# Approach toward *In Vitro*-Based Human Toxicity Effect Factors for the Life Cycle Impact Assessment of Inhaled Low-Solubility Particles

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**ABSTRACT:** Today's scarcity of animal toxicological data for nanomaterials could be lifted by substituting *in vivo* data with *in vitro* data to calculate nanomaterials' effect factors (EF) for Life Cycle Assessment (LCA). Here, we present a step-by-step procedure to calculate *in vitro*-to-*in vivo* extrapolation factors to estimate human Benchmark Doses and subsequently *in vitro*-based EFs for several inhaled nonsoluble nanomaterials. Based on mouse data, the *in vitro*-based EF of $TiO_2$ is between $2.76 \cdot 10^{-4}$ and $1.10 \cdot 10^{-3}$ cases/($m^2$/g·kg intake), depending on the aerodynamic size of the particle, which is in good agreement with *in vivo*-based EFs ($1.51 \cdot 10^{-4}$−$5.6 \cdot 10^{-2}$ cases/($m^2$/g·kg intake)). The EF for amorphous silica is in a similar range as for $TiO_2$, but the result is less robust due to only few *in vivo* data available. The results based on rat data are very different, confirming the importance of selecting animal species representative of human responses. The discrepancy between *in vivo* and *in vitro* animal data in terms of availability and quality limits the coverage of further nanomaterials. Systematic testing on human and animal cells is needed to reduce the variability in toxicological response determined by the differences in experimental conditions, thus helping improve the predictivity of *in vitro*-to-*in vivo* extrapolation factors.

**KEYWORDS:** *titanium dioxide, silica, cerium oxide, extrapolation factor, dosimetry, LCA*
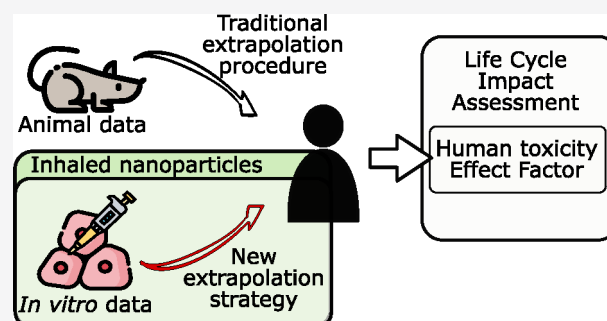
## INTRODUCTION

Nanotechnology has been recognized as one of the Key Enabling Technologies of the 21st century, thanks to its revolutionary applications in multiple sectors, ranging from energy to healthcare.[1] According to the International Organization for Standardization (ISO), nanomaterials are defined as materials "with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale".[2] In parallel to the enthusiasm for their novel functions, the inclusion of nanomaterials in products has also raised concerns about their potential impacts on the health of workers, consumers, and in general humans exposed to them along the product life cycle.[3]

Life Cycle Assessment (LCA) is the preferred methodology to assess the environmental impacts of nanoenabled products and compare them with existing alternatives, accounting for the negative but also positive impacts that a new technology may have on the overall environmental profile of the product (e.g., increased toxicity for humans but reduced greenhouse gas emissions).[4,5]

