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### **Paper:**

Burden, N., Aschberger, K., Chaudhry, Q., Clift, M., Fowler, P., Johnston, H., Landsiedel, R., Rowland, J., Stone, V. et. al. (2017). Aligning nanotoxicology with the 3Rs: What is needed to realise the short, medium and long-term opportunities?. *Regulatory Toxicology and Pharmacology*  
<http://dx.doi.org/10.1016/j.yrtph.2017.10.021>

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# Accepted Manuscript

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

Natalie Burden, Karin Aschberger, Qasim Chaudhry, Martin J.D. Clift, Paul Fowler, Helinor Johnston, Robert Landsiedel, Joanna Rowland, Vicki Stone, Shareen H. Doak



PII: S0273-2300(17)30336-7

DOI: [10.1016/j.yrtph.2017.10.021](https://doi.org/10.1016/j.yrtph.2017.10.021)

Reference: YRTPH 3968

To appear in: *Regulatory Toxicology and Pharmacology*

Received Date: 20 February 2017

Revised Date: 24 September 2017

Accepted Date: 19 October 2017

Please cite this article as: Burden, N., Aschberger, K., Chaudhry, Q., Clift, M.J.D., Fowler, P., Johnston, H., Landsiedel, R., Rowland, J., Stone, V., Doak, S.H., Aligning nanotoxicology with the 3Rs: What is needed to realise the short, medium and long-term opportunities?, *Regulatory Toxicology and Pharmacology* (2017), doi: 10.1016/j.yrtph.2017.10.021.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

3 opportunities?

4

5 **RUNNING HEAD**

6 Aligning nanotoxicology with the 3Rs

7

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

48

49 **KEY WORDS (max. 6)**

50 3Rs; alternative approaches; nanotoxicology; nanosafety; regulatory testing; *in vitro/in silico*

51

52 **ABBREVIATIONS**

53 AOP            Adverse outcome pathway

54 ECETOC        European Centre for Ecotoxicology and Toxicology of Chemicals

57	ITS-NANO	Intelligent Testing Strategy for Engineered Nanomaterials
58	NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in
59		Research
60	OECD	Organisation for Economic Cooperation and Development
61	QSAR	Quantitative Structure Activity Relationship
62	REACH	Registration, Evaluation, Authorisation & restriction of Chemicals
63	SCCS	European Scientific Committee on Consumer Safety
64	STIS	short-term inhalation study/studies
65	SUN	EU FP7 Project “Sustainable Nanotechnologies”
66		
67		
68		
69		

72 increasingly recognised over recent years. A nanomaterial can be defined as a material which has at  
73 least one dimension between 1 and 100 nm in diameter (ISO, 2008). However, there are currently  
74 multiple working definitions of a nanomaterial, which means that materials not specifically designed  
75 as nanomaterials can in some instances also be classified as “nano”, if for example they contain a  
76 fraction in the nano-sized range of >50% of the particle count, as per the EU Recommendation (EC,  
77 2011). There exists a vast array of different nanomaterials and forms that have been placed on the  
78 market for numerous applications across a wide range of sectors such as cosmetics, medicine,  
79 agriculture, food, textiles, electronics, packaging, and industrial chemicals (e.g. pigments (such as in  
80 paints) and construction chemicals; (Nowack, 2015)). Although the many advantages to their use are  
81 clear, concerns over their safety remain. In particular it will be useful to consider the following when  
82 identifying the potential risks associated with nanomaterials (Stone et al., 2016b):

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

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

97 (328/2012), the EU Cosmetics Regulation 1223/2009/EC and EU Food Additive Regulation (EC  
98 1333/2008). The European Food Safety Authority (EFSA) has also published Guidance on risk  
99 assessment of nanomaterials in food/feed and the European Commission's Scientific Committee on  
100 Consumer Safety (SCCS) has released Guidance on risk assessment of nanomaterials in cosmetics.  
101 The US FDA has also recently published Guidance for Industry Use of Nanomaterials in Food for  
102 Animals (FDA, 2015). Authorisations specifically referring to (nano)materials within size boundaries  
103 and/or specific forms may imply that each form of a nanomaterial used in regulated products will  
104 have to be tested for safety in its own right under the appropriate regulatory framework, even  
105 though some of these materials have been in production and use for many years. This approach  
106 could lead to extensive testing of different nanomaterial forms, resulting from for example from  
107 modifications to their size, geometry, and/or surface coatings. A desire to understand the behaviour  
108 of nanomaterials throughout their life cycle/value chain could also potentially contribute towards  
109 an increase in the amount of testing to understand the potential hazards to the consumer and the  
110 environment at different stages of the lifecycle. Generally, the toxicity testing of nanomaterials and  
111 bulk forms for regulatory purposes has been carried out primarily using a prescriptive list of animal  
112 studies which have been traditionally used in the risk assessment of chemicals (e.g. studies  
113 conducted in line with OECD Test Guidelines;  
114 <http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm>).

