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An integrated pathway based on *in vitro* data for the human hazard assessment of nanomaterials

Daina Romeo[a], Beatrice Salieri[b], Roland Hischier[b], Bernd Nowack[b], Peter Wick[a,*]

[a] Empa, Swiss Federal Laboratories for Materials Science and Technology, Particles-Biology Interactions Laboratory, Lerchenfeldstrasse 5, 9014 St. Gallen, Switzerland
[b] Empa, Swiss Federal Laboratories for Materials Science and Technology, Technology and Society Laboratory, Lerchenfeldstrasse 5, 9014 St. Gallen, Switzerland

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ABSTRACT

In line with the 3R concept, nanotoxicology is shifting from a phenomenological to a mechanistic approach based on *in vitro* and *in silico* methods, with a consequent reduction in animal testing. Risk Assessment (RA) and Life Cycle Assessment (LCA) methodologies, which traditionally rely on *in vivo* toxicity studies, will not be able to keep up with the pace of development of new nanomaterials unless they adapt to use this new type of data. While tools and models are already available and show a great potential for future use in RA and LCA, currently none is able alone to quantitatively assess human hazards (i.e. calculate chronic NOAEL or ED_{50} values). By highlighting which models and approaches can be used in a quantitative way with the available knowledge and data, we propose an integrated pathway for the use of *in vitro* data in RA and LCA. Starting with the characterization of nanoparticles' properties, the pathway then investigates how to select relevant *in vitro* human data, and how to bridge *in vitro* dose-response relationships to *in vivo* effects. If verified, this approach would allow RA and LCA to stir up the development of nanotoxicology by giving indications about the data and quality requirements needed in risk methodologies.

1. Introduction

Engineered nanomaterials (ENM) are synthesised particles with at least one dimension in the size range 1–100 nm, whose peculiar properties allow novel applications in many sectors, such as energy, electronics, health, chemistry, materials, textiles (Hulla et al., 2015; Nowack et al., 2012). In the last 30 years, the nanotechnology field has been following an exponential trend of development (Chen et al., 2008), and has been recognized as one of the Key Enabling Technologies of the 21st century (Tegart, 2004).

Together with the acknowledgement of the benefits of nanomaterials, there is also concern about eventual negative environmental and/or health impacts, since their wide use may presents a novel risk of involuntary exposure, and the same properties that make them innovative could determine a different toxicity compared to bulk materials (Srivastava et al., 2015; Klaine et al., 2012).The attention on potential toxic effects comes not only from a regulatory point of view (down-stream measures), but also from the proactive approach "Safer-by Design", which aims at selecting safer substances already during the development of new ENM (Schwarz-Plaschg et al., 2017; Morose, 2010). Identifying risks early and in an adequate manner, both in terms of exposure and toxic potential, can be achieved by combining the knowledge of nanotoxicology and general risk methodologies such as Risk Assessment (RA) or Life Cycle Assessment (LCA) (Rebitzer et al., 2004; Hetherington et al., 2014; Hengstler et al., 2006). Following the new paradigm for the toxicology of the 21st century (Andersen and Krewski, 2009), the nanotoxicology field is developing towards a mechanistic approach to toxicity, based on *in vitro* and *in silico* models (National Research Council, 2007). Thus, rather than observing the toxic effects of substances on animals, the focus shifts to: (i) understanding how toxicity is exerted, from a biochemical level up to a population (Burden et al., 2015), (ii) identifying which and to what extent the characteristics of the tested substances induce toxicity (Worth et al., 2017), (iii) developing new screening and predictive methods (Lamon et al., 2018). The translation of this vision to practice relies on the new tools and disciplines that aim at understanding and measuring toxicity from a mechanistic point of view, for example by developing *in vitro* models that are more predictive of *in vivo* effects (Andersen and Krewski, 2009). Undoubtedly this path is not free from technical and regulatory challenges (Hartung, 2010; Hartung, 2009; Berg et al., 2011), however it has set a direction that has influenced research by stimulating the development of alternative approaches in toxicology.

* Corresponding author.
E-mail addresses: Daina.Romeo@empa.ch (D. Romeo), Beatrice.Salieri@empa.ch (B. Salieri), Roland.Hischier@empa.ch (R. Hischier), Bernd.Nowack@empa.ch (B. Nowack), Peter.Wick@empa.ch (P. Wick).

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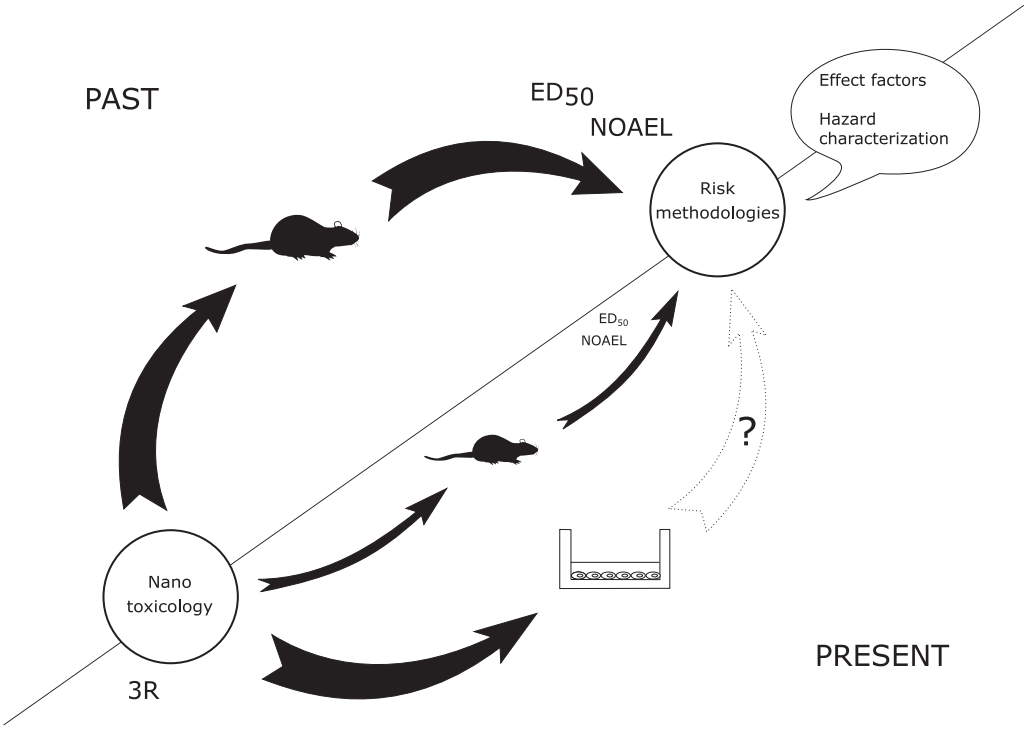

Fig. 1. The transition towards a mechanistic nanotoxicology determines a reduction in animal studies, which are the traditional data source of risk methodologies such as Risk Assessment and Life Cycle Assessment. At the same time, a growing amount of *in vitro* studies are being produced, but a way to use this new type of data in risk methodologies is still missing.

The effect of these changes is and will be a reduction of animal testing and an increasing availability and refinement of *in vitro* data and *in silico* tools, which are not yet implemented in LCA and RA (Fig. 1).