In LCA, impacts are calculated by linking all emissions occurring during a product life cycle to their corresponding characterization factors, which define the incidence of negative health/ecological effects caused by the emission of a substance. For toxicological impacts (on humans as well as on the ecosystem), the LCA community agreed on the use of USEtox as a common consensus model.[6] Within USEtox, a characterization factor is calculated as a combination of 1) a fate factor, which indicates how a substance is distributed in the environmental compartments following its emission; 2) an exposure factor, which describes the human uptake of the substance from the environmental compartments via multiple exposure pathways; 3) and an effect factor (EF), which relates the uptake of the substance to potential negative health effects.[7] USEtox and its calculation principles have been developed for organic chemicals and metal ions[6,8] and is thus not adequate for nanomaterials in its original setting.[9] A nanospecific fate model has been developed to calculate the fate factor for nanomaterials,[10] while the exposure factor is either calculated according to existing methodologies or disregarded.[11] The EF is calculated from animal toxicological studies using those extrapolation factors (e.g., the interspecies extrapolation factor) needed to convert the animal results to a
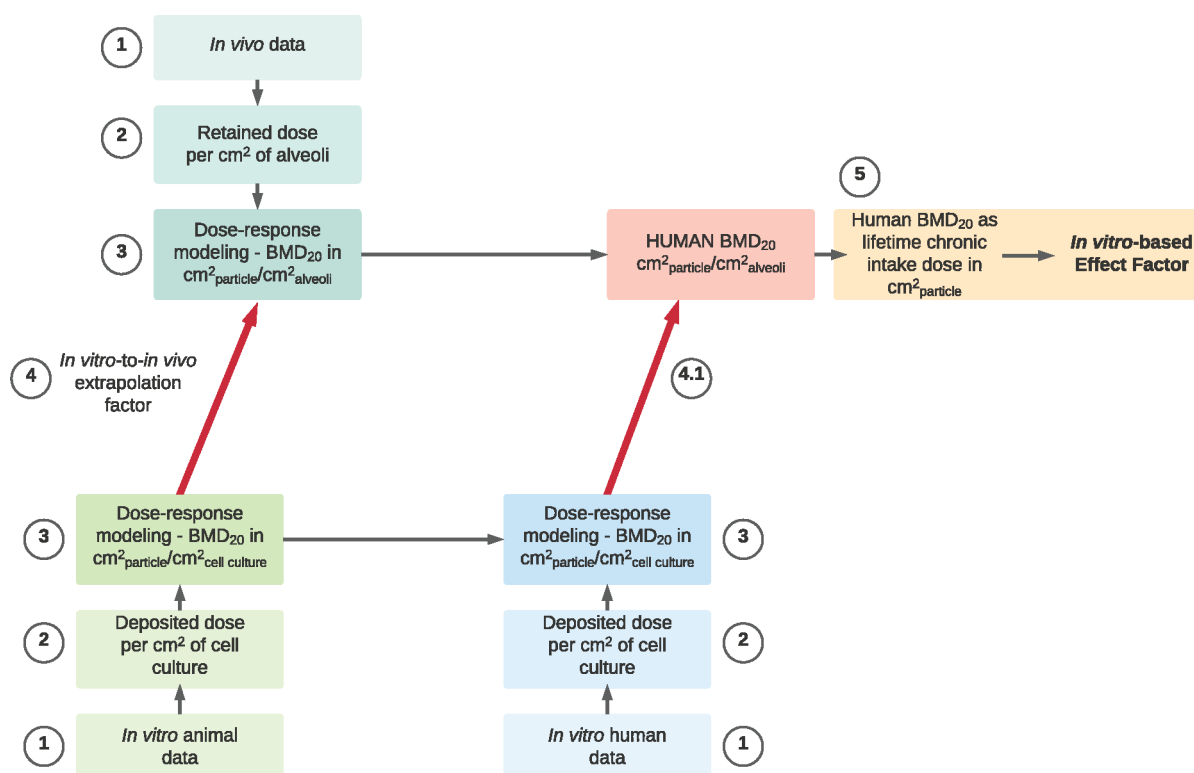
**Figure 1.** Five steps for the calculation of *in vitro*-based human EFs: (1) collection of *in vivo* and *in vitro* (from animal and human cells) data; (2) calculation of the deposited and retained dose per cm$^2$ of cell culture or lung corresponding to the *in vitro* and *in vivo* doses; (3) calculation of the BMD$_{20}$ from the dose−response curves; (4) derivation of the *in vitro*-to-*in vivo* extrapolation factors based on the ratio between animal and *in vitro* animal BMD$_{20}$ values and (4.1) use of the extrapolation factors to calculate the human BMD$_{20}$ from the *in vitro* human BMD$_{20}$; (5) calculation of the *in vitro*-based EF following the traditional extrapolation procedure.

human chronic ED$_{50}$, i.e., the lifetime dose generating a 50% increase in disease probability for humans.[6] Since these extrapolation factors have been obtained based on data for organic chemicals, their validity for nanomaterials is yet to be proven.[12,13] However, a bigger challenge lies upstream: animal testing is being reduced in favor of alternative methods, resulting in a scarcity of toxicological data compared to the number of newly developed nanomaterials.[14]

A potential solution to this could be to use *in vitro* data, i.e., the results of toxicological studies conducted on human cells, as the data pool for the calculation of human toxicity EFs, as suggested by several authors.[15,16] Salieri et al.[17] proposed an approach to calculate EFs for soluble nanoparticles from *in vitro* data, based on the fact that the toxic effects are mainly caused by the dissolved ions rather than the particle itself. This approach is though not fit for nonsoluble particles.

