupied molecular orbital energy, pristine size	
	(Halder, Melo, and Cordeiro 2020)	*In vitro*	Perturbation theory ML (PTML) based QSTR approach		Training and test set + external validation data	78 metal oxide NMs, *in vitro* Comet assay	Number of heavy atoms and the size of the NMs, graph density	Accuracy = 97.81%
Reproductive toxicity	(Ban et al. 2018)	*In vivo* (different rodents)	RF regression, Similarity network		Training data sets (from 20% to 100%) were randomly selected from original data sets, with 10 repetitions	Based on public data for male rodents; CNTs, and Ag and TiO_2 NPs		$R^2 = 0.624$, Root Mean Squared Error (RMSE) = 0.198
STOT – single exposure	(González-Durruthy et al. 2019)	*Ex vivo* (Wistar rats)	LDA and ANN based on linear and non-linear classification algorithms		Training and validation set	Prediction of mitotoxicity in rat kidney, 9 types of carbon nanotubes (CNTs), based on fractal SEM nanodescriptors		Specificity = 99.5% and sensitivity = 99.2%

(*Continued*)

Table 2. Continued.

Modeled endpoint	References	Study type	Model type	Feature selection performed	Computational validation performed	Study design / dataset	Selected descriptors for best model	Predictive performance of best model
STOT – repeated exposure	(Gajewicz et al. 2018)	*In vivo*	Decision tree model	Gini importance	Training and test sets, multiple splits	19 NM, No Observed Adverse Effect Concentration (NOAEC) from Short-term Inhalation Study (STIS), protein carbonylation and intrinsic oxidative potential (Ferric reducing ability of the serum assay)	Size, surface area, presence of a coating	Balanced accuracy = 0.8 for protein carbonylation and 1.0 for intrinsic oxidative potential
	(Bahl et al. 2019)	*In vitro / in vivo* (Wistar rats)	PCA with kNN, RF	RFE	LOOCV	11 NM (mainly SiO_2), predicted outcomes: macrophage assay and STIS, classification into active and passive	Zeta potential, redox potential and dissolution rate	Balanced accuracy = 0.82
	(Bahl et al. 2020)	*In vitro / in vivo* (Wistar rats)	Prediction model based on multivariate logistic regression with Firth's bias reduction method	Greedy search	LOOCV	14 NM from various material classes, categorization based on surface reactivity, predicted outcomes: macrophage assay and STIS, classification into active and passive	Carbonylation assay potentially in combination with either Ferric reducing ability of the serum assay or electron spin resonance assys with 5,5-dimethyl-1-pyrroline-N-oxide	Balanced accuracy = 0.89 for the macrophage assay and 0.83 for STIS
	(Marvin et al. 2017)	*In vitro / in vivo* (different rodents)	Bayesian network (network structure based on expert judgment)	/	Validated with independent data	Metal- and metal-oxide NMs, based on international expert consultation and the scientific literature (e.g. *in vitro / in vivo* data)	Elemental composition, surface coating, surface area, aggregation and particle size	Prediction accuracy = 0.72 for hazard potential and 0.71 for biological effect
	(Sheehan et al. 2018)	*In vitro / in vivo* (different rodents)	Bayesian networks (combined with weight-of-evidence with MCDA (multi-criteria decision analysis) methodology)	/	Validated with independent data	See Marvin et al. (Marvin et al. 2017)	See Marvin et al. (Marvin et al. 2017)	Prediction accuracy = 0.67
	(Drew et al. 2017)	*In vivo* (different rodents)	Dose-response-modeling, hierarchical clustering, RF regression (for initial potency class assignment) and RF classification (for prediction)	Mean squared error	External validation set	Data collected from 25 *in vivo* studies, various materials, pulmonary inflammation in rodents, materials classified into four potency groups	Density, surface area, and diameter	Balanced accuracy = 0.8
	(Furxhi and Murphy 2020)	*In vitro*	RF	Information gain analysis	k-fold cross validation and external validation set	Neurotoxicity prediction	Exposure dose and duration, toxicological assay, cell type, zeta potential	Accuracy = 0.98
	(Gernand and Casman 2014)	*In vivo* (different rodents)	Regression trees and RF	Mean variance reduction	10-fold cross-validation	Meta-analysis across 17 CNTs, polymorphonuclear neutrophils, macrophages, lactate dehydrogenase, and total protein modeled separately; reflect immune response and cell membrane damage and death	polymorphonuclear neutrophils + macrophages: median diameter, mass mode aerodynamic diameter, and cobalt content; lactate dehydrogenase: short median length, cobalt content; total protein: geometric variables, median length and median diameter, and mass mode aerodynamic diameter	R^2 between 0.83 for neutrophils and 0.95 for total protein
	(Gernand and Casman 2016)	*In vivo* (different rodents)	RF, Monte Carlo resampling technique	Mean variance reduction	Out-of-bag error	Meta-analysis of pulmonary nanoparticle toxicity, concentration of LDH or the number of PMNs in bronchoalveolar lavage fluid; CNT, TiO_2, SiO_2, ZnO, MnO	Varies with modeled material classes, total mass more important than all physicochemical parameters	R^2 = 0.97

most relevant. For occupational exposure, the inhalation route is frequently considered to be most relevant. For medical applications, direct injection of the NMs may also be important. Within one exposure route, the actual exposure scenario might also impact the results. As an example, studies targeting the inhalative route of exposure may be performed using inhalation, instillation or pharyngeal aspiration exposure as well as single or repeated doses. While repeated doses in an inhalation setting may reflect the natural occupational exposure more closely, other methods like instillation have the advantage of better control of the actual delivered amounts of particles (Kinaret et al. 2017a).