Traditionally, both RA and LCA use epidemiological or *in vivo* toxicological data to establish a dose-response assessment (Fig. 2). Risk assessment calls this step hazard characterization, while in LCA it is described by the so-called effect factor, as defined in the consensus model USEtox (Rosenbaum et al., 2008), applied as reference methodology for the assessment of human health impacts. Risk Assessment, faithful to its threshold approach, focuses on the maximum dose at which no adverse effect is observed (NOAEL, eventually derived from the LOAEL, lowest observed adverse effect level) (Organization, 1990), while LCA derives a linear dose-response curve from the dose generating an effect on 50% of the individuals (EC_{50} or ED_{50}) (Rosenbaum et al., 2008). Hence, when assessing the effects of ENM, both methods rely on classical nanotoxicological data to derive these toxicological dose descriptors. However, epidemiological studies of ENM are rare, and are available only when exposure has already caused an impact on human health, while animal data will become scarcer due to the transition occurring in the toxicology field.

To keep up with the pace of development of new (nano) materials, risk methodologies have to account for the shift in type and source of nanotoxicology data, and adapt accordingly. This methodological challenge can be proactively approached: without waiting for well-established non-animal methods and models, RA and LCA can already identify and express their new needs in terms of data and data quality (Mattsson and Simkó, 2017), to make sure they are met as the nanotoxicology field progresses towards more advanced *in vitro* and *in silico* models and a reduced use of animal testing.

Currently, there is not yet a complete strategy for a quantitative assessment of ENM human health impacts implementable with the available non-animal data and *in silico* models (Salieri et al., 2018; Burgdorf et al., 2019), i.e. a defined quantitative *in vitro in vivo* extrapolation (QIVIVE) procedure (Yoon et al., 2015). Previous studies focused on (i) single methods and their potential, without addressing all the requirements of LCA and RA (Gajewicz et al., 2012; Basei et al., 2019; Oomen et al., 2015), (ii) potential of future (i.e. not yet implementable) integrated approaches and frameworks (Sturla et al., 2014; Fadeel et al., 2018; Hristozov et al., 2016), and (iii) limitations and focus areas for future developments in the nanotoxicology field (Burden et al., 2017; Stone et al., 2016; Basei et al., 2019).

In our knowledge, all the strategies proposed for a quantitative ENM hazard assessment and effect factor calculation rely on future advancement of *in silico* tools or conceptual frameworks. Rather than producing an extensive review of available methods and models, we focus on a subset that addresses the choice and refinement of *in vitro* data, and the subsequent extrapolation to *in vivo* data. Building on the changes in the type of data produced in nanotoxicology and the requirements of RA and LCA, this work explores a pathway towards a QIVIVE of ENM, for a next-generation human toxicity assessment. We highlight how methods can support each other and which data are required for this integrated approach. Last, acknowledging the youth of the field, this paper pinpoints which quality requirements in nanotoxicology could accelerate the development of alternative strategies in human health impact assessment. The overall goal of this review is to provide the risk assessment and life cycle assessment communities with a potential way to implement *in vitro* data in hazard assessment, making it possible to provide feedback to the nanotoxicology community about the requirements, in terms of data and quality, of risk methodologies.

2. State of the art of the integration of *in vitro* data in RA and LCA

In the area of nanotoxicology, some attempts have been proposed to integrate *in vitro* data in RA and LCA, applying different methods and models.

Cheng et al. (2018) coupled pharmacokynetics and *in vitro* pharmacodynamics of gold nanoparticles using a probabilistic risk assessment approach. The pharmacodynamics was derived from *in vitro* toxicity dose-response curves for multiple submerged cell cultures, calculating the dose effectively reaching the cells using an *in vitro* dosimetry model. The ED_5 and ED_{10} values estimated from the dose-response relationship were used as internal doses from which the injected external doses are estimated, using a Physiologically-Based Pharmacokinetic model (PBPK). The obtained values representing the human equivalent dose generating the death of 5% and 10% of cells were compared with results from *in vivo* studies. As the authors point out,

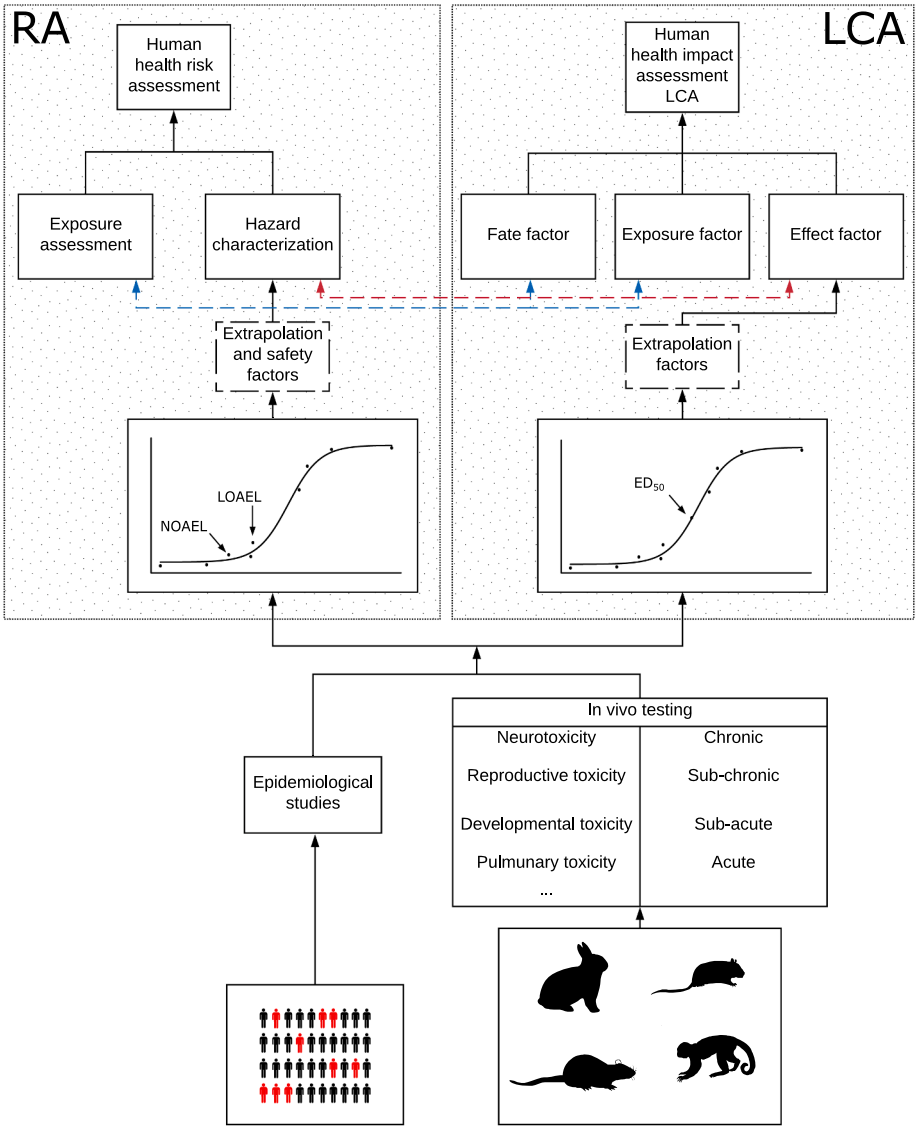

Fig. 2. The traditional human toxicity assessment in Risk Assessment and Life Cycle Assessment. Epidemiological data and *in vivo* animal data are used to derive the NOAEL and/or LOAEL values for RA, and the EC_{50} or ED_{50} values for LCA. Extrapolation and safety factors are applied in case of sub-optimal data, for example to account for differences in target species (from animal to human), duration of exposure, population variability. The result of the combination of the hazard characterization and the exposure assessment is the human health risk assessment, while human health impact assessment derives from the integration of fate, exposure and effect factors. Colored dashed arrows highlight the correspondence between RA and LCA steps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while their strategy provides interesting insights for risk assessment, the choice of cytotoxicity as *in vitro* endpoint is significantly different from sub-lethal, sub-chronic, or chronic endpoints traditionally evaluated in risk assessment, which hinders the predictivity of the proposed approach.