115 There are however increasing pressures to move away from using traditional toxicity testing where  
116 possible (EC, 2014). For example, there are emerging legislative bans on the use of animals in  
117 cosmetics testing, and there has been much debate within the field around whether the traditional  
118 testing strategies for chemical risk assessment are appropriate for nanomaterials (in a broad sense,  
119 and related to the suitability of specific assays)(Nel et al., 2013, Silbergeld et al., 2011, Stone et al.,  
120 2016a, Aschberger et al., 2016). For the sustainable development and use of nanomaterials, it is



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

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

139

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

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

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

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

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

177 Although the long-term effects of nanomaterial exposure remain a major safety concern, there are  
178 few inhalation laboratories equipped to carry out the time consuming and expensive sub-chronic  
179 (i.e. 90 day) or chronic (1.5 to 2 year) OECD inhalation tests, and thus there remains limited available  
180 animal data on the chronic effects of inhaled nanomaterials, e.g. (Ferin et al., 1992, Pothmann et al.,  
181 2015, Kasai et al., 2016). Furthermore, there is uncertainty when extrapolating from short-term *in*  
182 *vivo* studies to chronic effects due to limited knowledge regarding nanomaterial biokinetics and  
183 accumulation in the human body, and on the progression of short-term effects into adverse, chronic  
184 biological impacts.

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

198 nanomaterials for this purpose is a further concern regarding the testing necessary to determine  
199 dermal toxicity of nanomaterials. As many nanomaterials intended for dermal application are most  
200 likely to be found within cosmetic products, and cosmetics are no longer allowed to be tested on  
201 animals in many regions, viable alternatives to models of *in vivo* dermal exposure will be critical in  
202 coming years. In fact, the OECD has issued guidance on an integrated approach to testing and  
203 assessment (IATA; OECD 2014) which is based on alternative methods that should be employed  
204 when assessing the skin irritation and sensitisation potential of chemicals (OECD, 2014b; OECD  
205 2016a; OECD 2016b), and this IATA should be applied to nanomaterials.

206 The discussion on how to best assess the safety of NMs throughout their life-cycle may trigger the  
207 use of large numbers of animals and resources. Furthermore, insufficient knowledge on how stable  
208 nanomaterials are during transit within the body and their fate is adding to uncertainty around the  
209 utility of data generated in both *in vivo* and *in vitro* studies. Efforts have begun to investigate the  
210 stability/degradation of nanomaterials in relevant “body fluid” environments (e.g. (Kagan et al.,  
211 2010, Feliu et al., 2016)) and the influence that the formation of nanomaterial–protein complexes  
212 (which occurs following nanomaterial exposure, or during their transit in the body) has on the  
213 biological response (e.g. (Lundqvist et al., 2011), although there remains a lack of controlled studies  
214 which systemically address these questions. The plethora of nanomaterials/forms requiring  
215 investigation also means it is impractical to perform *in vivo* studies for every single  
216 nanomaterial/form. Furthermore, there are general questions being asked in aligned fields such as  
217 traditional chemical risk assessment, regarding whether data generated from animal studies really  
218 are the most appropriate means of predicting human hazards (Hartung, 2009).

219 There are also increasing business and legislative drivers towards the re-evaluation of the use of  
220 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 “21<sup>st</sup> 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* studies, 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

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

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

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

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



325           **3.2 In the medium term (5-10 years): Reduction of animal use through use of existing**  
326           **information, development of more robust, targeted *in vitro* approaches and more**  
327           **predictive computational models**

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

349 2015, Brown et al., 2007). Accordingly, for assessing the safety of these types of nanomaterial, the  
350 first key step would be identifying fibre length and diameter using (electron) microscopy. It would  
351 also be informative to assess the purity of samples through elemental analysis, as iron and nickel  
352 contaminants are known to contribute to CNT toxicity (Lam et al., 2004). This would be followed by  
353 assessment of *in vitro* macrophage responses (Wiemann et al., 2016) for HARN samples with  
354 physicochemical properties of concern (e.g. fibre length, metal content, diameter), followed by  
355 targeted *in vivo* testing to confirm *in vitro* findings, and fulfil data requirements (Stone et al., 2016a).