While *in vivo* approaches may yield highly valuable results, they are time- and cost-intensive and require ethical approval for each study performed. Therefore, avoiding and replacing *in vivo* methods whenever possible has been a major endeavor in the past years. *In vitro* methods form one important pillar in this replacement. Naturally *in vitro* models can only reflect complex biological processes partially. However, as long as these models show high predictivity for the true toxicological impacts, they are still sufficient for testing purposes. This predictivity may critically depend on the chosen cell model under study. Here, cell types which are expected to be in direct contact with the NMs like lung macrophages may be expected to be most predictive. Also, the carcinogenic nature of most cell lines should be regarded as these cells might react substantially different from healthy cells in the organism.

Another advantage of *in vitro* omics studies is that they can be performed in a high-throughput manner and thus allow for testing of a large number of NMs. Here, the investigation of different omics layers may lead to information complementing each other. Optimally, the data on different omics layers should be derived from the very same cells in a multi-omics setting. Time points for the different omics layers should also be chosen carefully. Here, as an example transcriptomics changes are expected to happen much faster than proteomics changes and thus the time points for sample collection should differ. Also, the choice of analysis method and devices used may drastically change omics results. Here, more recent methods usually aim at untargeted assessment of all analytes available in a sample while older techniques focused on pre-defined analytes in a targeted manner.

As mentioned previously, one of the major advantages with respect to omics data is that almost all journals require study authors to make the raw datasets belonging to a publication available. Therefore, a large amount of data is available in public databases, e.g. NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) for transcriptomic data or PRIDE archive (https://www.ebi.ac.uk/pride/archive/) for proteomic data. As ML models require large datasets with respect to the number of samples, reuse of this data to train or test models is of great value for developing robust approaches and has frequently been applied. In addition, meta-analyses of several omics-based studies may also broaden the understanding of molecular mechanisms and MoAs of NMs. Predictive ML models and other useful tools developed in the field of omics-based NM grouping are summarized in Tables 3 and 4, respectively.

From the literature review performed here, we identified a few models that aim to predict NM toxicity or grouping based on omics measurements (see Table 3). The models use different ML algorithms to predict either *in vitro* or *in vivo* outcomes or to directly suggest grouping on NMs. All models yield high predictive performances of at least 0.7 for the validation set. To reduce the number of descriptors, two studies use feature selection (Yanamala et al. 2019; Fortino et al. 2022) for reducing the parameters included in the final model thereby improving explainability.

In addition, multiple other tools have been developed or used to support omics-based analysis of NM toxicity. Kohonen et al. (Kohonen et al. 2017) developed a 'predictive toxicogenomics space' (PTGS) tool which yields a predictive signature for drug-induced liver injury (DILI). This tool has also recently been applied successfully to NMs (Kohonen et al. 2019). Serra et al. (Serra et al. 2019) created the INSIdE NANO tools which contextualizes transcriptomic changes of NMs with those induced by drugs, other types of chemicals and diseases. A similar approach is followed by Bahl et al. (Bahl et al. 2023) who developed PROTEOMAS, a harmonized proteomic workflow which is applied to a case study in a very similar fashion. In addition, tools calculating benchmark doses (BMDs) based on omics data are also useful to support NM toxicity evaluation and grouping. Proposals for such tools have been made by Halappanavar et al. (Halappanavar et al. 2019), Gromelski et al. (Gromelski et al. 2022) and Serra et al. (Serra et al. 2020b). Others have attempted to link physicochemical properties of NMs to observations of changes in omics data. Kinaret et al. (Kinaret et al. 2017b) used coexpression networks and Bannuscher et al.

Table 3. Predictive ML models for NM toxicity and grouping based on omics data.