The importance of choosing relevant *in vitro* endpoints and linking the *in vitro* dose to the corresponding external exposure dose was highlighted also by Forsby and Blaauboer (Forsby and Blaauboer, 2007). Their approach for the risk assessment of neurotoxicity of chemicals (not nanoparticles) requires the calculation of a set of endpoints that encompass cytotoxic, physiological, morphological and neuochemical effects. The assumption is that the lowest-dose showing any of these effects *in vitro* could be used as lowest observed effect level (LOEL) surrogate. This value is then coupled with a PBPK model to estimate the lowest observed effect dose (LOED), i.e. the external exposure dose producing a concentration in the blood and brain equal to the LOEL value calculated *in vitro*. In their study, the comparison of the estimated LOED with the corresponding values from *in vivo* experiments showed a good correlation (within one order of magnitude). While this approach seems promising, it is not specifically developed for ENM, and its applicability to this type of substances would have to be demonstrated.

A single work has so far addressed the use of *in vitro* data in Life Cycle Assessment (Salieri et al., 2020). The study estimated the *in vivo* ED_{50} values of a set of soluble nanoparticles from the *in vitro* and *in vivo* EC_{50} and ED_{50} of comparable known substances with the same mode of toxicity, using a Relative Potency Approach (explained in detail in Section 3). The main assumption of this methodology is that, if the *in vitro* dose-response curves of the test and reference substances are parallel, the ratio of the two substances' EC_{50} values in a subhuman system corresponds to the ratio in the human system (Calle and Zaighemi, 2000). The correlation between *in vitro* and *in vivo* data, without explicitly describing or modelling any process occurring between the cellular and whole organism level (e.g. the kinetics of the substances) is an assumption that however needs to be verified before this approach could be extensively implemented, and the integration

with other models is a possible solution highlighted by the authors as a way to further develop this reported approach.

3. A pathway for future-oriented hazard assessment of manufactured nanomaterials

As shown in Section 2, nanotoxicology a priori offers data, methods, and models that actually have the potential to support RA and/or LCA. However, since a single straightforward substitute of animal data does not exist yet, it is necessary to connect and integrate these different approaches/methods to calculate an EC_{50}, ED_{50}, NOAEL, or LOAEL in the absence of *in vivo* studies.

These models and approaches cover various aspects connected to the choice and refinement of *in vitro* data and their extrapolation to *in vivo* data. The choice of models to integrate is per se a subjective decision, that relies on the understanding of the available options and depends on the specific goal of the strategy. In our case, we aimed at selecting a strategy that was as much as possible implementable with the already available data. Therefore, inspired by the concept of Weight of Evidence (Linkov et al., 2009, 2015), we evaluated the readiness and potential for quantitative use of the models. The evaluation was performed considering as criteria the applicability range and the number and type of ENM included until now in each model. We then prioritized those models already covering a wide range of materials, considering the theoretical applicability range only in a second instance. This information was a support to the judgement on the integrability of different models and the selection of the strategy. The proposed pathway (Fig. 3) is rooted in the properties of nanomaterials, and relies on the use of *in vitro* human data. After choosing *in vitro* models and testing, as well as of relevant dose units, data from submerged *in vitro* cultures can be refined to account for the dose effectively reaching the cells via an *in vitro* dosimetry model (such as the one-dimensional Distorted Grid model). To move towards a higher representativeness of *in vivo* dose-response relationships, the *in vitro* data can be coupled with kinetic models such as PBPK models and the Multiple-Path Particle Dosimetry (MPPD) model, to link a response *in vitro* to the result of whole organism exposure. Even if with more constraints, a Relative Potency Factor Approach (RPF) can support those cases in which kinetic models are not available. The final outcomes are the respective values required within LCA and RA for the assessment of human health impacts. Each single step of this proposed pathway and the connected supporting models are presented in detail in the following sections.

3.1. Nanomaterial properties

The first indispensable step for the hazard assessment of ENM is to precisely know their physico-chemical properties (Gallagher et al., 2017). Since properties such as toxicity depend on ENM physico-chemical characteristics (e.g. size, surface area, shape), a precise characterization is needed to uniquely identify the substance under examination, and allow the reproducibility of the study. Often ENM are characterised in the form they have been purchased (e.g. nanopowder), however, the interaction with biological systems *in vivo* or *in vitro* will affect the characteristics of ENM, and therefore modify their properties (Warheit, 2008). For this reason, the characterization step has to be consistent with the type of test that will be conducted: for *in vitro* toxicological tests, the ENM should also be characterized in the exposure media, which should possibly mimic *in vivo* conditions (Murdock et al., 2008). For example, eventual agglomeration processes occurring in human blood should be replicated in the *in vitro* system, to increase

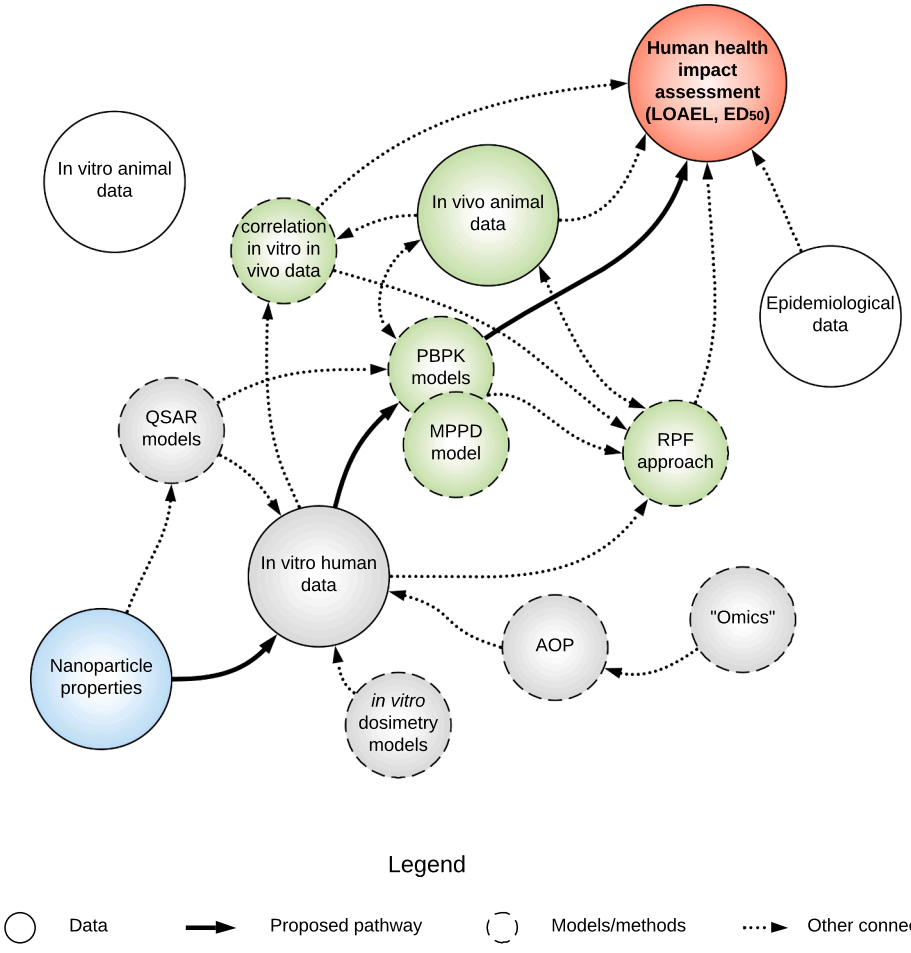

Fig. 3. Graphic representation of the proposed pathway for the assessment of human health impacts of ENM from non-animal data, in alignment with the new trends and developments in nanotoxicology. The importance of the properties of ENM (light blue) is described in Section 3.1. The selection of *in vitro* data (Section 3.2), in grey, includes the evaluation of Quantitative Structure-Activity Relationship models (QSAR), *in vitro* dosimetry models, Adverse Outcome Pathway (AOP), and "omics" technologies. The extrapolation from *in vitro* to whole organism level (Section 3.3), in green, includes the evaluation of the correlations between *in vitro* and *in vivo* data, Physiologically-Based Pharmacokinetic models (PBPK), the Multiple-Path Particle Dosimetry (MPPD) model, and the Relative Potency Factor. approach (RPF). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the representativity of the study; as the effects are caused by the agglomerated ENM, and not the pristine ENM, the new size of the ENM should be measured and reported.