Recently, we proposed that a combination of models could be used for the calculation of EFs from *in vitro* data,[18] and we developed a model to ease the application of this strategy for the specific case of inhaled spherical nanomaterials and their effects on the lung.[19] In this paper, we provide a proof of concept of the estimation of *in vitro*-to-*in vivo* extrapolation factors, and we use these preliminary factors to calculate *in vitro*-based EFs for titanium dioxide, amorphous silica, crystalline silica, and cerium oxide.

## ■ MATERIALS AND METHODS

**Overview of Methodology.** The calculation of *in vitro*-to-*in vivo* extrapolation factors and *in vitro*-based EFs follows multiple steps, depicted in Figure 1. The first step is the

collection of toxicity data from animal studies and from *in vitro* studies using animal and human cells (1). Then dosimetry models are applied to find the deposited doses per well area and the retained doses per alveoli area corresponding to the doses used *in vitro* and *in vivo* (2). The obtained doses are transformed into surface area doses, and a Benchmark Dose (BMD) is then calculated for each dose−response data set (3). The *in vitro*-to-*in vivo* extrapolation factors are then calculated as the ratio between the *in vivo* and *in vitro* animal data (4). A human Benchmark Dose is extrapolated from the *in vitro* human data using the *in vitro*-to-*in vivo* extrapolation factor, following the parallelogram approach, which states that the relationship between animal data and animal cell data is maintained also for humans and human cells (4.1).[20] Finally, the human toxicity EFs are calculated through the traditional extrapolation procedure from the USEtox methodology[6] (5).

The following low-solubility nanomaterials were included in this work: titanium dioxide, in the anatase, rutile, and P25 mixture (≈80% anatase 20% rutile) forms, cerium oxide, amorphous silica, and finally crystalline silica as a representative of a high-toxicity nanomaterial. Titanium dioxide data were grouped for the calculation of the extrapolation factor, to avoid the factor to be based on a single or few data points, while the EF was calculated for both the grouped and the single type TiO$_2$. For both cases, i.e., *in vitro* and *in vivo*, lung inflammation−the release of (pro-)inflammatory factors−was chosen as the relevant end point, since it is considered an important mode of action through which nanomaterials cause toxic effects; moreover, multiple studies showed a correlation between *in vitro* and *in vivo* indicators of inflammation,[21−24]

suggesting that *in vitro* tests may be able to measure early events leading to acute lung inflammation.[25] Acute inflammation may become chronic if the exposure is not halted and the inflammation resolved,[26] and more serious diseases such as lung fibrosis may develop.[27−29]

**Data Collection.** *In Vitro Data.* A literature search was conducted using Google Scholar and Scopus, using various combinations of the following keywords: "nanomaterial name", "*in vitro*", "inflammation", "toxicity", "macrophages"; to find data for human or animal cells, these additional keywords indicating the species or macrophages cell line were used: "mouse", "rat", "murine", "THP-1", "RAW264.7", "J774A.1", "HMDM", "NR8383". Moreover, the data set published in Romeo et al.[19] was also used as a data source.

The criteria for inclusion of data from a study were as follows: a) used a monoculture of human, rat, or mouse macrophages; b) tested spherical particles; c) tested the release of pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6, IL-8, MIP-2); d) included at least two doses plus negative control; e) included all parameters needed for the use of the Combined Dosimetry model CoDo, as described in Romeo et al.[19]

From 26 publications, we extracted 141 dose−response data sets, 59 using human cells, 35 for rat cells, and 47 for mouse cells.

*In Vivo Data.* *In vivo* data was collected from the literature and from the data set published in Romeo et al.[19] using a combination of the following keywords: "nanomaterial name", "rat", 'mouse', "*in vivo*", "toxicity", "lung", "inhalation". The inclusion criteria were as follows: a) rat or mouse as the animal; b) at least two doses tested in addition to the negative control; c) neutrophil (PMN) influx as a number or percentage in Bronchoalveolar Lavage Fluid (BALF) as the end point; d) the exposure time lasted at maximum 1 week; e) the postexposure time was at maximum 72 h if the particles were delivered via an intratracheal instillation; f) either the specific surface area of the particles or the primary particle diameter was reported.

155 dose−response data sets, 109 using rats and 46 using mice, were extracted from 30 publications.