Omics layer	References	Model type	Feature selection performed	Validation performed	Study design	Predictive performance of best model or proposed grouping
Transcriptomics	(Furxhi et al.)	Bayesian network		Internal 10-fold cross-validation + external test set + reliability validation set	• Eight different types of NM, certain pathways included into BN • Biological system: various human cell lines • Exposure: multiple concentrations and different time points	Depending on the endpoint, reliability ~0.9 – 1.0 for the test set and 0.7-0.9 for the validation set
Proteomics	(Yanamala et al. 2019)	HCA, LCA, L1-l2-norm, SVM, t-test	RFE	External validation set	Carboneous NMs, pulmonary toxicity (BALF) in mice • Biological system: BALF of male C57BL/6J mice • Exposure: oropharyngeal aspiration, two doses, two time points	Prediction accuracy = 0.9 – 1.0
	(Billing et al. 2020)	Fuzzy c-means algorithm, PCA			Fe_3O_4 nanoparticles doped with increasing amounts of cobalt, modeling inflammation, cytotoxicity and genotoxicity Biological system: BALF of female C57BL/6J mice Exposure: intracheal instillation, single dose regimen, two doses, two time points	PCA based on three protein signatures (NP response, NETosis, and NP response) as proposed grouping
Multiomics	(Fortino et al. 2022)	Bayesian information criterion, Similar Network Fusion, Logistic regression, RF	PCA, LASSO, varSelRF, GARBO	External validation set, 70% training and 30% test set	31 industrially relevant ENMs, metals / metal oxides and carboneous materials, mRNA, miRNA, proteins and protein corona, EC10, classification based on cytotoxicity and neutrophil infiltration • Biological system: BEAS-2B, Jurkat and THP-1 cells and BALF of female C57BL/6 mice • Exposure: *in vivo* – oropharyngeal aspiration, repeated dose regimen, one concentration, one time point, *in vitro* – one concentration, one time point	Accuracy ~1.0 for cytotoxicity and ~0.95 for *in vivo* toxicity in the best case

(Bannuscher et al. 2020b) and Karkossa et al. (Karkossa et al. 2019) used Weighted Gene Correlation Network Analysis (WGCNA) (Langfelder and Horvath 2008). Jagiello et al. developed a QSAR models for predicting transcriptomic pathway level response (Jagiello et al. 2021). A complete list of identified approaches with more details is shown in Table 4. In addition, this table also lists several meta-analyses which may provide useful datasets for further model development or validation.

6. The value of AI for supporting NAMs

AI is a subfield of computer science in which algorithms and models are developed to mimic cognitive functions of human intelligence, such as learning and problem-solving. AI can aid risk assessment of NMs in various ways, especially by automating and improving commonly used processes. The major field of classical ML mainly dealing with pattern recognition and predictive modeling has already been introduced in detail above. However, this type of modeling and pattern recognition is only one part of existing AI methods. Other applications of AI may also be relevant for risk assessment of NMs and are briefly introduced below.

6.1. Automated (meta)data extraction and linked data

One important task in risk assessment is to gather all available data on the NM under study. This is a difficult and time-consuming task as data might be spread across various databases, tables or even be only present in scientific publications as unstructured texts. AI may support this task in terms of data and text mining using automated information retrieval and Natural Language Processing (NLP). Different approaches for mining chemical and biological data have been developed in the past. Swain and Cole developed the ChemDataExtractor for automated extraction of chemical information from scientific literature (Swain and Cole 2016). Also tmChem (Leaman, Wei, and Lu 2015) can perform chemical named entity recognition from texts. In addition, CD-REST (Xu et al. 2016) is able to extract chemical-induced disease relations from literature. This automated data extraction may support integration of individual findings across multiple publications which was not obvious previously. Also, relationships between diseases and genes (Lever et al. 2019) or proteins and drugs (Zheng et al. 2019) could be identified by text mining approaches.

Table 4. Additional omics approaches that could support NM grouping.

Omics layer	References	Approach / Model type	Study design	Descriptors suitable for use in NM grouping
Transcriptomics	(Kohonen et al. 2017)	Predictive toxicogenomics space (PTGS) Unsupervised probabilistic component modeling	• Input: transcriptomic data of 1,300 compounds (CMap) and dose-dependent cytotoxicity data (NCI-60) • Prediction of chemically-induced pathological states in liver • PTGS composed of 1,331 genes distributed over 14 overlapping cytotoxicity-related gene space components • Biological system: MCF7, PC3 and HL60 cells • Exposure: one concentration, one time point	Predictive signature may be useful for NM-induced liver toxicity and approach may be used to find predictive signatures for other relevant endpoints
	(Serra et al. 2019)	INSIdE NANO Interaction network	• Input: over 3,000 biological entities including 28 NMs (mainly metaloxides) • Systems biology framework for contextualization of MoA of NMs • Infer knowledge on NMs from drugs, other types of chemicals and diseases • Biological system: various cell lines • Exposure: various exposure patterns	Biosignatures and similarities with other entities within the network
	(Serra et al. 2020b)	BMDx Benchmark Dose (BMD) Modeling	• Input: gene expression matrix and phenotype table • Computes BMDs, related values and IC50/EC50 estimations • Biological system: exemplified on liver tissue of Sprague-Dawley rats • Exposure: oral exposure, single- and repeated dose regimen, three doses, four time points	BMD based on transcriptional changes
	(Serra et al. 2020c)	TinderMix Time- and dose-response modeling for transcriptomics	• Input: Gene log fold changes • Simultaneously models the effects of time and dose on the transcriptome • Fits different integrated time and dose models to each gene, selects the optimal one, and computes time and dose effect map • Genes with time- and dose-dependent response • Biological system: liver tissue of Sprague-Dawley rats • Exposure: oral exposure, single- and repeated dose regimen, three doses, four time points	Identification of genes showing a dynamic (time-dependent) and dose-dependent response; responsive genes labeled according to integrated time and dose point of departure
	(Halappanavar et al. 2019)	BMDt (transcriptional) / BMDneu (apical – neutrophil influx) BMD modeling	• Input: Genome-wide lung transcriptomic responses for 10 different MWCNTs, 9 variants of nano-TiO_2 and one Carbon Black type - BMD values for transcriptional and apical endpoints • Biological system: lungs and BALF of female C57BL/6 mice • Exposure: intratracheal instillation, single dose regimen, two to five doses, one time point	BMD for transcriptional changes used to rank NMs by their potency (median BMD of all or most relevant pathways)
	(Marwah et al. 2019)	eUTOPIA Transcriptomic analysis workflow	• Processing, visualization and interpretation of results • R shiny app • Biological system: lung tissue and BALF of C57BL/6 mice and THP-1 cellsExposure: *in vivo* – oropharyngeal aspiration, repeated dose regimen, one concentration, one time point, *in vitro* – one dose, two time points	Differential gene expression results
	(Gromelski et al. 2022)	Nano-QSAR model	• Input: Length and diameter of MWCNTs • Web-based application that enables to predict the transcriptomic pathway-level response • Predict doses that initiate inflammation • Biological system: lung tissue of female C57BL/6 mice • Exposure: intratracheal exposure, three doses, two time points	BMD(L) value by AOP-anchored nano-QSAR model and expectations with respect to up- and downregulation of genes
	(Halappanavar et al. 2021a)	Review on AOPs	• Systematic review of nanotoxicology literature for identifying KEs of relevance to NMs • Development of nano-AOP database • Case studies using the database to describe KEs for AOPs	Description of effects related to certain KEs, possibility of calculating for instance BMDs
	(Kinaret et al. 2017b)	Coexpression networks	• Correlations between gene expression profiles and physicochemical properties • Significantly correlated genes selected • Gene-gene coexpression networks inferred • Genes in the network ranked based on previous correlations • Three different network response modules extrapolated (5,10 and 20 top-ranked genes) • GO terms progressively enriched across the response modules chosen • Biological system: lung tissue of C57BL/6 mice and THP-1 cells • Exposure: *in vivo* – oropharyngeal aspiration, repeated dose regimen, one concentration, one time point, *in vitro* – one concentration, two time points	List of most important genes and GO terms from coexpression networks and correlations with physicochemical properties

