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Aligning nanotoxicology with the 3Rs: What is needed to realise the short, medium and long-term opportunities?

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opportunities?

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Aligning nanotoxicology with the 3Rs

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their development and production for an expanse of applications. While the potential advantages of nanomaterials are clear, concerns over the impact of human and environmental exposure exist. Concerted, science-led efforts are required to understand the effects of nanomaterial exposure and ensure that protection goals are met. There is much on-going discussion regarding how best to assess nanomaterial risk, particularly considering the large number of tests that may be required. A plethora of forms may need to be tested for each nanomaterial, and risk assessed throughout the life cycle, meaning numerous acute and chronic toxicity studies could be required, which is neither practical nor utilises the current evidence-base. Hence, there is scientific, business, ethical and legislative drivers to re-consider the use of animal toxicity tests. An expert Working Group of regulators, academics and industry scientists were gathered by the UK's NC3Rs to discuss: i) opportunities being offered in the short, medium and long-terms to advance nanosafety, ii) how to align these advances with the application of the 3Rs in nanomaterial safety testing, and iii) shifting the focus of risk assessment from current hazard-based approaches towards exposure-driven approaches.

KEY WORDS (max. 6)

3Rs; alternative approaches; nanotoxicology; nanosafety; regulatory testing; *in vitro/in silico*

ABBREVIATIONS

AOP	Adverse outcome pathway
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals

ITS-NANO	Intelligent Testing Strategy for Engineered Nanomaterials
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research
OECD	Organisation for Economic Cooperation and Development
QSAR	Quantitative Structure Activity Relationship
REACH	Registration, Evaluation, Authorisation & restriction of Chemicals
SCCS	European Scientific Committee on Consumer Safety
STIS	short-term inhalation study/studies
SUN	EU FP7 Project “Sustainable Nanotechnologies”

increasingly recognised over recent years. A nanomaterial can be defined as a material which has at least one dimension between 1 and 100 nm in diameter (ISO, 2008). However, there are currently multiple working definitions of a nanomaterial, which means that materials not specifically designed as nanomaterials can in some instances also be classified as “nano”, if for example they contain a fraction in the nano-sized range of >50% of the particle count, as per the EU Recommendation (EC, 2011). There exists a vast array of different nanomaterials and forms that have been placed on the market for numerous applications across a wide range of sectors such as cosmetics, medicine, agriculture, food, textiles, electronics, packaging, and industrial chemicals (e.g. pigments (such as in paints) and construction chemicals; (Nowack, 2015)). Although the many advantages to their use are clear, concerns over their safety remain. In particular it will be useful to consider the following when identifying the potential risks associated with nanomaterials (Stone et al., 2016b):

- What are the potential consequences of nanomaterial exposure for human health and the environment?
- To what degree are humans actually exposed to nanomaterials (i.e., the likelihood that they pose a risk where there is a known hazardous potential)?
- What intrinsic and system-dependent physicochemical properties of nanomaterials confer their toxicity?
- What are the mechanism of actions underlying the toxicity of nanomaterials?
- What are the short and long-term effects of nanomaterial exposure (single, and repeated), and consequences of the bioaccumulation of insoluble and biopersistent nanomaterials?

Data on the hazard potential of nanomaterials is a necessary component of risk assessments (where information from both hazard and exposure assessment are combined to establish safe margins of exposure) and for classification and labelling purposes, to enable registration for marketing and sale.

(528/2012), the EU Cosmetics Regulation 1223/2009/EC and EU Food Additive Regulation (EC 1333/2008). The European Food Safety Authority (EFSA) has also published Guidance on risk assessment of nanomaterials in food/feed and the European Commission's Scientific Committee on Consumer Safety (SCCS) has released Guidance on risk assessment of nanomaterials in cosmetics. The US FDA has also recently published Guidance for Industry Use of Nanomaterials in Food for Animals (FDA, 2015). Authorisations specifically referring to (nano)materials within size boundaries and/or specific forms may imply that each form of a nanomaterial used in regulated products will have to be tested for safety in its own right under the appropriate regulatory framework, even though some of these materials have been in production and use for many years. This approach could lead to extensive testing of different nanomaterial forms, resulting from for example from modifications to their size, geometry, and/or surface coatings. A desire to understand the behaviour of nanomaterials throughout their life cycle/value chain could also potentially contribute towards an increase in the amount of testing to understand the potential hazards to the consumer and the environment at different stages of the lifecycle. Generally, the toxicity testing of nanomaterials and bulk forms for regulatory purposes has been carried out primarily using a prescriptive list of animal studies which have been traditionally used in the risk assessment of chemicals (e.g. studies conducted in line with OECD Test Guidelines; http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm).

There are however increasing pressures to move away from using traditional toxicity testing where possible (EC, 2014). For example, there are emerging legislative bans on the use of animals in cosmetics testing, and there has been much debate within the field around whether the traditional testing strategies for chemical risk assessment are appropriate for nanomaterials (in a broad sense, and related to the suitability of specific assays)(Nel et al., 2013, Silbergeld et al., 2011, Stone et al., 2016a, Aschberger et al., 2016). For the sustainable development and use of nanomaterials, it is

(Oberdorster et al., 2005). Other particle and fibre types, although not necessarily within the nanoscale, have been shown to cause adverse health effects in humans in the past (for example, asbestos, particulate air pollution and crystalline silica quartz). Thus, questions have been posed regarding whether exposure to nanomaterials could cause similar or more harmful effects, due to their small size and potential distribution patterns in the lung and other organs (Donaldson and Borm, 1998, Donaldson et al., 2010, Stoeger et al., 2006).

An expert Working Group of European regulators, academics and industry scientists led by the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) have identified the potential opportunities being offered in the short, medium and long-term to reduce the reliance on traditionally used animal toxicology tests whilst advancing the science of alternative testing strategies towards the risk assessment of nanomaterials. We also explore what is needed from the nanotoxicology community to ensure these endeavours are translated into genuine gains in the science and practice of nanomaterial safety assessment, and consider these issues in the wider legislative context. It is also important to note that the resulting recommendations may also be widely applicable to other areas of risk assessment that are seeking to move away from the use of animal toxicity tests (Burden et al., 2015).

2. The current landscape: *in vivo* testing strategies within the nanotoxicology field

Within the field, there is an increased desire to replace animal testing with alternative testing strategies when assessing nanomaterial toxicity. However there are a number of reasons why some animal toxicity tests will continue to be necessary in the risk assessment of nanomaterials and other (non-cosmetic) chemicals in the next five to ten years. Firstly, despite extensive research efforts,

associated with detection of small quantities of (unlabelled) nanomaterials. Whole organisms continue to be the most scientifically relevant test system as they are capable of capturing effects of nanomaterials after they have been absorbed and distributed (and possibly bio-processed) in the body. Furthermore, standard testing requirements in many regulations demand data from animal experiments, and risk assessors are most experienced, and have most confidence, in interpreting data from animal models. There is also insufficient knowledge of how results generated using non-animal methods compare with data from traditional *in vivo* tests, due to a lack of published studies focused on directly comparing effects seen using alternative models (e.g. *in vitro, in chemico*, invertebrate models) against those observed *in vivo* (e.g. (Snyder-Talkington et al., 2015, Landsiedel et al., 2014b, Krug, 2014)).

The majority of *in vivo* assessments undertaken so far have been intended to assess the effects of inhalation exposure to nanomaterials, as currently the primary populations at risk of exposure to nanomaterials are those working in industry, and thus occupational exposure via inhalation represents a high-priority group (Shatkin and Kim, 2015). Therefore to reflect this exposure route of concern, more pulmonary-orientated research than oral-based studies tends to be performed for nanomaterials (Stone et al., 2016a, Aschberger et al., 2016). Inhalation studies require specialised equipment and are more difficult and expensive to carry out than oral administration studies which are commonly used for other chemicals and products. Hazard assessment of nanomaterials has therefore largely utilised *in vivo* studies carried out using high dose intratracheal instillation, with post-exposure observation periods which are often selected to mimic accumulations resulting from chronic (low dose) exposure. The high doses tested and route of administration employed in these studies are not always relevant to human exposure scenarios, and can result in so-called “overload” of the test system (Morrow, 1988, Oberdorster et al., 2015). To address this, protocols such as short-term *in* vivo inhalation studies (STIS) have been developed and advanced, in order to increase

et al., 2014a). Adoption of the 3Hs protocol in research studies such as the EU funded Sustainable Nanotechnologies (SUN) project (www.sun-fp7.eu/) has reduced the time, financial and ethical implications associated with testing nanomaterial safety, but have not yet eradicated the need for longer term tests (Gosens et al., 2016).

Although the long-term effects of nanomaterial exposure remain a major safety concern, there are few inhalation laboratories equipped to carry out the time consuming and expensive sub-chronic (i.e. 90 day) or chronic (1.5 to 2 year) OECD inhalation tests, and thus there remains limited available animal data on the chronic effects of inhaled nanomaterials, e.g. (Ferin et al., 1992, Pothmann et al., 2015, Kasai et al., 2016). Furthermore, there is uncertainty when extrapolating from short-term *in vivo* studies to chronic effects due to limited knowledge regarding nanomaterial biokinetics and accumulation in the human body, and on the progression of short-term effects into adverse, chronic biological impacts.