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

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

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

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

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

437

### 438 **3.3 In the long term (10 years +): Replacement with accepted non-animal methods**

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

449 Exposure considerations will form an important component of such an integrated approach and  
450 could start to be addressed *in vitro* through the incorporation of barrier models, which have  
451 potential to allow for investigations into nanomaterial uptake and transport (Bachler et al., 2015,  
452 Braakhuis et al., 2015, Endes et al., 2015, Garcia-Garcia et al., 2005, George et al., 2015, Rothen-  
453 Rutishauser et al., 2007, Gordon et al., 2015). More complex *in vitro* models will also be important in  
454 providing information on barrier penetration and translocation capabilities, such as those which  
455 comprise more realistic and physiologically relevant systems than the traditional 2D/monolayer  
456 methods. This includes cultures of multiple cell types and growing cells in 3D, which has been  
457 demonstrated in the “ready to use” EpiDerm™ system, which more accurately mimics skin (although  
458 these types of commercial platforms tend to be expensive) (Wills et al., 2016). Also, the use of  
459 human or pig skin explants are used to estimate dermal uptake of nanomaterials (Monteiro-Riviere  
460 et al., 2013, Fabian et al., 2016). Three-dimensional tissue models demonstrate functional and  
461 metabolic properties that could be considered more representative of the *in vivo* environment, as  
462 recently suggested for the identification of eye irritation potential of nanomaterials (Kolle et al.,  
463 2016) . Consequently, biological response and outcomes seen in 3D and microfluidics models in  
464 relation to toxicity endpoints may be very different to those observed in 2D culture systems, which  
465 suggests that they may be more physiologically representative (Chapman et al., 2014, Hu et al.,  
466 2010, Clift et al., 2014, Snyder-Talkington et al., 2015, Ucciferri et al., 2014). An emphasis on using  
467 human cells and tissues in such models where possible will further increase their relevance in the  
468 assessment of human safety.

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

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

488

#### 489 **4. Key objectives to achieve the vision**

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

500

501 **5. Outlook**

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

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

517

518 Achieving the above will rely on:

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



523 regulators, to provide guidance on when they can accept non-traditional approaches and  
524 data (via case studies, to increase the efficiency of the case-by-case approach that is  
525 recommended); and to offer compromise between relying on new approaches and  
526 established methods of risk assessment, and adopting non-animal approaches. During the  
527 time in which data from both *in vivo* and non-animal tests is being produced, their  
528 concurrent consideration will help to maximise understanding of the merits and  
529 disadvantages of both approaches;

- 530 ■ Industry, to provide clarity about their needs and requirements, to support the steering of  
531 future research efforts.

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

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Key focus areas	Steps to enable focus areas
<p data-bbox="186 186 462 215"><u>Regulatory framework</u></p> <p data-bbox="186 329 592 862">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</p>	<ul style="list-style-type: none"> <li data-bbox="673 186 1364 291">▪ Developing methods to serve specific data requirements of decision-making frameworks.</li> <li data-bbox="673 329 1364 500">▪ Validation/standardisation of (alternative) test methods towards their use in hazard and risk assessment.</li> <li data-bbox="673 538 1364 710">▪ Increasing regulatory confidence in results from non-traditional methods (via guidelines, training, workshops, dialogue).</li> <li data-bbox="673 748 1364 1071">▪ Supporting risk assessors to understand the relevance and applicability of <i>in vitro</i> data for risk assessment, particularly as there will be a need for extensive resource and expertise to interpret and integrate data from various sources.</li> <li data-bbox="673 1110 1364 1281">▪ Adoption of a rationale to deal with uncertainties and limitations inherent to experimental models (both <i>in vitro</i> and <i>in vivo</i>).</li> <li data-bbox="673 1319 1364 1433">▪ Ensuring that uncertainty in the results is reflected clearly by risk assessors.</li> <li data-bbox="673 1471 1364 1643">▪ Applying a weight of evidence approach to consider all available evidence from different non-animal methods.</li> </ul>

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).