(*Continued*)

Table 4. Continued.

Omics layer	References	Approach / Model type	Study design	Descriptors suitable for use in NM grouping
	(Labib et al. 2016)	Transcriptional BMD	• Input: gene expression profiles for three MWCNTs • Significantly perturbed pathways categorized along KEs in lung fibrosis AOP • Benchmark doses (BMDs) calculated for each perturbed pathway • Overall transcriptional BMDs for each MWCNT derived • Biological system: Lung tissue of male and female C57BL/6 mice • Exposure: intratracheal instillation and pharyngeal aspiration, single dose, three to four doses, three to four time points	Overall transcriptional BMDs for each NM
	(Jagiello et al. 2021)	Nano-QSAR model	• Input: Transcriptomics data • Selection of relevant pathways for modeling • Generation of AOP-informed nano-QSAR model • Applied to MWCNT case study • Biological system: lung tissues of female C57BL/6 mice • Exposure: intratracheal exposure, single dose regimen, three doses, one time point	AOP-informed nano-QSAR modeling approach
	(Saarimäki et al. 2023)	AOP fingerprint	• Multi-step strategy to annotate AOPs is developed • Highlight relevant AOs for chemical exposures with strong *in vitro* and *in vivo* convergence • Supporting chemical grouping and other data-driven approaches • Panel of AOP-derived *in vitro* biomarkers for pulmonary fibrosis identified and experimentally validated • Biological system: lung tissue from male C57BL/6J mice and THP-1, A549, BEAS-2B and MRC9 cells • Exposure: *in vivo* – pharyngeal aspiration, single dose, four doses, four time points, *in vitro* – one concentration, one time point	AOP fingerprint and AOP-driven biomarkers
	(Williams and Halappanavar 2015)	Bi-clustering	• Input: transcriptional data for TiO_2 NMs, CB and CNTs • Meta-analysis on public microarray datasets for pulmonary diseases in mouse models following substance exposure • Similar gene expression profiles identified • Bi-clusters used for gene set enrichment analysis • Determination of disease significance of these data-driven gene sets • Biological system: meta-analysis on various murine lung disease models	Toxicity fingerprints for lung diseases (List of gene sets)
	(Halappanavar et al. 2015)	Transcriptional profiling	• Transcriptional profiling for different TiO_2 NM variants • Determination of differentially expressed genes • Biological system: lung tissue of female C57BL/6 mice • Exposure: intracheal instillation, single dose regimen, three doses, two time points	List of differentially expressed genes (DEGs)
	(Balfourier, Marty, and Gazeau 2023)	Meta-analysis	• Input: 56 GEO microarray datasets on 8 metals and 2 nonmetals • Forward selection of relevant properties • Biological system: various human cell lines	List of DEGs and pathway enrichment; clustering of NMs in meta-analysis
	(Saarimäki et al. 2021)	Meta-analysis	• Manually curated database on transcriptomic profiles on 101 NM-related datasets from GEO and Array express • Biological system: various cell lines and tissues from mice and rats	Curated and FAIRified collection of NM transcriptomics data including their physicochemical characteristics
	(Ghojavand, Bagheri, and Mesrian Tanha 2019)	Meta-analysis	• Publicly available microarray dataset treated by either Ag NPs or Ag ions • Protein-protein-interaction network analysis • Biological system: HepG2, Caco-2, MCF-7 and A549 cells • Exposure: one concentration, one time point	List of DEGs and up- and down-regulated hub genes
	(Nikota et al. 2016)	Meta-analysis, clustering	• Input: Seven toxicogenomics studies on mouse pulmonary responses for CNTs, carbon black, and TiO_2 NPs • mRNA profiles compared to publicly available datasets of 15 other mouse models of lung injury/diseases induced by various agents • Implications of NM-perturbed biological processes to disease pathogenesis in lungs • Biological system: lung tissue of different mouse strains Exposure: intratracheal instillation, inhalation and pharyngeal aspiration, single- and repeated dose regimen, one to four doses, one to seven time points	List of DEGs; clustering of traits in meta-analysis