Exposure assessments, which aid in the risk assessment process, are carried out with a focus on the release of nanomaterials over the life cycle of the products and actual aerosol concentrations in the air, with less focus on the determination of the internal body/circulating concentrations that result from such exposure (Pelclova et al., 2017). Furthermore, the patterns of exposure are likely to change over coming years as the industry grows. Although inhalation exposure to nanomaterials currently remains the primary portal of entry largely as a result of occupational exposure, effects on consumers following exposure via oral and dermal routes are becoming more relevant due to the wide array of potential applications possible for nanomaterials (e.g. in cosmetics, food or consumer products), and the increase in nanomaterials on the market. Few data are available as yet on uptake and effects through oral and dermal routes (Stone et al., 2016a), as particulate materials including nanomaterials are typically not often absorbed through intact skin (e.g. see SCCS, 2012). This is a

nanomaterials for this purpose is a further concern regarding the testing necessary to determine dermal toxicity of nanomaterials. As many nanomaterials intended for dermal application are most likely to be found within cosmetic products, and cosmetics are no longer allowed to be tested on animals in many regions, viable alternatives to models of *in vivo* dermal exposure will be critical in coming years. In fact, the OECD has issued guidance on an integrated approach to testing and assessment (IATA; OECD 2014) which is based on alternative methods that should be employed when assessing the skin irritation and sensitisation potential of chemicals (OECD, 2014b; OECD 2016a; OECD 2016b), and this IATA should be applied to nanomaterials.

The discussion on how to best assess the safety of NMs throughout their life-cycle may trigger the use of large numbers of animals and resources. Furthermore, insufficient knowledge on how stable nanomaterials are during transit within the body and their fate is adding to uncertainty around the utility of data generated in both *in vivo* and *in vitro* studies. Efforts have begun to investigate the stability/degradation of nanomaterials in relevant "body fluid" environments (e.g. (Kagan et al., 2010, Feliu et al., 2016)) and the influence that the formation of nanomaterial–protein complexes (which occurs following nanomaterial exposure, or during their transit in the body) has on the biological response (e.g. (Lundqvist et al., 2011), although there remains a lack of controlled studies which systemically address these questions. The plethora of nanomaterials/forms requiring investigation also means it is impractical to perform *in vivo s*tudies for every single nanomaterial/form. Furthermore, there are general questions being asked in aligned fields such as traditional chemical risk assessment, regarding whether data generated from animal studies really are the most appropriate means of predicting human hazards (Hartung, 2009).

There are also increasing business and legislative drivers towards the re-evaluation of the use of animal toxicity tests; for example risk assessments for the cosmetics/personal care products industry

223 in the development of novel nanomaterial ingredients if suitable alternative methods for gathering
224 safety data are not sought quickly. Other regulations stipulate that animal tests are only carried out
225 as a last resort, e.g. the European chemicals legislation REACH (Registration, Evaluation,
226 Authorisation & restriction of Chemicals), even though animal toxicity tests remain the standard
227 means to fill the information requirements.

228

229 **3. The vision: aligning the 3Rs with improved safety assessment of nanomaterials**

230 Creating an environment where the use of animals in nanotoxicology is refined, reduced and
231 replaced would help to address societal, business and legislative concerns, and could at the same
232 time could improve the science underlying the safety assessment of nanomaterials. However, a
233 systematic and focused shift towards this vision, and a clearly co-ordinated strategy to enable this
234 will be needed. There is currently an opportunity to create a scientifically-driven paradigm which
235 takes advantage of all the latest scientific and technological developments (Stone et al., 2016b,
236 Hussain et al., 2015) and applies them to promote a "21st century" approach to the risk assessment
237 of nanomaterials. Here we consider the opportunities currently available or under development that
238 within short, medium and long-term timeframes could allow these goals to be achieved.

239

240 **3.1 Immediately, and in the short term (0-5 years): Reduction and refinement of existing**
241 **animal models**

242 It is possible to immediately refine (i.e. minimise pain, suffering, distress or lasting harm) and reduce
243 the numbers of animal tests that are currently carried out to assess the safety of nanomaterials. For
244 example, the application of short-term inhalation studies (Landsiedel et al., 2014a), where rats are

247 further sub-chronic and chronic toxicity tests need to be carried out, and in this way would decrease
248 the number of longer term studies. Indeed, as more data from this type of study becomes available
249 it could be used as a screening and grouping tool and hence reduce the need for 90 day *in vivo*
250 studies altogether. It is worth noting that the progression of effects and chronic outcome may not be
251 detected in such a study e.g. those which result from biopersistence. Therefore it is crucial that
252 considerations around the fitness for purpose of short-term studies are made on a case by case basis
253 (as has been previously shown in (Ferin et al., 1992) and (Oberdörster et al., 1990)). There is also
254 potential to combine several endpoints within each animal study, and determine toxicity at both the
255 exposure site (e.g. lungs) and secondary target site (e.g. liver) to maximise the amount of
256 information obtained from each study (e.g. see (Gosens et al., 2015)). Inhalation studies have been
257 carried out which combine organ toxicity, genotoxicity and (albeit limited) biokinetic examinations
258 (Landsiedel et al., 2014a, Cordelli et al., 2017, Maser et al., 2015). Such an approach is frequently
259 applied to academic *in vivo s*tudies, as shown by several previous studies that have assessed a
260 number of biological responses (e.g. inflammation and oxidative stress) in order to better
261 understand the potential mechanisms underlying the adverse biological impact associated with
262 nanomaterials at different target sites (Cockburn et al., 2012, Poland et al., 2008, Shvedova et al.,
263 2005, Labib et al., 2016). Furthermore, European Commission-funded projects frequently perform *in*
264 *vivo* studies that share tissues between laboratories in order to enable assessment of toxicity at
265 several target sites in one study (e.g. (Kermanizadeh et al., 2016).

266 Increased incorporation of real-life exposure considerations when designing studies will aid in the
267 application of tiered approaches which can be used to prioritise or waive testing. This could mean
268 that nanomaterials are only tested in long-term animal studies if evidence (from *in vitro* testing) has
269 been gathered first which shows that there is a genuine potential risk. In this way assessments
270 would not only explore hazard potential but would also consider whether a) the nanomaterial is

justification for exposure-based waiving (an option under REACH guidelines). Such a concern-driven approach based on realistic exposure information is suggested by the EU-funded "Nano-safety cluster" (Oomen et al., 2014), and considerations of exposure are advised under the Scientific Committee on Consumer Safety (SCCS) Guidance on the safety assessment of nanomaterial in cosmetics, and European Food Safety Agency (EFSA) Guidance on the risk assessment of the applications of nanoscience and nanotechnologies in the food and feed chain. There would be great benefit in utilising evidence from clinical data on nanomaterial effects more widely, particularly to aid understanding around likely human exposure levels, and also when evaluating the predictive nature of both animal and non-animal approaches (see Table 1), although it is unclear how much of this information exists or is likely to be generated in this timeframe. Additional information could come from biomonitoring data from occupational settings, as well as initiatives that provide information on the exposure levels to nanomaterials that are possible following contact with, for example, different cosmetics and food products.

The addition of toxicokinetic analyses to short term *in vivo* studies could help with dose setting for subsequent chronic *in vivo* studies, as is the case for chemicals (Creton et al., 2012). Such analyses could be used to determine the relationship between internal exposure and systemic effects. This information is particularly important considering that internal exposure can be influenced by pre-absorption behaviour of the nanomaterial (e.g. agglomeration/aggregation (Pauluhn, 2010)), or the dose selected, as administration of excessively high doses may lead to higher or lower (agglomeration, and thus) exposures (Oberdorster et al., 2015). These effects highlight the importance of ensuring that the doses selected for testing are relevant to levels likely to be encountered by humans and the environment, and to enable cross-species extrapolation. To date, assessment of nanomaterial biodistribution has relied on the use of labelled (e.g. fluorescent, radioactive) nanomaterials (e.g. (Konduru et al., 2014). Fluorescence labels may produce artefacts in

approach cannot discriminate between particles or ions. Thus new approaches are required to enable the biodistribution of the diverse array of unlabelled nanomaterials to be performed (for example, the use of Coherent Anti-Stokes Raman Scattering (CARS) microscopy to image particle uptake by cells/tissues; (Johnston et al., 2015).