The characterization step is also needed to express the ENM dose in a unit that is relevant to its toxicity. In fact, while exposure doses are often reported in mass, the mass is not necessarily the driver of toxicity, but other characteristics, such as surface area, surface charge, shape, are responsible of the potential negative effects (Donaldson and Poland, 2013). This because toxicity is exerted by the Biologically Effective Dose (BED), i.e. the active agent that is directly associated to a response; on a practical level, the closer the specified dose converges with the BED, the more likely the dose-response association will be evident (Povey, 2000). With a well characterized substance, the mass-based dose can be converted to the BED, which, for example, has been shown to affect the correlation between *in vitro* and *in vivo* responses (Rushton et al., 2010).

A good characterization of ENM provides a robust basis for any subsequent test, supports an initial idea about the potential mode of toxicity and the classification of ENM, and provides all the data necessary to express their dose in units relevant to their toxicity.

3.2. Selection of in vitro data

In vitro tests assess the response of isolated cells, organoids or tissues to the exposure to ENM, i.e. their pharmacodynamics. Unlike *in vivo* testing, *in vitro* assays are cheaper, faster, and can use human cells, avoiding in this way the critical point of extrapolating from species to species (Fröhlich et al., 2014). Moreover, they are better fit to study and explain the mechanism of toxicity, since they can describe the interaction of particles and living beings on a molecular and cellular level. At the same time, however, *in vitro* data do not represent a systemic response, especially when only one cell type is used, which limits the direct use of these data in RA and LCA. To overcome this limitation, more complex systems are being developed, reflecting the tri-dimensional structure of organs (Fitzgerald et al., 2015), and the real exposure of cells (Fröhlich et al., 2014). This shows that the field has not yet reached a mature state, and that in the future we can expect *in vitro* models to be more representative of tissues/organs and their interaction with ENM (Wick et al., 2014).

3.2.1. AOP and "omics" technologies support the choice of endpoints

A challenge for the use of *in vitro* data is the choice of cells and endpoints, since a wide range of physiological, morphological, and chemical effects can be measured at cellular level, for different cell types (Jones and Rowland-Yeo, 2013). Such a selection should be guided by the knowledge of the kind of data that are required by RA and LCA: the focus is preferably on chronic effects caused by a lifetime exposure (Rosenbaum et al., 2008). While in the future more complex models could be more predictive of chronic exposure effects (Comfort et al., 2014), currently most *in vitro* tests show acute and sub-acute responses obtained by one or a few repeated doses, rather than the effect of a long-term low-dose exposure. The aim then would be to select those endpoints that show non-lethal injuries or disruptions in cell functioning that could be attributed to an early phase of a chronic response rather than an acute toxic response such as death (Comfort et al., 2014). As a consequence, cell viability tests, which are performed in great numbers, are only partially informative, as they represent a critical acute response obtained at relatively high doses.

Identifying the early cellular effects potentially leading to a disease is one of the focus points of the Adverse Outcome Pathway (AOP), a framework that maps the path that, from a molecular initiating event (MIE) and through a variable number of key events, leads to an adverse outcome at organism or population level (Villeneuve et al., 2014). A quantitative AOP identifies all the necessary and causally-interlinked steps at molecular, cellular, organ level that will lead to an adverse effect, and reports quantitatively the relationships between these steps, the exposure doses, and the time, obtaining for each step a dose-(time)-response curve (Burden et al., 2015). AOPs have the potential to support the development of predictive toxicity models and the selection of early biomarkers and assays predictive of adverse outcomes (Lee et al., 2015).

A promising resource in the establishment and application of AOPs are "omics" technologies, which is a generic term for all those "methods that aim to analyse complex biological samples by focusing on a complete set of biomolecules, e.g. the whole genome (genomics), transcriptome (transcriptomics), proteome (proteomics) or metabolome (metabolomics)" (Brockmeier et al., 2017). Through the production of high-content databases, "omics" technologies provide information about the complete genetic or molecular profiles of perturbed living systems, including the correlations and dependencies occurring between molecular components (Schneider and Orchard, 2011). Their use lies both in the determination of MIE and key events, and in proposing biomarkers for particles toxicity screening (Vinken, 2019). The "omics profile" can also be used comparatively, to classify the effect of ENM with respect to other chemicals, drugs, and diseases (Serra et al., 2019).

Whereas from a regulatory perspective the standard remains the use of classical toxicity tests (Labib et al., 2015), the use of these data is gaining acceptance, with a growing number of studies using "omics" data to identify modes of toxicity. However, the development of AOPs relevant for ENM is still in an initial qualitative phase, where the focus is on the identification of key events, but no information is available about the relationship existing between them (Gerloff et al., 2017). Halappanavar et al. (2019) screened *in vitro* and *in vivo* data about ENM toxicity and assigned the reported biological events to potential key events and adverse outcomes. Interestingly, most key events were linked to chronic inflammation and oxidative stress. The available data did not allow the development of quantitative AOP, but only a qualitative identification of key events. A few other attempts have been done towards quantitative AOPs (Conolly et al., 2017; Zgheib et al., 2019; Maxwell et al., 2014), but there is still no standardized approach to AOP quantification, and the application of different methodologies has shown diverging results (Zgheib et al., 2019), which suggests that the path to quantitative AOPs is still long.

3.2.2. Realistic doses and in vitro dosimetry

The choice of the doses used in *in vitro* tests is crucial for the relevance of the tests for RA and LCA. As seen before, these methodologies investigate chronic effects caused by environmental exposure, which should guide the selection of *in vitro* doses. A dose as coherent as possible to expected environmental exposure will show effects more realistic than high doses that more easily produce cytotoxic effects, characteristic of acute toxicity (Kawata et al., 2009). Thus, the *in vitro* dose should represent the fraction of the environmental concentration effectively reaching the target cells (Balduzzi et al., 2004). When this internal dose is not directly available, it can be obtained by using models that simulate the kinetic of the particles in the body (as explained in Section 3.3.2).

The next step is to assure that the cells in the *in vitro* system are effectively exposed to such dose (Drasler et al., 2017). For chemicals soluble in the exposure media of submerged systems, the cellular dose corresponds to the concentration in the media. ENM, on the other hand, are not dissolved in the media, and are therefore affected by solution dynamics via agglomeration, settling, and diffusion processes, and by chemical interaction with the media (e.g. change of surface and surface charge) (Hinderliter et al., 2010). Due to these interactions, the concentration of the particles in the media not necessarily corresponds to the dose that reaches the cells, and the assumption of *in vitro* systems of proportionality between the concentration of a substance in the media and the cellular dose does not hold true for ENM (Teeguarden et al., 2014).