(*Continued*)

Table 4. Continued.

Omics layer	References	Approach / Model type	Study design	Descriptors suitable for use in NM grouping
Proteomics	(Bahl et al. 2023)	PROTEOMAS Analysis workflow	• Proteomic analysis workflow for harmonized meta-analyses • Exemplified using 25 datasets from PRIDE archive on lung-specific alterations • Biological system: meta-analyses on various cell lines, lung tissue and BALF of humans, mice and rats	List of altered proteins and pathway enrichment; clustering of traits in meta-analysis
	(Basak, Vracko, and Witzmann 2016)	Computation of similarity index	• Input: MWCNTs and TiO_2 nanobelts • Biological system: co-culture of Caco-2/HT29-MTX cells • Exposure: two concentrations, two time points	Similarity index telling how similar abundances of a certain protein are between two conditions
	(Varsou et al. 2018)	toxFlow Model-based connectivity mapping	• Web-tool integrating physicochemical, omics and biology information for read-across prediction • Based on gene set variation analysis • Biological system: Applied in a case study based on 129 protein corona fingerprints for 84 gold NMs to predict cell association with human A549 cells (R_{LOO}^2 = 0.97)	Integrated network of physicochemical, omics and biology information data for read-across prediction
Metabolomics	(Enea et al. 2019)	PCA and OPLS-DA	• Input: Gold nanospheres versus gold nanostars in rat liver • Biological system: liver tissue of Wistar rats • Exposure: intraveneous administration, single dose, one dose, one time point	Discriminating metabolites
Multiomics	(Shin et al. 2021)	PCA and kNN	• Input: 11 NMs with different core materials • Transcriptomics, proteomics and metabolomics • Afterwards 19 drugs applied to see how they affect nanotoxicity effects • Biological system: BV2 cells • Exposure: two concentrations, one time point	Predictive signature
	(Canzler and Hackermüller 2020)	multiGSEA	• Tool to perform sequential gene set enrichment analysis integrating multiple omics layers • Biological system: exemplified on HeLa cells • Exposure: one concentration, one time point	Pathway enrichment across omics levels
	(Bannuscher et al. 2020b)	Weighted Gene Correlation Network Analysis (WGCNA)	• Input: Proteomics and metabolomics for 7 NMs • Biological system: rat lung macrophage cell line NR8383 Exposure: three concentrations, one time point	Correlations between omics and physicochemical properties and identification of key drivers
	(Karkossa et al. 2019)	Weighted Gene Correlation Network Analysis (WGCNA)	• Input: Proteomics and metabolomics for 11 NMs • Biological system: rat epithelial cell line RLE-6TN • Exposure: one concentration, one time point	Correlations between omics and physicochemical properties and identification of key drivers
	(Dumit et al. 2023)	Meta-analysis, RF	• Meta-analysis across publicly available proteomic and transcriptomic data on MWCNTs • Biological system: various cell lines and lung tissue / BALF data from human, mouse and rat	Identification of most discriminant HALLMARK pathways, prediction of expected behavior with respect to inducing a fibre-specific MoA
	(Martens et al. 2018)	WikiPathways	• Make omics data interoperable with the AOP-Wiki	Mapping of omics data to AOPs
	(Cai et al. 2018)	Multi-hierarchical nano-SAR profiling	• Input: Fe_2O_3 NMs • Heatmap correlating metabolomic and proteomic results to seven basic physicochemical properties • Metabolic pathways from MetaboAnalyst software for metabolomics and KEGG pathways for proteomics • Biological system: THP-1 cells • Exposure: one concentration, one time point	Correlations between omics, physicochemical properties and toxicity outcomes

Similar approaches may also be used in the field of nanotoxicology. Especially, with recent developments in the field of Large Language Models (LLMs) this field is expected to be of major relevance in future research. Integration of such LLMs with knowledge graphs may further support data retrieval and storage in a structured way. This may also aid the curation of databases with respect to toxicological results as well as metadata. In addition, linking different databases holding certain information on NMs may also be facilitated by LLMs due to automated recognition of similar terms and alternative naming. As an example, AI may be useful for linking NM-specific information stored in databases like eNanoMapper (Jeliazkova et al. 2015) to omics databases like GEO or PRIDE in an efficient way even if the naming schema and underlying ontologies differ. In the broader context of risk assessment, toxicity information may be automatically integrated with other information such as their intended use or information based on different exposure scenarios. This may also be useful in terms of prioritization of NMs to be investigated and ML models may be developed specifically for this task. Additionally, if trained well, AI models can evaluate multiple risk factors and dependencies between them simultaneously. This may be of high value when evaluating complex mixtures of NMs or chemicals.