A further area of importance is the current efforts to evaluate, improve and validate current standard *in vitro* test systems for nanomaterial hazard assessment. There is an appreciation that approaches which already have associated OECD Test Guidelines are not always appropriate for nanomaterial testing, and thus there are ongoing activities to address these issues to recommend protocols developed specifically for nanomaterial evaluation (Doak et al., 2012, Pfuhler et al., 2013, OECD, 2014a, Oesch and Landsiedel, 2012, Rasmussen et al., 2016). These efforts will help to redress the problems associated with the relevance and reliability of current *in vitro* assays for nanomaterials, but new test systems may still be required, as it is unlikely that the current models are able to adequately report on all mechanisms leading to adverse effects potentially induced by nanomaterials (Doak et al., 2012, Hirsch et al., 2011). Building knowledge about the mode of action of nanomaterial toxicity (i.e. the cellular and molecular processes driving pathogenicity) will enable informed, evidence based *in vitro* models to be identified, which can be used in the first instance to screen for nanomaterial toxicity and could reduce the number of nanomaterials taken forward for *in vivo* testing. There is also scope to apply knowledge of how other non-nano-sized particles and fibres behave, to identify and inform which responses are of most importance and interest when assessing nanomaterial hazard. The OECD has recommended a testing strategy for assessment of skin irritation and sensitisation which uses models of varied complexity, including *in vitro* and *in chemico* test systems (OECD, 2014b), OECD 2016a, OECD 2016b). These protocols have not been widely applied to nanomaterial risk assessment (e.g. for eye irritation testing see (Kolle et al., 2016), but

3.2 In the medium term (5-10 years): Reduction of animal use through use of existing information, development of more robust, targeted *in vitro* approaches and more predictive computational models

There is scope to leverage existing information to prioritise nanomaterials for testing. One way to achieve this is through grouping, to allow the utilisation of read-across approaches and provide justification for waiving of tests. There is however recognition within the field that the grouping of nanomaterials is complicated and cannot be reliably carried out based on properties such as chemical composition, size or surface coating alone, as the links between these and any adverse biological impacts are complex (Braakhuis et al., 2016). Thus, there has been a need to categorically identify the most appropriate and relevant factors which causally lead to apical endpoints. Currently the most straightforward comparison that can be made is to the bulk counterpart of a nanomaterial, for which there usually exists documented evidence on toxicity and also information on human exposure (Cockburn et al., 2012). So far, a robust structure activity relationship and a good correlation between *in vitro* and *in vivo* studies have been identified for asbestos fibres and carbon nanotubes (Poland et al., 2008, Brown et al., 2007) and work is ongoing to establish such correlations for other types of nanomaterial. Accordingly, existing knowledge on the intrinsic and system-dependent physicochemical properties of nanomaterials which confer toxicity can support evidence based, tiered approaches to testing their pathogenicity. For example in the case of high aspect ratio nanomaterials (HARNs) such as carbon nanotubes (CNTs) fibre length has been correlated to both *in vivo* effects (e.g. inflammation), with increasing fibre length (>5µm) causing greater toxicity (Donaldson et al., 2010). The HARN concept has not yet been adopted for two-dimensional materials, like graphene. This effect has also been observed *in vitro* when macrophages

2013, Brown et al., 2007). Accordingly, for assessing the safety of these types of nanomaterial, the first key step would be identifying fibre length and diameter using (electron) microscopy. It would also be informative to assess the purity of samples through elemental analysis, as iron and nickel contaminants are known to contribute to CNT toxicity (Lam et al., 2004). This would be followed by assessment of *in vitro* macrophage responses (Wiemann et al., 2016) for HARN samples with physicochemical properties of concern (e.g. fibre length, metal content, diameter), followed by targeted *in vivo* testing to confirm *in vitro* findings, and fulfil data requirements (Stone et al., 2016a).

Quantitative Structure Activity Relationship (QSAR) models that can be used for prediction of nanomaterial exposure-dose-responseare currently under development for metal-based nanomaterials (Kleandrova et al., 2014, Winkler et al., 2014). There have also been significant efforts in the field focusing on QSAR models and physiologically based pharmaco-kinetics (PBPK) models to predict *in vivo* nanomaterial exposure hazards for human and aquatic organisms developed in FP7 European projects including SUN, ENPRA, MARINA and MODENA-COST, designed to provide a basis for in *vitro / in vivo* extrapolations (IVIVE)(Speck-Planche et al., 2015, Puzyn et al., 2011, Winkler et al., 2013, Lin et al., 2016, Carlander et al., 2016, Li et al., 2016). However, whilst such computational models can complement experimental work (Horev-Azaria et al., 2011) they cannot, at this time, replace it and there has been limited success in facilitating IVIVE (Lin et al., 2016). For example, as the extrinsic properties of nanomaterials dynamically change according to the biological environment, correlation of *in vivo/in vitro* test results with their pristine structure and/or intrinsic properties (i.e. the classic (Q)SAR approach) is insufficient. Quantitative Structure-Property Relationships (QSPR) therefore need to be established and also represent an area of increasing focus requiring further development as our understanding of nanomaterial behaviour in complex biological environments improves (Winkler et al., 2013, Hristozov et al., 2014). Thus, at this time

The enormous diversity of nanomaterials and models (e.g. mammalian cells, rodents, humans, aquatic organisms, terrestrial organisms, plants, bacteria) that must be considered is a barrier to the fast development of QSARs (Kleandrova et al., 2014). As such, high throughput (HTP) automated systems which can be used to fill data gaps are desirable to enable the generation of sufficiently predictive QSAR models. Relating material properties to biological outcomes will also be useful in read-across approaches, and the large body of data recently released from the OECD (www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm) had potential to contribute relevant information on major nanomaterials that could form part of the reference base for improved read-across (Foss Hansen et al., 2016). Recently a decision making framework for the grouping and testing of nanomaterials for human health assessments has been proposed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" (DF4nanoGrouping; (Arts et al., 2015, Arts et al., 2016)) which aims to ensure that *in vivo* studies are only performed where there are specific data needs, i.e. when read-across cannot be performed, or when the data supporting read-across is not sufficient. The grouping process proposed considers information such as exposure, the characteristics responsible for the functionality of the nanomaterials (e.g. uptake and system-dependent properties including solubility, agglomeration, dispersibility), and cellular effects (i.e. mechanisms of action), and the link between these factors and apical endpoints. Work is ongoing to build confidence in this strategy (RIVM, JRC, and ECHA, 2016; OECD 2016c); other factors that will benefit from further investigation within a grouping approach include: a) the physicochemical characteristics known to drive biological interactions (including shape and surface area of the nanomaterial); b) the ability of the nanomaterial to enter different cellular compartments (thus allowing for the possibility of a variety of biological responses); and c) the number of nanoparticles interacting with cells. The intention is

Expanding the use of *in vitro* approaches that are specifically targeted towards the fulfilment of data requirements could be possible within this time frame. These would include HTP systems to provide information on nanomaterial physicochemical characteristics, hazards and exposure for use in risk assessment (as envisioned by the ITS-NANO framework (Stone et al., 2014)). This requires a shift towards the use of robust, systematic and comprehensive *in vitro* test platforms that provide an indication of uptake and biological effects of nanomaterials specifically over a range of toxicity endpoints, and consideration of how multiple tests can be integrated to allow for accurate predictions of each endpoint (Clift et al., 2011, Stone et al., 2009, DeLoid et al., 2017). In the medium-term such information will be gained through the application of currently used *in vitro* cell-based test systems (e.g., those applied in chemical toxicity tests and used in the nanotoxicology field currently, as reviewed in (Hartung and Sabbioni, 2011)) or adaptations thereof. In combination with data from high throughput screening this approach will help to build confidence in the use of cell-based systems and will contribute to gaining useable knowledge about the biological reactivity of nanomaterials, as well as a better understanding of their toxicological mechanisms. These platforms may also be used as tools in the early screening of candidate nanomaterials to help ensure that potential to induce toxicity is detected and further understood prior to a substance being administered in animal tests (Clift et al., 2014). The animal tests may then be avoided completely if problematic substances are flagged by these screens, or any necessary animal tests could then be better designed and refined. In addition, innovative technologies which utilise microfluidics, such as "lung-on-a-chip" micro-devices that can accurately replicate specific conditions within the human lung (Huh et al., 2012), and those which could mimic passage of nanomaterials from the gut through blood vessels to the liver (such as (Kim et al., 2016) or that developed in the inlivetox project: http://www.inlivetox.eu/), are becoming available and have potential to contribute useful physiologically relevant information. Concomitant to such progression within cell based *in vitro*

development of nano-specific *in vitro* tests, it is conceivable that in the medium-term useful models that are currently available may have progressed towards validation.

While efforts in each of these areas are ongoing, it is important that investment continues into refining and reducing the numbers of animals used in the *in vivo* tests that remain mandatory, and from which information will be used to inform the utility of the new/adapted *in vitro* approaches. For example, developing short-term studies for routes other than inhalation (e.g., short term studies for oral administration are being developed as part of the EU-funded project SUN), and improving the technical aspects of STIS, particularly as aerosol generation and characterisation is demanding. Moreover, it is challenging to model actual lung burdens resulting from aerosol inhalation *in vitro*. However, strides have been taken to close this gap, for example in a recent publication where the occupational exposure of an inhalatory dose of carbon nanotubes could be mimicked based upon their physicochemical characteristics (Chortarea et al., 2015).