	<p>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 <i>vitro/in vivo</i>. 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.</p> <ul style="list-style-type: none"> <li>▪ Development of advanced analytical techniques to ascertain levels of exposure.</li> </ul>
<p><u>IVIVE (<i>in vitro</i> to <i>in vivo</i> extrapolation)</u></p> <p>Increased understanding of extrapolation between different <i>in vivo</i> and <i>in vitro</i> models (both <i>in vivo</i> vs. <i>in vitro</i> and between different <i>in vitro</i> models)</p>	<ul style="list-style-type: none"> <li>▪ Selection of relevant concentrations in <i>in vitro</i> models.</li> <li>▪ Identification of appropriate positive controls/“benchmark” nanomaterials, and comparable studies undertaken using them; this would be useful in potency ranking for hazard identification.</li> <li>▪ Incorporation of toxicokinetic aspects into tests to enable consistent assurance that nanomaterials are being taken up, and reaching targets and leading to systemic exposure.</li> <li>▪ Cross-talk between <i>in vivo</i> and <i>in vitro</i> scientists and a culture shift away from treating each in isolation; this</li> </ul>

	<p>through targeted investment into developing and better understanding the utility of 3D models, fluidic dynamic models and multi-cellular cultures.</p> <ul style="list-style-type: none"> <li>▪ Development of <i>in vitro</i> models that allow repeat-dosing to be performed.</li> <li>▪ Taking into account the utility of other emerging technologies that can provide at least a part of the evidence, such as 'omics'.</li> <li>▪ Enhanced investigation of mode of action of nanomaterial toxicity.</li> </ul>
<p><u>Validation</u></p> <p>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 <i>in vitro</i> approaches utilise cells of human origin</p>	<ul style="list-style-type: none"> <li>▪ For a), generation of sufficient <i>in vivo</i> 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).</li> <li>▪ 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</li> </ul>

<p><u>Mode of action/AOPs</u></p> <p>Adaptation of current standard <i>in vitro</i> approaches and improved test item preparation, dosing, and understanding of toxicity mechanisms; followed by utilisation of the mechanistic data they provide to build AOPs</p>	<p>food additives.</p> <ul style="list-style-type: none"> <li>▪ Concerted efforts to target areas where current <i>in vitro</i> methods are not adequate (e.g. alveolar absorption), where the entire range of toxicological responses that would be seen <i>in vivo</i> are not captured (e.g. lung toxicity), and on better mimicking the realistic exposure situation including consideration around relevant delivery techniques.</li> <li>▪ Dedicated programmes of work and entering of relevant AOPs into the AOP Wiki.</li> </ul>
<p><u>Publication standards</u></p> <p>Raised publication standard so that only high quality, relevant and comparable information is generated in <i>in vitro</i> studies</p>	<ul style="list-style-type: none"> <li>▪ Widespread implementation of standardised protocols e.g. which ensure consistency in cell lines used, facilitated by ring trials.</li> <li>▪ 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 <i>in vitro</i> effects on a whole organism level e.g. incorporation of components which reflect distal effects caused following local absorption.</li> <li>▪ Definition and dissemination of scopes and limitations of the tests including open recognition by</li> </ul>

	aspects, and determination of how the predictive capabilities of <i>in vitro</i> systems could be utilised in these situations.
<u>QSARs/<i>in silico</i> models</u>  Necessary characteristics and essential levels of complexity incorporated into computational models	<ul style="list-style-type: none"><li>▪ Extensive collaborations between toxicologists, mathematicians and theoretical physicists will produce useable, reliable models.</li><li>▪ Expansion of the use of high throughput systems which will enable data gaps to be filled more quickly.</li></ul>

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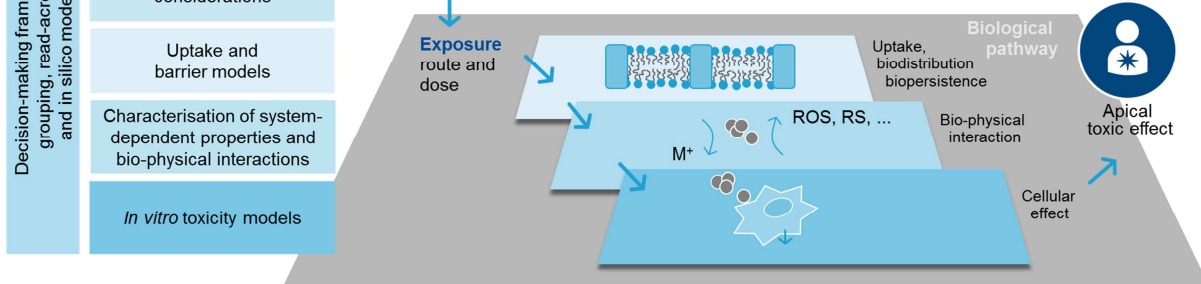
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911 opportunities outlined in section 3. The boxes on the left hand side detail the tools that are  
912 necessary towards a) ensuring that intrinsic properties and nanomaterial life cycle are considered in  
913 the prioritisation of nanomaterials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

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- 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.

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