6.2. Data curation

Data curation is of utmost importance for developing reliable approaches and models for NM toxicity (Alves et al. 2021). However, if performed manually this is a highly time-consuming task with numerous challenges as shown in various projects before. AI may provide useful tools to facilitate this task (Martín et al. 2023). Here, anomaly detection is one of the most well-known information enrichment techniques which enables identification of outliers or patterns differing from the rest of the data in an automated way. Suitable models may significantly speed-up the identification of inconsistencies in the data. In addition, NLP may also improve the detection of data gaps in registration dossiers for NMs on the market in the context of regulatory process optimization by automated screening strategies. At the same time, recommendations based on historical data may automatically be generated. In addition to advantages in time consumption, AI methods are also less prone to errors compared to humans processing large amounts of data, given that the input data is of high quality. While not every published study can be considered as high quality, AI methods can directly support the identification of quality issues and assess the data quality. Anomaly detection is one very prominent example of data quality checks and is frequently supported by computational tools like Isolation Forests (Liu et al. 2008) or autoencoders (Shvetsova et al. 2021).

6.3. Image analysis

Another field which provides great opportunities for the application of AI is the field of image analysis. Automated image analysis may be enabled by deep learning approaches like convolutional neural networks. An example of such an approach has been provided by Aversa et al. (Aversa et al. 2018) who automatically identified NMs in Transmission Electron Microscopy/Scanning Electron Microscope images and derived their size and number. In addition, Karatzas et al. (2020) used deep learning models to predict the effects of NMs on Daphnia magna. Similar approaches may also be derived from videos instead of images. However, the main challenge for image recognition is that large datasets need to be labeled before training the model.

6.4. Support during omics data analysis

Omics data are especially useful in the context of AI as usually many datasets are publicly available and, in addition, integration of NM-specific data with other chemicals or traits is comparatively easy. Thus, they allow to obtain more comprehensive insights into the underlying biological consequences of NM treatment. One of the most common applications of AI to omics data is the identification of potential biomarkers (Michelhaugh and Januzzi 2022). LLMs, in turn, could also support the interpretation of the identified biomarkers by quickly searching for existing literature and extracting relevant information and context. Similarly, one may also elucidate information on perturbed molecular pathways, affected targets or common patterns induced by treatment with different NMs. These developments are supported by the fact that LLMs like chatGPT can access databases such as GenBank, Ensembl and Gene Ontology, KEGG, Reactome, GEO or ArrayExpress thereby directly being able to connect the various information stored in these resources (Ng et al. 2023). The integration across different omics layers is another field which may be supported by AI. Commonly, ML and Deep Learning

algorithms are applied in the context of multi-omics integration (Picard et al. 2021; Biswas and Chakrabarti 2020). In predictive ML models, omics data may also be used to infer links between physicochemical properties, molecular changes and toxicity which may support regulatory decision making or SSbD strategies. Especially, explainability is an important area of research allowing for new insights into outcomes of AI models thereby probably enhancing their acceptance in the field of toxicology (Santorsola and Lescai 2023).

Apart from all these advantages, AI models are highly dependent on the quality and amount of underlying data. Training AI models requires large datasets of high-quality data which are relevant for the studied question and are not subject to biases. One important limiting factor is data availability. Therefore, the implementation of the FAIR (findable, accessible, interoperable and reusable) principles is a key factor in the development of reliable AI models (Jeliazkova et al. 2021).

7. Remaining challenges and future requirements

7.1. Data availability

The first and most critical challenge in developing robust NM grouping frameworks is the limited availability of data. Any grouping approach can only be generalizable to different kinds of NMs if it was developed based on NMs belonging to different classes and property combinations. This requires many datasets to be available and to be comparable to each other in a way that they can be integrated. Especially, ML models can only make robust predictions if they have been trained on large sets of data containing as much variability as possible (Duan, Edwards, and Dwivedi 2019). Although high-throughput omics technologies are facilitating the generation of ever larger training data sets even by academic researchers, no single project will be able to generate sufficiently extensive datasets, and data reuse is the only option. In order to allow proper data reuse, the FAIR data principles (Jeliazkova et al. 2021; Wilkinson et al. 2016) should be considered when publishing any results. Briefly, the principles refer to e.g. clear and harmonized use of persistent identifiers, and that data as well as corresponding metadata can easily be found by both humans and machines at the same time. Data should also be accessible, either openly or through means of authorization or authentication depending on the need for restriction or not. Furthermore, data should be interoperable in order to allow for integration across highly diverse data sets. Overall, the principles guide in terms of making data reusable in the sense that descriptions of the data and metadata are sufficiently detailed to allow for reproducibility, automated assessments of, for example, data quality and large-scale integration allowing for novel interpretation opportunities.