Here, we present two ways to calculate the dose effectively reaching the cells: Quantitative Structure-Activity Relationship (QSAR) models,

and the one-dimensional Distorted Grid (DG) model.

Quantitative Structure-Activity Relationship models are computer-assisted (*in silico*) methods that identify those characteristics of the physico-chemical structure of ENM that are related to a biological activity, property or effect, with the aim of predicting such activity by only knowing the selected descriptors (Schultz et al., 2003). One of the activities that have been modelled is the uptake of ENM by human cells (Chau and Yap, 2012; Fourches et al., 2010; Ghorbanzadeh et al., 2012; Epa et al., 2012; Ojha et al., 2019). The data source of all the studies was a database of 109 nanoparticles with the same superparamagnetic core, and different coatings (Weissleder et al., 2005). The uptake was studied for different cell types, including pancreatic cancer cells (Chau and Yap, 2012; Fourches et al., 2010; Ghorbanzadeh et al., 2012; Epa et al., 2012; Ojha et al., 2019), endothelial cells (Epa et al., 2012), and macrophages (Ojha et al., 2019). The determinants of cellular uptake were properties linked to the chemical formulas of the coating groups, such as lipophilicity, magnetic properties, size of the nanoparticles. All models showed a good performance in terms of sensitivity and specificity.

An interesting consideration after an evaluation of these models is that exogenous parameters, such as those relative to the interaction with the exposure media, were not taken into the account, and since some of these variables were constant over the dataset, their influence on cellular uptake is not captured. This is the case for the type of exposure media, the dose, and the exposure time (Raies and Bajic, 2016). This restricts the applicability domain of the models; considering that the dose influences the uptake (Singh and Ramarao, 2013), the uptake predicted by these QSARs can be considered reliable only for the same doses used to build the models.

For the models to capture the contribution of more variables, and provide previsions for a larger class of nanomaterials, more complete datasets are necessary (Tong et al., 2005). The requirement of large amounts of data to be built and validated is one of the limitations of QSAR models (Forest et al., 2019). To accelerate the solution of this issue, modelers and experimentalists can collaborate to make sure that experimental data provide all the features required for QSAR, such as a large enough sample size, and a complete particle characterization (Puzyn et al., 2010). Up to now, the available QSARs should be used only when the case study falls in the applicability domain of the model, and important limitations such as the lack of dose-dependent uptake functions should be taken into account.

In vitro dosimetry models allow the calculation of the dose of ENM effectively reaching the cells in submerged *in vitro* cultures, to compare biological responses to exposure doses more physiologically-relevant than the ENM concentration in the media (Cohen et al., 2015). The one-dimensional Distorted Grid (DG) model (DeLoid et al., 2015) (Matlab code available for free as supplementary software of (DeLoid et al., 2017)) simulates sedimentation and diffusion processes of suspended particles to calculate their transport over time. It represents an advancement with respect to the In vitro Sedimentation, Diffusion and Dosimetry (ISDD) model (Hinderliter et al., 2010) and the updated volumetric centrifugation method (VCM) ISDD (Cohen et al., 2014).

The DG model is able to simultaneously simulate the behavior of a polydisperse suspension of soluble or non soluble particles, considering the characteristics of the media, of the particles, and of the experiment (Table 1). It also accounts for the adsorption of particles on the cells, which determines the level of re-suspension of particles deposited at the bottom of the system. The output is the fraction, mass, surface area, or number of particles/agglomerates moving vertically through the media and reaching cells over time, expressed as absolute values or concentrations. The model has been validated by comparison with experimental data for multiple ENM suspensions; a protocol to prepare and characterize the nanomaterials is also available to assure a standardised calculation of all needed parameters (DeLoid et al., 2017).

Table 1
The input parameters and the output of the One-dimensional Distorted Grid model.

One-dimensional Distorted Grid Model	
Inputs	
System parameters	Height of suspension column
	Media density
	Media viscosity
	Temperature
ENM parameters	Concentration in mass
	Density of ENM
	Effective density of agglomerates
	Diameter of suspended ENM
	Solute ENM fraction
Outputs	
Deposited ENM over time	Concentration of particles
	Mass of particles
	Number of particles
	Surface area of particles

3.2.3. In summary, the choice of in vitro endpoints and doses

The choice of endpoints and doses is a fundamental step for the use of *in vitro* data (Park et al., 2009). Even if AOPs are providing new knowledge about the development of toxicity over time and across different levels of biological organization, the field is still in an early stage. Waiting for quantitative indications, for now the choice of *in vitro* endpoints relies on the knowledge about the mode of toxicity of the nanoparticle (e.g. oxidative stress induction), the type of data preferred in RA and LCA, and, more generally, the experts' experience. While the amount taken up by the cells would be a more precise dose, our analysis of models state of development showed that QSAR uptake models have, for now, a too limited range of application to be used consistently, making the DG model a preferable solution for *in vitro* dosimetry (Table 2).

By combining the characterization of nanoparticles with the DG model, we obtain a dose-response curve *in vitro* where the dose is expressed as biologically effective dose reaching the cells, and the response is one or more endpoints that are chosen by expert judgement to be a good indicator of chronic, sub-chronic or sub-acute effects. Even if still reliant on arbitrary choices, not overlooking any of these points provides, for a well characterized ENM, a more precise evaluation of *in vitro* responses, and high potential for integration with the next steps of our proposed pathway.

3.3. From in vitro to whole organism level

Linking a cellular response to an *in vivo* response is a challenge for which a standard approach has not being identified yet. One strategy is to verify whether there is any correlation between *in vitro* and *in vivo* results. Another approach couples the information about pharmacodynamics obtained by *in vitro* tests with pharmacokinetics modelling. Last, the Relative Potency Factor Approach allows the estimate of *in vivo* ENM potency (i.e. the dose that yields a given level of response) from

Table 2
The theoretical applicability range of the models and approaches that can be used to select or refine *in vitro* data, and the type of particles that are currently covered.[1]Labib et al. (2015), [2]Gerloff et al. (2017), [3]Worth et al. (2017).

Model	Applicability range	Currently covered ENM
AOP	Any ENM (disease specific)	Lung fibrosis from carbon nano-tubes[1]
		Liver fibrosis from metal oxides[2]
QSAR uptake models	Any ENM	Coated iron oxide[3]
DG model	Spherical ENM	Any spherical ENM