3.3 In the long term (10 years +): Replacement with accepted non-animal methods

In the long-term many sectors have a desire to move away completely from using animal toxicity tests towards the use of scientifically and regulatory accepted non-animal approaches which bear greater relevance to humans. Like traditional *in vivo* tests, each non-animal method has its own merits and disadvantages, and it is unlikely that one cell-based assay or computational model will ever replace an existing animal test on a 1:1 basis. Thus, the most appropriate methodologies will need to be applied in an integrated assessment and testing strategy (Landsiedel, 2015), which includes weight of evidence considerations. This will negate the use of a predefined test battery even with suitable *in vitro* methods at hand. This will also mean that data packages may need to be

Exposure considerations will form an important component of such an integrated approach and could start to be addressed *in vitro* through the incorporation of barrier models, which have potential to allow for investigations into nanomaterial uptake and transport (Bachler et al., 2015, Braakhuis et al., 2015, Endes et al., 2015, Garcia-Garcia et al., 2005, George et al., 2015, Rothen-Rutishauser et al., 2007, Gordon et al., 2015). More complex *in vitro* models will also be important in providing information on barrier penetration and translocation capabilities, such as those which comprise more realistic and physiologically relevant systems than the traditional 2D/monolayer methods. This includes cultures of multiple cell types and growing cells in 3D, which has been demonstrated in the "ready to use" EpiDerm™ system, which more accurately mimics skin (although these types of commercial platforms tend to be expensive) (Wills et al., 2016). Also, the use of human or pig skin explants are used to estimate dermal uptake of nanomaterials (Monteiro-Riviere et al., 2013, Fabian et al., 2016). Three-dimensional tissue models demonstrate functional and metabolic properties that could be considered more representative of the *in vivo* environment, as recently suggested for the identification of eye irritation potential of nanomaterials (Kolle et al., 2016) . Consequently, biological response and outcomes seen in 3D and microfluidics models in relation to toxicity endpoints may be very different to those observed in 2D culture systems, which suggests that they may be more physiologically representative (Chapman et al., 2014, Hu et al., 2010, Clift et al., 2014, Snyder-Talkington et al., 2015, Ucciferri et al., 2014). An emphasis on using human cells and tissues in such models where possible will further increase their relevance in the assessment of human safety.

Determining whether the endpoints or biomarkers measured within *in vitro* tests are truly driving the key events that result in adverse effects at an organism level would be facilitated by an increased understanding of mechanisms/modes of action; sufficient acquisition of this type of knowledge

paradigm would also help to identify research and data gaps in the toxicity pathways triggered by harmful nanomaterials. This has started to be explored e.g. see (Wang et al., 2015), and under the auspices of the EU's MARINA, NanoSafetyCluster and ITS-NANO (Stone et al., 2014) projects. Application of pathways-based approaches has the potential to improve mechanistic understanding of nanomaterial effects (Nel et al., 2013), and advance the development and implementation of non-animal methods to determine whether substances are likely to cause the key events that result in adverse outcomes. Again, it is crucial that an exposure element is captured in such an activity, a feature not encompassed by the current AOP paradigm. Reliable and advanced *in silico* models, if progressed through the availability of more hazard and physicochemical data generated for example by high throughput systems, could also offer huge benefits to the field in the long-term, and will be key tools for predicting the likelihood of different nanomaterials to induce the key events within toxicity pathways. Large-scale efforts towards such modelling approaches have already been initiated, with projects such as the COST Action TD1204 Modelling Nanomaterial Toxicity (MODENA): http://www.cost.eu/COST_Actions/mpns/Actions/TD1204.

4. Key objectives to achieve the vision

The ultimate aspiration of aligning the 3Rs principles with nanotoxicology is the efficient and reliable risk assessment of nanomaterials through application of a focused, exposure-driven integrated approach which utilises data from animal studies only where it genuinely adds value and concentrates testing on specific scientific questions, feeding back into safe-by-design nanomaterials. Table 1 outlines the expert group's perspective on the key focus areas resulting from the short, medium and long-term goals and the necessary steps to enable this vision, while Figure 1 summarises the major scientific considerations needed in approaching these objectives. It is worth

(Nowack et al., 2016, Sotiriou et al., 2016, Ding et al., 2017).

5. Outlook

This broad level analysis focuses on how the application of non-animal methods could drive advances in the field of nanotoxicology and the potential next steps to achieve this. The proposals have widespread applicability and are relevant across multiple sectors. By prioritising attention on the key focus areas identified in section 4 we recommend that the toxicology community work together to:

- Evaluate and acknowledge the limitations and uncertainties of all *in vivo* and *in vitro* approaches, both traditional and alternative;
- Provide clarity as to which potential effects can be adequately covered in safety assessment and which potential effects require further research;
- Appreciate that there will never be a single system that is suitable for all nanomaterials - different models/frameworks/integrated approaches (some of which are already available) covering different aspects of several nanomaterials, will prove helpful; ultimately a battery of approaches will cover most nanomaterials;
- Design exposure-driven integrated approaches/decision-making frameworks first then seek the methods that provide the appropriate data for this specific purpose.

Achieving the above will rely on:

- Academic scientists to work on systematically addressing the data gaps identified here, and strategically focus and align research;

- Regulators, to provide guidance on when they can accept non-traditional approaches and data (via case studies, to increase the efficiency of the case-by-case approach that is recommended); and to offer compromise between relying on new approaches and established methods of risk assessment, and adopting non-animal approaches. During the time in which data from both *in vivo* and non-animal tests is being produced, their concurrent consideration will help to maximise understanding of the merits and disadvantages of both approaches;
- Industry, to provide clarity about their needs and requirements, to support the steering of future research efforts.

Finally, the output of these discussions will most likely translate into tangible impacts on the reduction, refinement and replacement of animals with 1) the engagement and support from scientific organisations such as the NC3Rs that is complementary to the efforts of the OECD's Working Party on Nanotechnology, and 2) open, face-to-face discussion and collaboration which incorporates dialogue between all relevant stakeholders (regulators, legislators, funders, industry and academics).

References

ARTS, J. H., HADI, M., IRFAN, M. A., KEENE, A. M., KREILING, R., LYON, D., MAIER, M., MICHEL, K., PETRY, T., SAUER, U. G., WARHEIT, D., WIENCH, K., WOHLLEBEN, W. & LANDSIEDEL, R. 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). *Regul Toxicol Pharmacol,* 71**,** S1-27.

ARTS, J. H., IRFAN, M. A., KEENE, A. M., KREILING, R., LYON, D., MAIER, M., MICHEL, K., NEUBAUER, N., PETRY, T., SAUER, U. G., WARHEIT, D., WIENCH, K., WOHLLEBEN, W. & LANDSIEDEL, R.

ASCHBERGER, K., CHRISTENSEN, F. M., RASMUSSEN, K., JENSEN, K. A., XING, B., VECCHIS, C. D. &
SENESI, N. 2016. Feasibility and challenges of human health risk assessment for engineered nanomaterials. *Engineered Nanoparticles and the Environment: Biophysicochemical Processes and Toxicity,* Chapter 21, 409-441.

BACHLER, G., LOSERT, S., UMEHARA, Y., VON GOETZ, N., RODRIGUEZ-LORENZO, L., PETRI-FINK, A., ROTHEN-RUTISHAUSER, B. & HUNGERBUEHLER, K. 2015. Translocation of gold nanoparticles across the lung epithelial tissue barrier: Combining in vitro and in silico methods to substitute in vivo experiments. *Part Fibre Toxicol,* 12**,** 18.

BRAAKHUIS, H. M., KLOET, S. K., KEZIC, S., KUPER, F., PARK, M. V., BELLMANN, S., VAN DER ZANDE, M., LE GAC, S., KRYSTEK, P., PETERS, R. J., RIETJENS, I. M. & BOUWMEESTER, H. 2015. Progress and future of in vitro models to study translocation of nanoparticles. *Arch Toxicol,* 89**,** 1469-95.

BRAAKHUIS, H. M., OOMEN, A. G. & CASSEE, F. R. 2016. Grouping nanomaterials to predict their potential to induce pulmonary inflammation. *Toxicol Appl Pharmacol,* 299**,** 3-7.

BROWN, D., KINLOCH, I., BANGERT, U., WINDLE, A., WALTER, D., WALKER, G., SCOTCHFORD, C., DONALDSON, K. & STONE, V. 2007. An in vitro study of the potential of carbon nanotubes and nanofibres to induce inflammatory mediators and frustrated phagocytosis. *Carbon,* 45**,** 1743–1756.

BURDEN, N., SEWELL, F. & CHAPMAN, K. 2015. Testing Chemical Safety: What Is Needed to Ensure the Widespread Application of Non-animal Approaches? *PLoS Biol,* 13**,** e1002156.

CARLANDER, U., LI, D., JOLLIET, O., EMOND, C. & JOHANSON, G. 2016. Toward a general physiologically-based pharmacokinetic model for intravenously injected nanoparticles. *International journal of nanomedicine,* 11**,** 625.

reconstructed skin assay and correlation with 2D dose responses. *Mutagenesis,* 29**,** 165-73.

CHORTAREA, S., CLIFT, M. J., VANHECKE, D., ENDES, C., WICK, P., PETRI-FINK, A. & ROTHEN-RUTISHAUSER, B. 2015. Repeated exposure to carbon nanotube-based aerosols does not affect the functional properties of a 3D human epithelial airway model. *Nanotoxicology,* 9**,** 983-93.

CLIFT, M. J., ENDES, C., VANHECKE, D., WICK, P., GEHR, P., SCHINS, R. P., PETRI-FINK, A. & ROTHEN-RUTISHAUSER, B. 2014. A comparative study of different in vitro lung cell culture systems to assess the most beneficial tool for screening the potential adverse effects of carbon nanotubes. *Toxicol Sci,* 137**,** 55-64.