7.2. Unique identification of NMs

Another central question in the case of NMs, which is directly related to the FAIR data principles, is how to uniquely identify a specific NM. Each NM has a multitude of different physicochemical properties which may also change during their lifecycle. Small changes in few of these properties may have large impacts on the toxicity outcome. Even for benchmark materials, variations may occur if they are obtained from different batches. Therefore, it is important to know exactly which NF was studied in a certain experiment in order to be able to interpret and reuse data. Due to the complexity of NMs, this is, however, not straightforward. Some attempts have been made to structure the nomenclature in such a way that each NM can be uniquely identified, e.g. the NInChI (Lynch et al. 2020) or labeled with a unique identifier even to the level of new batches, e.g. the European Materials Registry (van Rijn et al. 2022). These first approaches will have to be fine-tuned in the future and, importantly, also must be established within the community such that this information will be provided along with published data.

7.3. Reliability of data

Reliability of studies on NMs is another important factor. The main challenge for NMs is their handling during experiments. Especially, dispersion stability is a major issue as usually NMs are highly reactive and thus tend to agglomerate or interact with components in their surrounding environment like proteins from the medium they are dispersed in. The best way of dispersing NMs is still a matter of debate and thus is handled differently among researchers. In addition, there are no standardized ways on how to perform certain measurements of physicochemical properties or toxicity assays. Many of the OECD test guidelines are not yet adapted for NMs (Rasmussen et al. 2019; Bleeker 2023). For some of the

toxicological assays, interferences of the assays with the tested NMs may occur and suitable replacements are not always available at the time being. All these uncertainties, missing adaptations to NMs and lacking standardizations lead to high variability of the resulting measurements and contradictory results in existing literature and renders integration of results from different studies a difficult task. In addition, only few NMs have been tested in *in vivo* studies and thus adapting NAMs such that they reflect the *in vivo* situation is not trivial.

In silico methods critically depend on data quality of the underlying experimental data. While especially ML models are capable of capturing very complex relations and interactions, they can, by definition, not be better than the underlying data. Thus, if the experimental results are not reliable, also developed models cannot be of high quality. Instead, data with low technical error levels allow for models which can reliably detect underlying patterns in the data. Quality criteria for NM experimental data have been introduced (Marchese Robinson et al. 2016; Comandella et al. 2020; Basei et al. 2022; Shao, Beronius, and Nymark 2023) and can guide scientists as well as modelers in judging the reliability of experimental data and thus allowing for subsequent robust model development.

7.4. Standardization and regulatory acceptance of omics data

Some of the uncertainties induced by grouping based on physicochemical properties and toxicity data alone can be overcome by adding omics data as these add an additional layer of mechanistic understanding. However, omics data often lack sufficient comparability. This is mainly because different measurement devices and analysis pipelines are in use. Therefore, also in the case of omics data harmonization and standardization of data reporting and analysis workflows are of utmost importance. The transcriptomic (OECD 2021a) and metabolomic (OECD 2021b) reporting frameworks developed by the OECD are highly valuable tools in this context. These frameworks define certain information that needs to be provided for transcriptomic and metabolomic experiments thereby supporting the implementation of the FAIR data principles.

7.5. Obtaining robust models

Finally, the question on how to combine and compare NM-related data in order to obtain robust models is another important aspect for grouping of NMs. Here, different aspects need to be taken into consideration: 1) Different measures of similarity exist and can be applied. A number of tools for quantifying similarity between NMs were recently compared by Jeliazkova et al. (Jeliazkova et al. 2022b) However, the different measures have different advantages and disadvantages and may eventually lead to different results. The use of high-dimensional omics datasets may even pose additional challenges in this regard; 2) Instead of focusing solely on NMs, integration with other more extensively studied effects like those from drugs, other types of chemicals or diseases may be supporting the acceptance of a certain grouping hypothesis as they may provide additional insights. While this is complicated with regards to physicochemical properties as those may be very different between NMs and other traits, on the level of omics data this approach seems to be reasonable. ML models like those based on transfer learning may be very valuable in this context; 3) AI in general is expected to form an important pillar in the generation of robust models as it will not be feasible to search, evaluate and integrate the huge and ever-increasing amount of data available manually; and 4) Once ML models have been developed on well-standardized data and methods, extensive validation is indispensable. The quality of a developed model may also be assessed based on certain criteria. For QSAR models, the OECD has published recommendations for determining the quality of developed models (OECD 2014b) as well as a corresponding QSAR assessment framework (OECD 2023). Such evaluation criteria are urgently needed in order to guarantee the use of high-quality models. These evaluation criteria may be used or adapted for more general ML and omics-based approaches to assess their robustness.