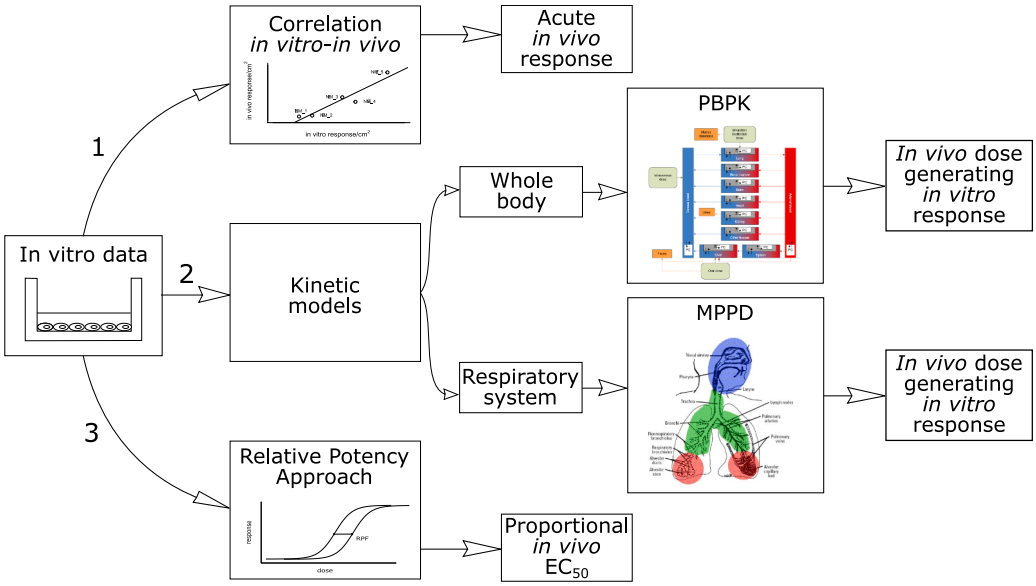

Fig. 4. The three analyzed ways to bridge a cellular response to *in vivo* conditions, and their outcome at the current level of knowledge. Option 1 (Section 3.3.1): investigate the correlation of *in vitro* and *in vivo* responses. Currently correlations have been found only for acute inflammatory responses to inhaled nanoparticles. Option 2 (Section 3.3.2): use kinetic models to link a response *in vitro* to the external doses generating such response, i.e. an external dose-*in vitro* response curve. Option 3 (Section 3.3.3): the relative potency approach can be used to estimate a response *in vivo* if the necessary conditions of this method are verified. Adapted with permission from (Oberdörster et al., 2005 and Carlander et al., 2018).

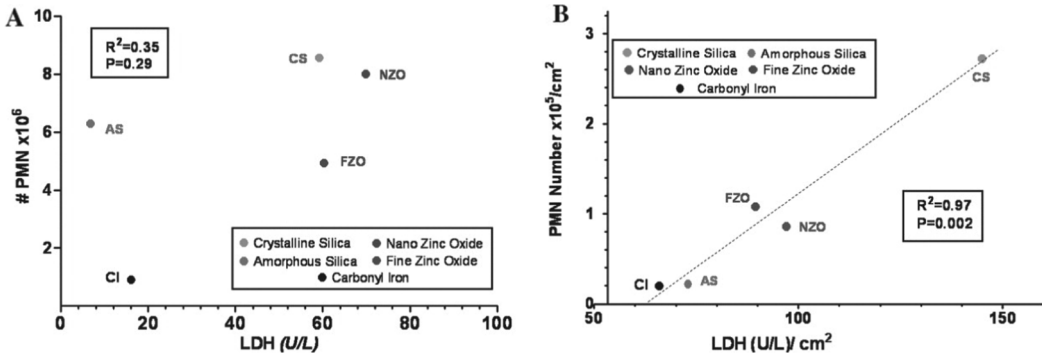

Fig. 5. The effect of the choice of dose unit, either mass (A) or particle surface area (B), on the correlation of *in vivo* and *in vitro* responses. A: "*In vivo* (number of PMNs in rat lung lavage) vs. *in vitro* (release of LDH in rat alveolar macrophage + rat type 2 cell-line co-culture) correlation, using the highest measured response elicited with high doses of the different particles" (Rushton et al., 2010). B: "*In vivo* (number of PMNs/cm2 in rat lung lavage) vs. *in vitro* (release of LDH/cm2 in rat alveolar macrophage + rat type 2 cell-line co-culture) correlation, using the highest response per unit particle surface area" (Rushton et al., 2010). Adapted with permission from Rushton et al. (2010).

the comparison with the potency of a better characterized reference substance using subhuman data (e.g., subcellular, cellular, animal) (Calle and Zaighemi, 2000). The three options and the type of data they can generate are presented in Fig. 4.

3.3.1. Correlation of *in vitro* and *in vivo* data

To assert the predictivity of *in vitro* tests for *in vivo* responses to ENM exposure, a correlation should exist between the results of these two tests (Sayes et al., 2007). Coherently with the fact that most *in vitro* studies represent an acute or sub-acute response, the reference *in vivo* endpoints also assess the acute/sub-acute toxicity of nanoparticles, with a particular focus on lung inflammation (Rushton et al., 2010; Sayes et al., 2007; Han et al., 2012; Duffin et al., 2007).

To verify the existence of a correlation between the responses *in vivo* and *in vitro*, it is fundamental to set a criterion for comparison, i.e. define which doses and responses are assumed to correspond (Han et al., 2012). For example, the considered dose can be the exposure level (Sayes et al., 2007), or the amount of particles associated to the cells (Teeguarden et al., 2014), and can be expressed in mass or surface area (Duffin et al., 2007), while the choice of endpoints to compare is guided by the knowledge on nanoparticles mechanisms of toxicity (Rushton et al., 2010; Lu et al., 2009).

The experimental tests provide, for each particle and according to the chosen criteria, a dose-response curve *in vitro* and *in vivo*; to assess the existence of a correlation for multiple particles, these values need to be combined. To reduce the number of variables, each response can be normalized per unit dose, providing in this way an estimation of the potency of each nanoparticle (Rushton et al., 2010). Since each point with a different slope in a dose-response curve has a different normalized response, Han et al. (2012) proposed to select the point corresponding to the steepest slope, i.e. the maximum response per unit of dose. Following this strategy, the dose-response curve is simplified to a single value representing the most sensitive response, which is induced at medium doses. The correlation is then investigated by comparing the *in vivo* and *in vitro* marginal responses of all the selected nanoparticles.

The results of these studies highlight the effect of expressing the dose and the normalized response in mass dose or in a unit closer to the BED, usually the surface area. Mass unit doses generally showed no correlation (Sayes et al., 2007; Hong et al., 2013), while statistically-significant linear relationships unveiled when the doses were expressed as particle surface area (Rushton et al., 2010; Duffin et al., 2007; Han et al., 2012). In particular, Rushton and colleagues (Rushton et al., 2010) used both original data and the data from Sayes et al. (Sayes et al., 2007) (for which no correlation was found using mass-based doses), and showed good correlations with surface area doses (Fig. 5). Interestingly, all the assessed *in vitro* endpoints were reliable predictors of the *in vivo* effect, even if with different levels of correlation. Even if the assays used different cell lines (or cell-free systems), they were all selected to show the effects of oxidative stress, which is one of the mechanisms of toxicity of nanoparticles (Gerloff et al., 2017).

These studies showed that *in vitro* tests can be used as a predictive tool for acute *in vivo* effects, if relevant endpoints and dose units are selected. Such correlations are not demonstrated for chronic *in vivo* effects, for which comparable *in vitro* tests are rarely available. The

advancement of *in vitro* systems towards set-ups that allow for chronic testing, such as the chronic *in vitro* model for dermal exposure to silver nanoparticles (Comfort et al., 2014), could provide the data to assess the predictivity of *in vitro* tests for chronic effects *in vivo*.

3.3.2. Reaching the target organ: kinetic models

While *in vitro* tests describe the pharmacodynamics of ENM, the pharmacokinetics and respiratory tract dosimetry fields investigate the fate of ENM in the body, to determine whether and in which dose the ENM will come in contact with organs and tissues after the exposure to an external dose (Meibohm and Derendorf, 1997). PBPK models are originally developed in pharmacology to map the distribution of drugs in the whole organism over time, as determined by absorption, distribution, metabolism, and excretion (ADME) processes (Jamei, 2016; Baud, 1998). On the other hand, respiratory tract dosimetry models, such as the MPPD model, restrict their focus to the respiratory system, modelling the deposition and clearance of inhaled particles (Lamon et al., 2019).