CLIFT, M. J., GEHR, P. & ROTHEN-RUTISHAUSER, B. 2011. Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. *Arch Toxicol,* 85**,** 723-31.

COCKBURN, A., BRADFORD, R., BUCK, N., CONSTABLE, A., EDWARDS, G., HABER, B., HEPBURN, P., HOWLETT, J., KAMPERS, F., KLEIN, C., RADOMSKI, M., STAMM, H., WIJNHOVEN, S. & WILDEMANN, T. 2012. Approaches to the safety assessment of engineered nanomaterials (ENM) in food. *Food Chem Toxicol,* 50**,** 2224-42.

CORDELLI, E., KELLER, J., ELEUTERI, P., VILLANI, P., MA-HOCK, L., SCHULZ, M., LANDSIEDEL, R. & PACCHIEROTTI, F. 2017. No genotoxicity in rat blood cells upon 3- or 6-month inhalation exposure to CeO2 or BaSO4 nanomaterials. *Mutagenesis,* 32**,** 13-22.

CRETON, S., SAGHIR, S. A., BARTELS, M. J., BILLINGTON, R., BUS, J. S., DAVIES, W., DENT, M. P., HAWKSWORTH, G. M., PARRY, S. & TRAVIS, K. Z. 2012. Use of toxicokinetics to support chemical evaluation: Informing high dose selection and study interpretation. *Regul Toxicol Pharmacol,* 62**,** 241-7.

12**,** 355-371.

DING, Y., KUHLBUSCH, T. A., VAN TONGEREN, M., JIMENEZ, A. S., TUINMAN, I., CHEN, R., ALVAREZ, I. L., MIKOLAJCZYK, U., NICKEL, C., MEYER, J., KAMINSKI, H., WOHLLEBEN, W., STAHLMECKE, B., CLAVAGUERA, S. & RIEDIKER, M. 2017. Airborne engineered nanomaterials in the workplace-a review of release and worker exposure during nanomaterial production and handling processes. *J Hazard Mater,* 322**,** 17-28.

DOAK, S. H., MANSHIAN, B., JENKINS, G. J. & SINGH, N. 2012. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. *Mutat Res,* 745**,** 104-11.

DONALDSON, K. & BORM, P. J. 1998. The quartz hazard: a variable entity. *Ann Occup Hyg,* 42**,** 287-94.

DONALDSON, K., MURPHY, F. A., DUFFIN, R. & POLAND, C. A. 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol,* 7**,** 5.

EC 2011. *Commission Recommendation of 18 October 2011 on the definition of nanomaterial (Text with EEA relevance)(2011/696/EU)*.

EC 2014. *Publications Office of the European Union,* Alternative methods for regulatory toxicology – a state-of-the-art review. JRC91361.

ENDES, C., MUELLER, S., KINNEAR, C., VANHECKE, D., FOSTER, E. J., PETRI-FINK, A., WEDER, C., CLIFT, M. J. & ROTHEN-RUTISHAUSER, B. 2015. Fate of cellulose nanocrystal aerosols deposited on the lung cell surface in vitro. *Biomacromolecules,* 16**,** 1267-75.

FABIAN, E., OESCH, F., OTT, K., LANDSIEDEL, R. & VAN RAVENZWAAY, B. 2016. A protocol to determine dermal absorption of xenobiotica through human skin in vitro. *Arch Toxicol*.

FDA 2015. Guidance for Industry Use of Nanomaterials in Food for Animals, 220.

degeneration and the fate of inorganic nanoparticles. *Chem Soc Rev,* 45**,** 2440-57.

FERIN, J., OBERDORSTER, G. & PENNEY, D. P. 1992. Pulmonary retention of ultrafine and fine particles in rats. *Am J Respir Cell Mol Biol,* 6**,** 535-42.

FOSS HANSEN, S., HJORTH, R., SKJOLDING, L. M., BOWMAN, D. M., MAYNARD, A. & BAUNA, A. 2016. A critical analysis of the environmental dossiers from the OECD sponsorship programme for the testing of manufactured nanomaterials. *Environmental Science: Nano,* In press.

GARCIA-GARCIA, E., GIL, S., ANDRIEUX, K., DESMAELE, D., NICOLAS, V., TARAN, F., GEORGIN, D., ANDREUX, J. P., ROUX, F. & COUVREUR, P. 2005. A relevant in vitro rat model for the evaluation of blood-brain barrier translocation of nanoparticles. *Cell Mol Life Sci,* 62**,** 1400-8.

GEORGE, I., VRANIC, S., BOLAND, S., COURTOIS, A. & BAEZA-SQUIBAN, A. 2015. Development of an in vitro model of human bronchial epithelial barrier to study nanoparticle translocation. *Toxicol In Vitro,* 29**,** 51-8.

GORDON, S., DANESHIAN, M., BOUWSTRA, J., CALONI, F., CONSTANT, S., DAVIES, D. E., DANDEKAR, G., GUZMAN, C. A., FABIAN, E., HALTNER, E., HARTUNG, T., HASIWA, N., HAYDEN, P., KANDAROVA, H., KHARE, S., KRUG, H. F., KNEUER, C., LEIST, M., LIAN, G., MARX, U., METZGER, M., OTT, K., PRIETO, P., ROBERTS, M. S., ROGGEN, E. L., TRALAU, T., VAN DEN BRAAK, C., WALLES, H. & LEHR, C. M. 2015. Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. *ALTEX,* 32**,** 327-78.

GOSENS, I., CASSEE, F. R., ZANELLA, M., MANODORI, L., BRUNELLI, A., COSTA, A. L., BOKKERS, B. G., DE JONG, W. H., BROWN, D. & HRISTOZOV, D. 2016. Organ burden and pulmonary toxicity of nano-sized copper (II) oxide particles after short-term inhalation exposure. *Nanotoxicology,* 10**,** 1084-1095.

Comparative hazard identification by a single dose lung exposure of zinc oxide and silver nanomaterials in mice. *PLoS One,* 10**,** e0126934.

HAHN, D., WIEMANN, M., HAASE, A., OSSIG, R., ALESSANDRINI, F., MA-HOCK, L., LANDSIEDEL, R., NERN, M., VENNEMANN, A., DRIESSEN, M., LUCH, A., DOPP, E. & J, S. 2014. Toxicological Effects of Metal Oxide Nanomaterials. *In:* WOHLLEBEN, W. K., TAJ; SCHNEKENBURGER, J; LEHR, C (ed.) *Safety of Nanomaterials along Their Lifecycle.* CRC Press.

HARTUNG, T. 2009. Toxicology for the twenty-first century. *Nature,* 460**,** 208-12.

HARTUNG, T. & SABBIONI, E. 2011. Alternative in vitro assays in nanomaterial toxicology. *Wiley Interdiscip Rev Nanomed Nanobiotechnol,* 3**,** 545-73.

HIRSCH, C., ROESSLEIN, M., KRUG, H. F. & WICK, P. 2011. Nanomaterial cell interactions: are current in vitro tests reliable? *Nanomedicine (Lond),* 6**,** 837-47.

HOREV-AZARIA, L., KIRKPATRICK, C. J., KORENSTEIN, R., MARCHE, P. N., MAIMON, O., PONTI, J., ROMANO, R., ROSSI, F., GOLLA-SCHINDLER, U., SOMMER, D., UBOLDI, C., UNGER, R. E. & VILLIERS, C. 2011. Predictive toxicology of cobalt nanoparticles and ions: comparative in vitro study of different cellular models using methods of knowledge discovery from data. *Toxicol Sci,* 122**,** 489-501.

HRISTOZOV, D. R., GOTTARDO, S., CINELLI, M., ISIGONIS, P., ZABEO, A., CRITTO, A., VAN TONGEREN, M., TRAN, L. & MARCOMINI, A. 2014. Application of a quantitative weight of evidence approach for ranking and prioritising occupational exposure scenarios for titanium dioxide and carbon nanomaterials. *Nanotoxicology,* 8**,** 117-131.

HU, T., KHAMBATTA, Z. S., HAYDEN, P. J., BOLMARCICH, J., BINDER, R. L., ROBINSON, M. K., CARR, G. J., TIESMAN, J. P., JARROLD, B. B., OSBORNE, R., REICHLING, T. D., NEMETH, S. T. & AARDEMA, M. J. 2010. Xenobiotic metabolism gene expression in the EpiDermin vitro 3D human epidermis model compared to human skin. *Toxicol In Vitro,* 24**,** 1450-63.

induced pulmonary edema in a lung-on-a-chip microdevice. *Sci Transl Med,* 4**,** 159ra147.

HUSSAIN, S. M., WARHEIT, D. B., NG, S. P., COMFORT, K. K., GRABINSKI, C. M. & BRAYDICH-STOLLE, L. K. 2015. At the crossroads of nanotoxicology in vitro: past achievements and current challenges. *Toxicological Sciences,* 147**,** 5-16.

ISO 2008. *International Organization for Standardization (ISO) Technical Specification (ISO/TS) 27687:2008; Nanotechnologies—Terminology and definitions for nano-objects—Nanoparticle, nanofibre and nanoplate*.