8. Conclusion

8.1. ML Models for NM grouping

ML is a valuable tool supporting NM grouping. Especially, the ability to extract the most important parameters describing toxicity in an automated, objective fashion is of great use. The extracted properties can then be used to determine the similarity of NMs. ML models can aid NM grouping approaches in several ways: 1) ML models are able to derive information and patterns from complex and high-dimensional datasets which cannot be easily detected by human inspection. Thus, they may

capture more complex interactions between physical, chemical, and biological properties and can potentially enable more accurate classification and grouping of NMs; 2) ML models can predict the behavior of NMs under different conditions which may reduce time and cost when assessing safety, toxicity, and environmental impact of NMs and may guide SSbD approaches; 3) ML models are very time and cost efficient. Once a model is trained, incoming data for new NMs can usually be processed very quickly; and 4) ML can also aid the discovery of new properties or applications of NMs by identifying common patterns or correlations in large datasets. In addition, combining supervised and unsupervised approaches may also be important. While the prediction task of models is usually based on labeled data and supervised approaches, the advantage of unsupervised methods is that they can also use the large pool of unlabeled data to search for patterns or reduce dimensionality. As dataset size is of high relevance with respect to developing robust ML models with a large applicability domain, this is a very useful and recommended strategy.

8.2. Omics for NM grouping

Usually, NM grouping approaches are initially based on measured physicochemical properties or sometimes calculated descriptors. The advantage of directly linking physicochemical properties with toxicity is that one obtains information on how NMs need to be manipulated to make them safer, e.g. in SSbD approaches. However, limitations with respect to the generalizability and applicability domain have frequently been discussed and can also be observed from the different models presented in this review. This is because the importance of certain properties largely varies with the material types and shapes under study. Also, similar effects may be obtained from NMs with very different physicochemical properties. Omics approaches are very well suited to overcome these limitations as they yield additional mechanistic insights which can support NM grouping. Nevertheless, there are a few challenges which need to be overcome in order to successfully integrate omics results into NM grouping approaches. Especially, FAIRification of NM omics data, harmonization and standardization of measurements and analyses workflows, the definition of similarity and validation of findings are major fields in which improvement is still required in order to achieve robust models and regulatory acceptance. Once these hurdles are successfully tackled, omics approaches are a very promising source of information for supporting NM grouping. This is confirmed by the first approaches which obtained very good predictive performance when including omics data in the prediction models for NM toxicity. Thereby, omics approaches hold a number of benefits for NM grouping approaches: 1) Omics data provide detailed information about biological effects and interactions of NMs. This may aid the formulation and testing of a grouping hypothesis and thus support regulatory bodies in making informed decisions regarding the safety of NM; 2) While grouping approaches based on physicochemical properties suffer from the fact that these properties may change depending on their surrounding medium as well as over time, omics provide a more direct read-out of the actual state of the NM that was seen by the cells. In combination with ML, patterns and relationships not evident from traditional analysis methods may be detected; and 3) If well performed and analyzed, omics experiments are directly comparable among studies and thus well suited for meta-analyses. This does not only hold true within studies on different NMs but instead meta-analyses including other traits like drugs, other types of chemicals and so on are also possible. The fact that a huge number of such omics datasets is publicly available including raw data as well as metadata, is very promising for the implementation of robust ML models, whose performance largely depends on the amount of available data.

8.3. AI for NM grouping

AI in general will be unavoidable in the context of developing reliable grouping approaches to cope with the wealth of information available. The complexity of the task of finding suitable NAMs for NM toxicity assessment leads to a high number of potential applications of different AI methods. Especially NLP is expected to have a great influence on future data processing and retrieval. The following tasks have high potential for support by NLP: (1) automated review and data extraction from scientific literature which may also include automated generation of databases; (2) finding patterns, trends and inconsistencies in research papers or datasets; (3) standardization of terminologies; (4) compliance of research documents with regulatory standards; and (5) automated annotation and generation of metadata within databases. However, while AI may

provide very helpful tools for obtaining information in an efficient and objective manner, human judgment will be of utmost importance and should not be underestimated. This may especially be important in terms of plausibility considerations, contextual interpretation, decision making, quality checks or results with high uncertainties.

8.4. Recommendations for future research investment

In order to implement ML and omics techniques successfully in the process of NM grouping, standardization of test methods including dispersion protocols and quality assessment followed by appropriate data curation of produced data are urgently needed in order to allow comparison between results from different studies. In line with that, raw data should be made available to the community once they are published and they should be accompanied by sufficient metadata. This may be supported by further refinement of file and model sharing formats, ontologies, terminologies and data quality assessment tools specific for the needs in the field of NMs. Along these lines, it is also of high relevance to find a solution for unambiguous naming of NMs which allows direct comparison of the data. New developments in AI, especially in the field of LLMs can aid the curation and linkage of existing databases. More reliable and comparable data will automatically support the implementation of more robust ML models with a larger applicability domain. At the same time, it will be necessary to enhance the explainability of ML models in order to derive a grouping hypothesis that can also be tested for validation purposes. Here, ML algorithms need to be explored and adjusted with respect to methods and insights that allow for interpretation of the outcome. While it is important to increase the explainability of the model itself, also unraveling the underlying MoA will support the hypothesis formulation. Here, more omics studies on NMs and meta-analyses will be highly useful for extracting predictive signatures that can be used in hazard assessment. Once predictive signatures and thus relevant transcripts, proteins or metabolites have been identified, targeted testing of only these entities will be possible allowing for high-throughput screening.

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Data availability statement

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