The application of these models is double. If the environmental exposure levels to a substance are known, through a kinetic model it is possible to calculate how the substance distributes over time among the organs; on the contrary, applying these models following the reverse dosimetry concept allows to calculate the external dose causing a certain concentration in a specific organ or tissue at a certain time (Chen et al., 2010). Such approaches can support the use of *in vitro* testing in RA and LCA by (i) determining *in vitro* doses that are coherent with environmental exposure levels, (ii) calculating the external dose that generates a response *in vitro* that is relevant for RA and LCA (e.g. extrapolate an EC_{50} or ED_{50} *in vivo* from an EC_{50} *in vitro*), (iii) allowing the comparison and modelling of human kinetics from animal kinetic models (Louisse et al., 2016).

PBPK models estimate the distribution of chemicals inside the body by modelling ADME processes. In the model, the human or animal body is simplified as a set of compartments interconnected by the blood circulatory system, and the distribution of ENM in each compartment over time is modelled by a system of differential equations. The processes that regulate the fate of ENM in the organism are described by physiological parameters, such as the blood flow rate and the organs size, and particle-specific parameters, such as the permeability of organ membranes and the excretion rate (Table 3) (Jones and Rowland-Yeo, 2013).

Compared to traditional chemicals, the distribution of ENM to the organs is not driven only by the transfer of the ENM from the blood through the membrane, but also by the active uptake by the cells of the mononuclear phagocytic system (MPS) (Yuan et al., 2019). The MPS is composed of phagocytic cells such as Kupffer cells, macrophages, and monocytes, which are heterogeneously distributed in the organs (mainly in liver and spleen), and have an immune response function (Gustafson et al., 2015).

The ability of the MPS to recognise and phagocytize a nanoparticle depends on the surface of the particle, for example particles coated with specific proteins or antibodies (opsonins) are more easily recognized than ENM coated with polyethylene glycol (PEG) (Yang et al., 2014). The MPS uptake rate depends also on the level of saturation of these cells and on the concentration of the ENM (Lin et al., 2016; Liang et al., 2016). The dose-dependency of the MPS uptake process affects the equilibrium of the whole system, meaning that different exposure levels will produce a different distribution of ENM in the organs (Lin et al., 2016). This increases the complexity of nano-PBPK models, since the particle-dependent parameters describing the MPS processes are not constant, but are function of the dose.

Nanoparticle-specific parameters are currently extrapolated from *in vivo* studies. To derive MPS parameters valid for a range of exposure levels, different doses should be tested *in vivo* (Lin et al., 2016); this seems unrealistic with the reduction trend in animal testing. However, a solution could be to test a single dose similar to environmental levels, to assure to obtain realistic parameters. To avoid animal testing, a possibility is to use *in vitro* systems that are able to mimic the transport of chemicals or ENM in specific parts of the body (for example membrane models (Aengenheister et al., 2018)) and, using *in vitro-in vivo* extrapolation (IVIVE) approaches, to derive the parameters needed in human PBPK models (Yuan et al., 2019). Whereas this approach seems promising for conventional chemicals, the obstacle with ENM is that their characteristics are modified by the interaction with the biological system, and the ENM reaching an organ can be very different from the ENM that were administered (Caracciolo et al., 2017).

The modification of the ENM in the body represents an additional complication for the use of PBPK models in RA and LCA; in fact, most PBPK models are developed to study the effect of potential nanodrugs administered intra-venously (IV), and not of environmental exposure via inhalation, ingestion or skin contact (Yuan et al., 2019). Except for models where all parameters had been fitted from *in vivo* inhalation or ingestion studies (Li et al., 2016; Sweeney et al., 2015), other models simply extended the IV PBPK models by adding a new compartment for the specific route of exposure (Bachler et al., 2015; Carlander et al., 2016; Bachler et al., 2013). The assumption in these cases is that once inside the body, the transport between compartments will be the same regardless of the entrance point, and therefore parameters calculated for IV administration will be valid also for other exposure routes (Li and Reineke, 2011). This does not mean that the ADME profile will be constant for every exposure route, but that the ENM that reach the circulatory system will follow the same behavior. The validity of this assumption is not certain: on an empirical level, PBPK models developed by fitting only route-dependent parameters were not always successful (Carlander et al., 2016); from a mechanistic perspective, there are indications that the protein corona of ENM varies depending on the exposure route, affecting the fate of the ENM (Kreyling et al., 2014).

The Multiple-Path Particle Dosimetry model (MPPD), available for free at https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304, was developed in 1995 to calculate the

Table 3
The input parameters and the output of the Physiologically-Based Pharmacokinetic Model. Due to the differences in modelling the MPS system, the authors using each one of the MPS parameters are reported. [1]: Liang et al. (2016), [2]: Lin et al. (2016), [3]: Lin et al. (2016), [4]: Cheng et al. (2018), [5]: Li et al. (2016), [6]: Carlander et al. (2016), [7]: Carlander et al. (2018), [8]: Bachler et al. (2013), [9]: Bachler et al. (2015), [10]: Bachler et al. (2015), [11]: Li et al. (2013).

Physiologically-Based Pharmacokinetic Model	
Inputs	
Physiological parameters	Body weight
	Organs weight
	Cardiac output
	Blood flow to organs
	Volume of blood in organs
Nano-specific parameters	Tissue:plasma distribution
	Permeability coefficients
	Biliary/urinary excretion rates
Nano-specific parameters of MPS system	Max uptake rate constant[1,2,3,4,5,6,7]
	Time to reach half of max uptake rate[1,2,3,4]
	Hill coefficient[1,2,3,4]
	Release rate constant[1,2,3,4,6,7,11]
	Uptake capacity per tissue weight[1,2,3,4,5,6,7]
	Uptake constant[8,9,10,11]
	Migration rate inactive MPS cells[11]
Experimental parameters	Exposure dose
	Exposure time
Outputs	
Amount and concentration of ENM over time	In organs
	In organ tissue
	In organ blood
	In organ MPS

Table 4
The input parameters and the output of the Multiple-Path Particle Dosimetry model. Parameters marked with the symbol ∗ have default values provided.

Multiple-Path Particle Dosimetry model	
Inputs	
Airway morphometry	Species∗
	Type of model∗
	Functional residual capacity (FRC)∗
	Upper respiratory tract (URT) volume∗
Aerosol properties	Particle density
	Particles diameter
	Aspect ratio (lenght/diameter)∗
	Inhalability factor (y/n)
	Geometric standard deviation (GSD) of diameter∗
	Equivalent diameter model for irregular-shaped particles (y/n)
Exposure conditions	Constant/Variable exposure
	Clearance (y/n)
Outputs	
Deposited particles	Acinar and Lobar deposition distribution
	Deposition in airway regions
	Regional deposition per particle diameter
	Regional distribution over time
Cleared particles	Clearance in airway regions

deposition of inhaled particles in the airways (Anjilvel and Asgharian, 1995). The MPPD is based on physical and physiological parameters to model sedimentation, diffusion and impaction processes (Table 4). The total amount of deposited particles is affected by the heterogeneous structure of the lungs, the physical characteristics of the inhaled particles, the clearance processes and the air flow.

The model was updated in 2016, and the improvements to the original model and the expanded potential of the new version are reported by Miller et al. (2016). First of all, while the original model was developed for the rat lung (Anjilvel and Asgharian, 1995), it has been expanded to model humans (Asgharian et al., 2001), pigs (Asgharian et al., 2016), monkeys (Asgharian et al., 2012), mice, sheep, and rabbits (Miller et al., 2016), by providing the physiological parameters of each species. Moreover, the new MPPD can model heterogeneous mixtures with up to four subsets of particles with different characteristics (e.g. fractions with dissimilar diameter, density). The model has become more flexible, by allowing the user to modify the standard clearance parameters derived from poorly soluble particles, in this way extending the use to any type of particles and conditions (e.g. diseased subjects with compromised clearance). Last, the results, expressed in mass, can be normalized per unit surface area, per unit time, per unit time per unit area.