JOHNSTON, H., POJANA, G., ZUIN, S., JACOBSEN, N. R., MOLLER, P., LOFT, S., SEMMLER-BEHNKE, M., MCGUINESS, C., BALHARRY, D., MARCOMINI, A., WALLIN, H., KREYLING, W., DONALDSON, K., TRAN, L. & STONE, V. 2013. Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges. *Crit Rev Toxicol,* 43**,** 1-20.

JOHNSTON, H. J., MOURAS, R., BROWN, D. M., ELFICK, A. & STONE, V. 2015. Exploring the cellular and tissue uptake of nanomaterials in a range of biological samples using multimodal nonlinear optical microscopy. *Nanotechnology,* 26**,** 505102.

KAGAN, V. E., KONDURU, N. V., FENG, W., ALLEN, B. L., CONROY, J., VOLKOV, Y., VLASOVA, II, BELIKOVA, N. A., YANAMALA, N., KAPRALOV, A., TYURINA, Y. Y., SHI, J., KISIN, E. R., MURRAY, A. R., FRANKS, J., STOLZ, D., GOU, P., KLEIN-SEETHARAMAN, J., FADEEL, B., STAR, A. & SHVEDOVA, A. A. 2010. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. *Nat Nanotechnol,* 5**,** 354-9.

KASAI, T., UMEDA, Y., OHNISHI, M., MINE, T., KONDO, H., TAKEUCHI, T., MATSUMOTO, M. & FUKUSHIMA, S. 2016. Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. *Part Fibre Toxicol,* 13**,** 53.

STOLGER, T., LOFT, S., MOLLER, P., TRAN, L. & STONE, V. 2016. A Multilaboratory
Toxicological Assessment of a Panel of 10 Engineered Nanomaterials to Human Health--
ENPRA Project--The Highlights, Limitations, and Current and Future Challenges. *J Toxicol Environ Health B Crit Rev,* 19**,** 1-28.

KERMANIZADEH, A., POJANA, G., GAISER, B. K., BIRKEDAL, R., BILANICOVA, D., WALLIN, H., JENSEN, K. A., SELLERGREN, B., HUTCHISON, G. R., MARCOMINI, A. & STONE, V. 2013. In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. *Nanotoxicology,* 7**,** 301-13.

KIM, H. J., LI, H., COLLINS, J. J. & INGBER, D. E. 2016. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proceedings of the National Academy of Sciences,* 113**,** E7-E15.

KLEANDROVA, V. V., LUAN, F., GONZALEZ-DIAZ, H., RUSO, J. M., SPECK-PLANCHE, A. & CORDEIRO, M. N. 2014. Computational tool for risk assessment of nanomaterials: novel QSTR-perturbation model for simultaneous prediction of ecotoxicity and cytotoxicity of uncoated and coated nanoparticles under multiple experimental conditions. *Environ Sci Technol,* 48**,** 14686-94.

KOLLE, S. N., SAUER, U. G., MORENO, M. C., TEUBNER, W., WOHLLEBEN, W. & LANDSIEDEL, R. 2016. Eye irritation testing of nanomaterials using the EpiOcular eye irritation test and the bovine corneal opacity and permeability assay. *Part Fibre Toxicol,* 13**,** 18.

KONDURU, N. V., MURDAUGH, K. M., SOTIRIOU, G. A., DONAGHEY, T. C., DEMOKRITOU, P., BRAIN, J. D. & MOLINA, R. M. 2014. Bioavailability, distribution and clearance of tracheally-instilled and gavaged uncoated or silica-coated zinc oxide nanoparticles. *Part Fibre Toxicol,* 11**,** 44.

KRUG, H. F. 2014. Nanosafety research--are we on the right track? *Angew Chem Int Ed Engl,* 53**,** 12304-19.

framework for risk assessment of multi-walled carbon nanotubes. *Part Fibre Toxicol,* 13**,** 15.

LAM, C. W., JAMES, J. T., MCCLUSKEY, R. & HUNTER, R. L. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol Sci,* 77**,** 126-34.

LANDSIEDEL, R. 2015. Concern-driven integrated approaches for the grouping, testing and assessment of nanomaterials. *Environ Pollut*.

LANDSIEDEL, R., FABIAN, E., MA-HOCK, L., VAN RAVENZWAAY, B., WOHLLEBEN, W., WIENCH, K. & OESCH, F. 2012. Toxico-/biokinetics of nanomaterials. *Arch Toxicol,* 86**,** 1021-60.

LANDSIEDEL, R., MA-HOCK, L., HOFMANN, T., WIEMANN, M., STRAUSS, V., TREUMANN, S., WOHLLEBEN, W., GROTERS, S., WIENCH, K. & VAN RAVENZWAAY, B. 2014a. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. *Part Fibre Toxicol,* 11**,** 16.

LANDSIEDEL, R., SAUER, U. G., MA-HOCK, L., SCHNEKENBURGER, J. & WIEMANN, M. 2014b. Pulmonary toxicity of nanomaterials: a critical comparison of published in vitro assays and in vivo inhalation or instillation studies. *Nanomedicine (Lond),* 9**,** 2557-85.

LI, D., MORISHITA, M., WAGNER, J. G., FATOURAIE, M., WOOLDRIDGE, M., EAGLE, W. E., BARRES, J., CARLANDER, U., EMOND, C. & JOLLIET, O. 2016. In vivo biodistribution and physiologically based pharmacokinetic modeling of inhaled fresh and aged cerium oxide nanoparticles in rats. *Particle and fibre toxicology,* 13**,** 45.

LIN, Z., MONTEIRO-RIVIERE, N. A., KANNAN, R. & RIVIERE, J. E. 2016. A computational framework for interspecies pharmacokinetics, exposure and toxicity assessment of gold nanoparticles. *Nanomedicine,* 11**,** 107-119.

ACS Nano, 5**,** 7503-9.

MA-HOCK, L., TREUMANN, S., STRAUSS, V., BRILL, S., LUIZI, F., MERTLER, M., WIENCH, K., GAMER, A. O., VAN RAVENZWAAY, B. & LANDSIEDEL, R. 2009. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. *Toxicol Sci,* 112**,** 468-81.

MASER, E., SCHULZ, M., SAUER, U. G., WIEMANN, M., MA-HOCK, L., WOHLLEBEN, W., HARTWIG, A. & LANDSIEDEL, R. 2015. In vitro and in vivo genotoxicity investigations of differently sized amorphous SiO2 nanomaterials. *Mutat Res Genet Toxicol Environ Mutagen,* 794**,** 57-74.

MONTEIRO-RIVIERE, N. A., SAMBERG, M. E., OLDENBURG, S. J. & RIVIERE, J. E. 2013. Protein binding modulates the cellular uptake of silver nanoparticles into human cells: implications for in vitro to in vivo extrapolations? *Toxicol Lett,* 220**,** 286-93.

MORROW, P. E. 1988. Possible mechanisms to explain dust overloading of the lungs. *Fundam Appl Toxicol,* 10**,** 369-84.

NEL, A. E., NASSER, E., GODWIN, H., AVERY, D., BAHADORI, T., BERGESON, L., BERYT, E., BONNER, J. C., BOVERHOF, D., CARTER, J., CASTRANOVA, V., DESHAZO, J. R., HUSSAIN, S. M., KANE, A. B., KLAESSIG, F., KUEMPEL, E., LAFRANCONI, M., LANDSIEDEL, R., MALLOY, T., MILLER, M. B., MORRIS, J., MOSS, K., OBERDORSTER, G., PINKERTON, K., PLEUS, R. C., SHATKIN, J. A., THOMAS, R., TOLAYMAT, T., WANG, A. & WONG, J. 2013. A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment. *ACS Nano,* 7**,** 6422-33.

NOWACK, B., BOLDRIN, A., CABALLERO, A., HANSEN, S. F., GOTTSCHALK, F., HEGGELUND, L., HENNIG, M., MACKEVICA, A., MAES, H., NAVRATILOVA, J., NEUBAUER, N., PETERS, R., ROSE, J., SCHAFFER, A., SCIFO, L., VAN LEEUWEN, S., VON DER KAMMER, F., WOHLLEBEN, W., WYRWOLL, A. & HRISTOZOV, D. 2016. Meeting the Needs for Released Nanomaterials Required for Further Testing-The SUN Approach. *Environ Sci Technol,* 50**,** 2747-53.

Disposal to the Environment. *In:* VIANA, M. (ed.) *Indoor and Outdoor Nanoparticles: Determinants of Release and Exposure Scenarios.* Springer International Publishing Switzerland

OBERDORSTER, G., CASTRANOVA, V., ASGHARIAN, B. & SAYRE, P. 2015. Inhalation Exposure to Carbon Nanotubes (CNT) and Carbon Nanofibers (CNF): Methodology and Dosimetry. *J Toxicol Environ Health B Crit Rev,* 18**,** 121-212.

OBERDÖRSTER, G., FERIN, J., FINKELSTEIN, J., WADE, P. & CORSON, N. 1990. Increased pulmonary toxicity of ultrafine particles. 2. Lung lavage studies. *Journal of Aerosol Science,* 21**,** 384-387.

OBERDORSTER, G., OBERDORSTER, E. & OBERDORSTER, J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect,* 113**,** 823-39.

OECD 2014a. GENOTOXICITY OF MANUFACTURED NANOMATERIALS : REPORT OF THE OECD EXPERT MEETING. *Series on the Safety of Manufactured Nanomaterials,* 43.

OECD 2014b. New Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation. *Series on Testing and Assessment,* 203.

OECD 2016a. Guidance Document on the Reporting of Defined Approaches to be used within Integrated Approaches to Testing and Assessment. *Series on Testing and Assessment*, 255.

OECD 2016b. Guidance Document of the Reporting of Defined Approaches and Individual Information sources to be Used within Integrated Approaches to Testing and Assessment (IATA) for Skin Sensitisation. *Series on Testing and Assessment*, 256.

OECD 2016c. Categorization of Manufactured Nanomaterials. *Series on Testing and Assessment*, 66.

OESCH, F. & LANDSIEDEL, R. 2012. Genotoxicity investigations on nanomaterials. *Arch Toxicol,* 86**,** 985-94.

OOMEN, A. G., BOS, P. M., FERNANDES, T. F., HUND-RINKE, K., BORASCHI, D., BYRNE, H. J., ASCHBERGER, K., GOTTARDO, S., VON DER KAMMER, F., KUHNEL, D., HRISTOZOV, D.,

the NanoSafety Cluster Working Group 10. *Nanotoxicology,* 8**,** 334-48.

PAULUHN, J. 2010. Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures. *Toxicol Sci,* 113**,** 226-42.

PELCLOVA, D., ZDIMAL, V., KACER, P., ZIKOVA, N., KOMARC, M., FENCLOVA, Z., VLCKOVA, S., SCHWARZ, J., MAKEŠ, O. & SYSLOVA, K. 2017. Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers. *Nanotoxicology,* 11**,** 52-63.

PEPTU, C., ROTARU, R., IGNAT, L., HUMELNICU, A. C., HARABAGIU, V., PEPTU, C. A., LEON, M. M., MITU, F., COJOCARU, E., BOCA, A. & TAMBA, B. I. 2015. Nanotechnology approaches for pain therapy through transdermal drug delivery. *Curr Pharm Des,* 21**,** 6125-39.

PFUHLER, S., ELESPURU, R., AARDEMA, M. J., DOAK, S. H., MARIA DONNER, E., HONMA, M., KIRSCH-VOLDERS, M., LANDSIEDEL, R., MANJANATHA, M., SINGER, T. & KIM, J. H. 2013. Genotoxicity of nanomaterials: refining strategies and tests for hazard identification. *Environ Mol Mutagen,* 54**,** 229-39.

POLAND, C. A., DUFFIN, R., KINLOCH, I., MAYNARD, A., WALLACE, W. A., SEATON, A., STONE, V., BROWN, S., MACNEE, W. & DONALDSON, K. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol,* 3**,** 423-8.

POTHMANN, D., SIMAR, S., SCHULER, D., DONY, E., GAERING, S., LE NET, J. L., OKAZAKI, Y., CHABAGNO, J. M., BESSIBES, C., BEAUSOLEIL, J., NESSLANY, F. & REGNIER, J. F. 2015. Lung inflammation and lack of genotoxicity in the comet and micronucleus assays of industrial multiwalled carbon nanotubes Graphistrength((c)) C100 after a 90-day nose-only inhalation exposure of rats. *Part Fibre Toxicol,* 12**,** 21.

cytotoxicity of metal oxide nanoparticles. *Nature nanotechnology,* 6**,** 175-178.

RASMUSSEN, K., GONZALEZ, M., KEARNS, P., SINTES, J. R., ROSSI, F. & SAYRE, P. 2016. Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. *Regul Toxicol Pharmacol,* 74**,** 147-60.

RICHARZ, A.-N., MADDEN, J., MARCHESE ROBINSON, R., LUBINSKI, L., MOKSHINA, E., URBASZEK, P., KUZ'MIN, V. E., PUZYN, T. & MTD, C. 2015. Development of computational models for the prediction of the toxicity of nanomaterials. . *Perspectives in Science,* 3**,** 27-29.

RIVM, JRC & ECHA. 2016. Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance Elements to consider.

ROTHEN-RUTISHAUSER, B., MUHLFELD, C., BLANK, F., MUSSO, C. & GEHR, P. 2007. Translocation of particles and inflammatory responses after exposure to fine particles and nanoparticles in an epithelial airway model. *Part Fibre Toxicol,* 4**,** 9.

SCCS. 2012. OPINION ON Zinc Oxide (nano form). SCCS/1489/12.

SHATKIN, J. A. & KIM, B. 2015. Cellulose nanomaterials: life cycle risk assessment, and environmental health and safety roadmap. *Environmental Science: Nano,* 2**,** 477-499.

SHVEDOVA, A. A., KISIN, E. R., MERCER, R., MURRAY, A. R., JOHNSON, V. J., POTAPOVICH, A. I., TYURINA, Y. Y., GORELIK, O., AREPALLI, S., SCHWEGLER-BERRY, D., HUBBS, A. F., ANTONINI, J., EVANS, D. E., KU, B. K., RAMSEY, D., MAYNARD, A., KAGAN, V. E., CASTRANOVA, V. & BARON, P. 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol,* 289**,** L698-708.

SILBERGELD, E. K., CONTRERAS, E. Q., HARTUNG, T., HIRSCH, C., HOGBERG, H., JACHAK, A. C., JORDAN, W., LANDSIEDEL, R., MORRIS, J., PATRI, A., POUNDS, J. G., DE VIZCAYA RUIZ, A., SHVEDOVA, A., TANGUAY, R., TATARAZAKO, N., VAN VLIET, E., WALKER, N. J., WIESNER, M.,

nanomaterials. *ALTEX,* 28**,** 230-41.

SNYDER-TALKINGTON, B. N., DONG, C., ZHAO, X., DYMACEK, J., PORTER, D. W., WOLFARTH, M. G., CASTRANOVA, V., QIAN, Y. & GUO, N. L. 2015. Multi-walled carbon nanotube-induced gene expression in vitro: concordance with in vivo studies. *Toxicology,* 328**,** 66-74.

SOTIRIOU, G. A., SINGH, D., ZHANG, F., CHALBOT, M. C., SPIELMAN-SUN, E., HOERING, L., KAVOURAS, I. G., LOWRY, G. V., WOHLLEBEN, W. & DEMOKRITOU, P. 2016. Thermal decomposition of nano-enabled thermoplastics: Possible environmental health and safety implications. *J Hazard Mater,* 305**,** 87-95.

SPECK-PLANCHE, A., KLEANDROVA, V. V., LUAN, F. & DS CORDEIRO, M. N. 2015. Computational modeling in nanomedicine: prediction of multiple antibacterial profiles of nanoparticles using a quantitative structure–activity relationship perturbation model. *Nanomedicine,* 10**,** 193-204.

STOEGER, T., REINHARD, C., TAKENAKA, S., SCHROEPPEL, A., KARG, E., RITTER, B., HEYDER, J. & SCHULZ, H. 2006. Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. *Environ Health Perspect,* 114**,** 328-33.

STONE, V., JOHNSTON, H. & SCHINS, R. P. 2009. Development of in vitro systems for nanotoxicology: methodological considerations. *Crit Rev Toxicol,* 39**,** 613-26.

STONE, V., JOHNSTON, H. J., BALHARRY, D., GERNAND, J. M. & GULUMIAN, M. 2016a. Approaches to Develop Alternative Testing Strategies to Inform Human Health Risk Assessment of Nanomaterials. *Risk Anal,* 36**,** 1538-50.

STONE, V., MILLER, M. R., CLIFT, M. J., ELDER, A., MILLS, N. L., MØLLER, P., SCHINS, R. P., VOGEL, U., KREYLING, W. G. & JENSEN, K. A. 2016b. Nanomaterials vs ambient ultrafine particles: an opportunity to exchange toxicology knowledge. *Environ Health Perspect*.