The MPPD model is widely used for the study of inhaled particles, including ENM (Kuempel et al., 2015), and its applications encompass the comparison of cell doses causing inflammation *in vivo* and *in vitro* (Teeguarden et al., 2014), the derivation of intake doses from dose-response relationships expressed per mass deposited or mass retained in the lungs (Buist et al., 2017), and the extrapolation of human exposure levels from animal data in risk assessment (Ji and Yu, 2012).

3.3.3. Relative potency factor approach

The relative potency concept is used to express the effect of a substance of interest in relationship to the effect of another substance used as standard reference (Jones et al., 1988). The relative potency factor RPF indicates the dose needed of a substance to generate the same effect as a given dose of a reference substance; usually it is calculated as the ratio between the EC_{50} values of the reference substance and a substance "A" of interest (Villeneuve et al., 2000):

$$RPF = \frac{EC_{50reference}}{EC_{50A}} \quad (1)$$

A relative potency factor is valid only if the following conditions are verified: (i) the substances share the same mechanism of toxicity, (ii) their dose-response curves are parallel, and thus, displaced along the x-axis, (iii) they have an equal maximum achievable response (Putzrath, 1997). Since different endpoints have different dose-response curves (Devito et al., 1994), the relative potency factor is assay-specific (i.e. the relative potency might vary depending on the assay), which makes the choice of endpoint(s) a critical decision (Villeneuve et al., 2002). If the two substances have a common slope, the assumption is that the one of interest behaves like a dilution or concentration of the standard compound (Villeneuve et al., 2002).

The relative potency factor approach has been used in risk assessment by assuming that the relative potency of a substance does not change between human and subhuman systems (Calle and Zaighemi, 2000), i.e. that:

$$\frac{d_{reference}}{d_A} = \frac{D_{reference}}{D_A} \quad (2)$$

With $d_{reference}$ and d_A the doses at subhuman level of respectively the reference and investigated substances, and $D_{reference}$ and D_A the corresponding doses at human level. The relative potency factor is an indirect bioassay: if the required conditions are verified, the assumption of constant relative potency factor can be used to estimate the dose generating a given response at human level (Calle and Zaighemi, 2000), as:

$$d_A = d_{reference} \cdot \frac{D_A}{D_{reference}} \quad (3)$$

It is worth noting that the relative potency factor at human level, i.e. the ratio of the exposure doses of two substances generating a certain effect, inherently includes the contribution of pharmacokinetics to toxicity, while the relative potency obtained from *in vitro* data only considers the pharmacokinetics *in vitro* system, and not in the whole human organism.

3.3.4. In summary, the choice of in vitro-in vivo extrapolation method

In vivo-in vitro correlations, PBPK models, the MPPD model, and the relative potency factor approach can all be used to link a response *in vitro* to an effect at whole organism level. The models have been developed for various ENM (Table 5), however not all of them fulfill the requirements of LCA and RA. The correlation between *in vivo* and *in vitro* results is (for now) verified only for acute responses, which limits its direct use in LCA and RA. However, it highlights how toxic effects are related to the biologically effective dose, such as the surface area dose, showing the importance of the characterization of ENM and the use of models that support this unit (e.g. *in vitro* dosimetry models). The use of the relative potency factor approach depends on the availability of a reference substance that satisfies all the necessary conditions, and on the verification of the assumption of constant relative potency between biological systems. PBPK models have already been used to complement *in vitro* data for RA (Cheng et al., 2018), however the models available for environmental exposure are still scarce (Yuan et al., 2019). The development of new models suffers from the lack of quantitative information about the transformation of ENM in the body, and the effect that this has on ADME processes (Carlander et al., 2016). The MPPD model is an established resource in the field of inhalation studies; it can be readily used for ENM, but its application is limited to the respiratory system and exposure via inhalation (Anjilvel and Asgharian, 1995). Interestingly, both PBPK and MPPD models can support the choice of *in vitro* doses coherent with environmental exposure levels, by estimating the concentrations in human organs and tissues. By applying a reverse dosimetry approach to the EC_{50}, NOAEL, or LOAEL values obtained from *in vitro* assays, the responses *in vitro* can be extrapolated to external exposure doses. Eventually, a relative potency approach can be integrated to derive a

Table 5
The theoretical applicability range of the models and approaches that can be used to extrapolate *in vitro* data to *in vivo* data, and the type of particles that are currently covered. [1]Rushton et al. (2010), [2]Monteiller et al. (2007), [3]Péry et al. (2009), [4]Bachler et al. (2015), [5]Carlander et al. (2016), [6]Bachler et al. (2013), [7]Lankveld et al. (2010), [8]Sweeney et al. (2015), [9]Li et al. (2013), [10]Li et al. (2014), [11]Wenger et al. (2007), [12]Lin et al. (2016), [13]Bachler et al. (2015), [14]Cheng et al. (2018), [15]Mager et al. (2012), [16]Liang et al. (2016), [17]Lin et al. (2008), [18]Li et al. (2016), [19]Carlander et al. (2018), [20]Bi et al. (2018), [21]Lin et al. (2015), [22]Salieri et al. (2020).

Model	Applicability range	Currently covered ENM
Correlation *in vitro-in vivo*	Any ENM	Low-toxicity Low-solubility nanoparticles (acute effects)[1,2]
PBPK models	Any ENM	Technetium carbon nano-particles [3] $TiO_{4,5}$ Silver [6,7] Iridium [8] PAA-peg [5,9,10,11] Gold [5,12,13,14,15] non-soluble nanoparticles [5] Cd-based QD [16,17] CeO[18,19] SPION[20] ZnO[21]
MPPD model	Spherical ENM	Any spherical ENM
Relative potency factor	Any ENM	CuO, ZnO, Silver[22]

systemic *in vivo* response from the tissue- or organ-specific response obtained *in vitro*.

4. Conclusions

Based on our evaluation combined with the analysis of the status of available *in vitro* and *in silico* models, we proposed a pathway for the estimation of *in vivo* NOAEL, LOAEL, EC_{50} or ED_{50} to use in RA and LCA. Starting from a well characterized ENM, the pathway bases the selection of *in vitro* data on risk methodologies requirements, AOP qualitative indications, and experts' knowledge. The application of *in vitro* dosimetry (e.g. through the DG model) is advised for submerged cell cultures. Last, kinetic models (PBPK and MPPD) support the extrapolation to *in vivo* responses.

This new combined use of already existing models is the result of connecting the knowledge of nanotoxicology to the needs of risk methodologies, with the goal of addressing the reduction in animal testing not only from a nanotoxicological perspective, but also with a proactive action from RA and LCA. In fact, since producers (nanotoxicology) and users (risk methodologies) of data do not correspond, an early collaboration can foster a positive feedback loop where data requirements are efficiently met. Risk methodologies can indicate a range of realistic doses to use *in vitro*, based on known environmental concentrations. On the other hand, nanotoxicology can identify the dose-dependency of adverse effects, by providing dose-response curves from which to derive LOAEL or EC_{50} values. When using submerged cell cultures, the application of an *in vitro* dosimetry model should become common practice, by measuring the parameters needed by the model. A similar consideration applies for the parameters and physico-chemical properties required by kinetic models, which could be produced along with toxicity data.

Even though the pathway is, for now, a theoretical proposal, one of the selection criteria was the readiness of models for quantitative use, allowing the pathway to be tested with currently available data. This paves the way for future studies and collaborations, which can apply and refine this strategy to accelerate the evaluation of ENM by RA and LCA methodologies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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