875 HRISTOZOV, D., HUND-RINKE, K., JOHNSTON, H., MARCOMINI, A., PANZER, O., RONCATO, D.,
876 SABER, A. T., WALLIN, H. & SCOTT-FORDSMAND, J. J. 2014. ITS-NANO--prioritising
877 nanosafety research to develop a stakeholder driven intelligent testing strategy. *Part Fibre*
878 *Toxicol,* 11**,** 9.
879 TANTRA, R., OKSEL, C., PUZYN, T., WANG, J., ROBINSON, K. N., WANG, X. Z., MA, C. Y. & WILKINS, T.
880 2015. Nano(Q)SAR: Challenges, pitfalls and perspectives. *Nanotoxicology,* 9**,** 636-42.
881 UCCIFERRI, N., COLLNOT, E. M., GAISER, B. K., TIRELLA, A., STONE, V., DOMENICI, C., LEHR, C. M. &
882 AHLUWALIA, A. 2014. In vitro toxicological screening of nanoparticles on primary human
883 endothelial cells and the role of flow in modulating cell response. *Nanotoxicology,* 8**,** 697-
884 708.
885 WANG, X., DUCH, M. C., MANSUKHANI, N., JI, Z., LIAO, Y. P., WANG, M., ZHANG, H., SUN, B., CHANG,
886 C. H., LI, R., LIN, S., MENG, H., XIA, T., HERSAM, M. C. & NEL, A. E. 2015. Use of a pro-
887 fibrogenic mechanism-based predictive toxicological approach for tiered testing and decision
888 analysis of carbonaceous nanomaterials. *ACS Nano,* 9**,** 3032-43.
889 WIEMANN, M., VENNEMANN, A., SAUER, U. G., WIENCH, K., MA-HOCK, L. & LANDSIEDEL, R. 2016. An
890 in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of
891 nanomaterials. *J Nanobiotechnology,* 14**,** 16.
892 WILLS, J. W., HONDOW, N., THOMAS, A. D., CHAPMAN, K. E., FISH, D., MAFFEIS, T. G., PENNY, M. W.,
893 BROWN, R. A., JENKINS, G. J., BROWN, A. P., WHITE, P. A. & DOAK, S. H. 2016. Genetic
894 toxicity assessment of engineered nanoparticles using a 3D in vitro skin model (EpiDerm).
895 *Part Fibre Toxicol,* 13**,** 50.
896 WINKLER, D. A., BURDEN, F. R., YAN, B., WEISSLEDER, R., TASSA, C., SHAW, S. & EPA, V. C. 2014.
897 Modelling and predicting the biological effects of nanomaterials. *SAR QSAR Environ Res,* 25**,**
898 161-72.

current status and future potential. *Toxicology,* 313**,** 13-23.

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Key focus areas	Steps to enable focus areas
<u>Regulatory framework</u> Framework established to enable implementation of alternative non-animal methods into risk assessment and acceptance, with built-in recognition that it is likely that no single method for hazard assessment or physicochemical data will suffice in isolation	▪ Developing methods to serve specific data requirements of decision-making frameworks. ▪ Validation/standardisation of (alternative) test methods towards their use in hazard and risk assessment. ▪ Increasing regulatory confidence in results from non-traditional methods (via guidelines, training, workshops, dialogue). ▪ Supporting risk assessors to understand the relevance and applicability of *in vitro* data for risk assessment, particularly as there will be a need for extensive resource and expertise to interpret and integrate data from various sources. ▪ Adoption of a rationale to deal with uncertainties and limitations inherent to experimental models (both *in vitro* and *in vivo*). ▪ Ensuring that uncertainty in the results is reflected clearly by risk assessors. ▪ Applying a weight of evidence approach to consider all available evidence from different non-animal methods.

Hazard prediction Accurate predictions of toxicity that can be confidently linked to physicochemical properties (not only material properties of the pristine material but also functionality of the nanomaterial, e.g. bio-physical interactions of the nanomaterial with its environment (e.g. body fluids))	▪ Adoption of a dual approach: hypothesis driven studies which test if a particular nanomaterial property impacts on toxicity, and studies which compare the toxicity of panels of nanomaterials. These parallel approaches will aim to identify which properties confer toxicity. ▪ Production and easier access to series of systematically altered nanomaterials (e.g. different nanomaterials of the same material with one characteristic altered to enable hypothesis-driven studies to be performed; although we recognise this could prove challenging). ▪ Standardisation of measurements and methods used for nanomaterial characterisation. ▪ Continuation of data sharing on the characterisation of nanomaterials and hazard information in order to document properties and make connections to adverse outcomes, as is taking place within certain EU projects (via round-robin exercises, etc.). ▪ Pooling existing toxicity and physicochemical data and analysing trends to enable predictions, providing the data is comparable and reliable (i.e. all variables are kept the same).

	particulate materials (e.g. silica or asbestos) which are deemed "representative" (dependent on the nanomaterial being studied) and the use of appropriate positive controls to relate the effects of the nanoforms in *vitro*/*in vivo*. This involves ensuring that knowledge already in existence in other areas of particle toxicology is utilised to help build knowledge within the discipline of nanotoxicology. ▪ Development of advanced analytical techniques to ascertain levels of exposure.
<u>IVIVE (*in vitro* to *in vivo* extrapolation)</u> Increased understanding of extrapolation between different *in vivo* and *in vitro* models (both *in vivo* vs. *in vitro* and between different *in vitro* models)	▪ Selection of relevant concentrations in *in vitro* models. ▪ Identification of appropriate positive controls/"benchmark" nanomaterials, and comparable studies undertaken using them; this would be useful in potency ranking for hazard identification. ▪ Incorporation of toxicokinetic aspects into tests to enable consistent assurance that nanomaterials are being taken up, and reaching targets and leading to systemic exposure. ▪ Cross-talk between *in vivo* and *in vitro* scientists and a culture shift away from treating each in isolation; this

	through targeted investment into developing and better understanding the utility of 3D models, fluidic dynamic models and multi-cellular cultures. ▪ Development of *in vitro* models that allow repeat-dosing to be performed. ▪ Taking into account the utility of other emerging technologies that can provide at least a part of the evidence, such as 'omics'. ▪ Enhanced investigation of mode of action of nanomaterial toxicity.
<u>Validation</u> Consensus reached on how best to validate non-animal approaches: against a) animal or b) human data, considering that human is the species in question, and many *in vitro* approaches utilise cells of human origin	▪ For a), generation of sufficient *in vivo* data, to enable comparisons. This should only be carried out when necessary, in situations where the data are critical and meaningful (i.e. ensuring that exposure and test nanomaterial are well characterised, although considering the multitude of possible nanomaterials and exposure routes, this will be difficult to achieve, but may be aided by grouping approaches). ▪ For b), exploitation of clinical/biomonitoring information (i.e. from the welding/mining/tattooing industries), gathering information from workplaces and environments where nanomaterials are used, and building knowledge of precisely the

	food additives.
Mode of action/AOPs Adaptation of current standard *in vitro* approaches and improved test item preparation, dosing, and understanding of toxicity mechanisms; followed by utilisation of the mechanistic data they provide to build AOPs	▪ Concerted efforts to target areas where current *in vitro* methods are not adequate (e.g. alveolar absorption), where the entire range of toxicological responses that would be seen *in vivo* are not captured (e.g. lung toxicity), and on better mimicking the realistic exposure situation including consideration around relevant delivery techniques. ▪ Dedicated programmes of work and entering of relevant AOPs into the AOP Wiki.
Publication standards Raised publication standard so that only high quality, relevant and comparable information is generated in *in vitro* studies	▪ Widespread implementation of standardised protocols e.g. which ensure consistency in cell lines used, facilitated by ring trials. ▪ Studies designed with consideration of the scientific question e.g. relevant delivery methods used and toxicologically relevant endpoints assessed, accounting for system dependent material properties, and consideration of *in vitro* effects on a whole organism level e.g. incorporation of components which reflect distal effects caused following local absorption. ▪ Definition and dissemination of scopes and limitations of the tests including open recognition by

	aspects, and determination of how the predictive capabilities of *in vitro* systems could be utilised in these situations.
<u>QSARs/*in silico* models</u> Necessary characteristics and essential levels of complexity incorporated into computational models	▪ Extensive collaborations between toxicologists, mathematicians and theoretical physicists will produce useable, reliable models. ▪ Expansion of the use of high throughput systems which will enable data gaps to be filled more quickly.

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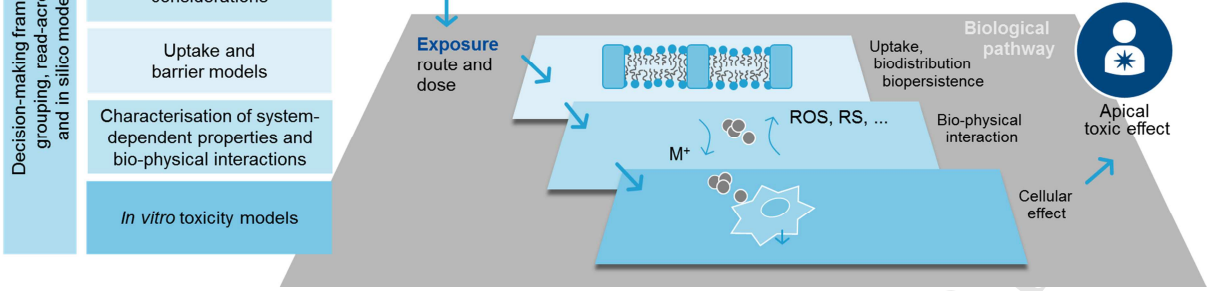
opportunities outlined in section 3. The boxes on the left hand side detail the tools that are necessary towards a) ensuring that intrinsic properties and nanomaterial life cycle are considered in the prioritisation of nanomaterials taken forward into hazard testing, and b) the successful utilisation of non-animal, mechanistic approaches to predict apical toxic effects. Figure adapted from that presented at the second International Congress on Safety of Engineered Nanoparticles and Nanotechnologies (SENN) 2015, Helsinki, Finland by R. Landsiedel.



- Application of non-traditional, alternative methods could improve nanosafety assessment
- There are many short, medium and long-term opportunities to apply the 3Rs within nanotoxicology
- Key focus areas and steps needed to ensure genuine gains are identified.