**Simulation of Particle Deposition and Retention.** For *in vitro* data, the Combined Dosimetry model CoDo was used to simulate the deposition of the particles on the cells, determined by sedimentation and diffusion processes.[19] For *in vivo* data, when the particles were administered via inhalation, the Multiple-Path Particle Dosimetry model (MPPD)[30,31] was used to calculate the amount of particles retained in the animal alveoli, while for instillation, we assumed 100% deposition in the lung. The parameters used for both models are reported in the Supporting Information. Whenever possible, the retained dose was preferred to the deposited dose as it has been shown to better correlate with the effects measured in the animal.[32] Both *in vitro* and *in vivo* deposited/retained doses were normalized by the surface area of the cell culture well or the animal alveoli, respectively.

**Calculation of Benchmark Doses.** The Benchmark Dose (BMD) approach was chosen due to its recognition in the risk assessment and LCA communities as a way to determine toxicological dose descriptors from dose−response data sets.[33−35] In this approach, a dose−response curve is fit on the data, and the dose causing a certain response over the control (the Benchmark Response (BMR)) is identified, together with the uncertainty of such a value.[33]

Since the surface area was identified in multiple studies as a more relevant dose metric than mass,[36,37] the deposited/retained doses were transformed from mass to surface area doses using the specific surface area (SSA) of the particles; when not reported, the SSA was calculated from the primary particle diameter of the particles by assuming a perfectly spherical shape. A Benchmark Response (BMR) of 20% was chosen for the BMD calculation, done with the PROAST software.[38,39] The percentage of neutrophils in BALF was considered a quantal response, while other end points were considered continuous responses. Whereas Pennington et al.[34] proposed the use of the $ED_{10}$ or $BMD_{10}$ for the linear extrapolation of risk at low doses when calculating an EF (in place of the $ED_{50}$), we chose a $BMD_{20}$, equivalent to the $ED_{20}$, since such change is considered a sign of low inflammation[40,41] and still resides in the low-dose region of the dose−response curve.[42]

**Calculation of *In Vitro*-to-*In Vivo* Extrapolation Factors.** The calculation of *in vitro*-to-*in vivo* extrapolation factors is done in parallel for each nanomaterial, for rat and mouse animal and cell lines and for the two *in vivo* end points (number of PMN and PMN percentage). For each group of data, we calculated the ratio between each combination of *in vivo* and *in vitro* $BMD_{20}$ values. A nonparametric bootstrapping procedure was applied to estimate the distribution of the median *in vivo*−*in vitro* ratio. The use of the median is more robust compared to the mean for non-normal distributions.[43] Then, we removed the outliers according to the 1.5·IQR rule, which identifies as outliers those points that have a distance from the 0.25 and 0.75 quantiles of at least 1.5 times the interquartile range (IQR).[44] The *in vitro*-to-*in vivo* extrapolation factor estimated via the bootstrapping procedure is the arithmetic mean of the estimated population of ratios, after the removal of outliers.

**Calculation of Human Toxicity EFs from *In Vitro* Data.** For each particle, the calculation of the *in vitro*-based EFs was done following these steps:

1. Calculate the median $BMD_{20}$ from *in vitro* human data via nonparametric bootstrapping;
2. Multiply by the *in vitro*-to-*in vivo* extrapolation factor to obtain the human $BMD_{20}$ in dose per $cm^2$ lung;
3. Multiply by the human alveoli surface area to obtain the total retained dose in the lung;
4. Divide by the retention rate to find the intake dose. The retention rates were calculated via the MPPD model for particles with an aerodynamic diameter between 10 nm and 1 $\mu$m; since the retention rate is not constant over time, a 7-day continuous exposure was chosen (same exposure limit as for the selection of animal studies). The maximum and minimum rates were then used to obtain a range of intake doses;
5. Divide by 7 to find the daily intake dose;
6. Divide by 5 to extrapolate from subacute $BMD_{20}$ to chronic $BMD_{20}$ with the extrapolation factor from Vermeire et al.;[45]
7. Convert to lifetime intake by multiplying by 365 days and 70 years;
8. Convert the lifetime chronic $BMD_{20}$ from $cm^2_{particle}$ lifetime intake to $(m^2_{particle}/g_{particle})\cdot kg_{intake}$, so that the unit of the EF will be consistent with the one of published EFs (see the SI for conversion steps);
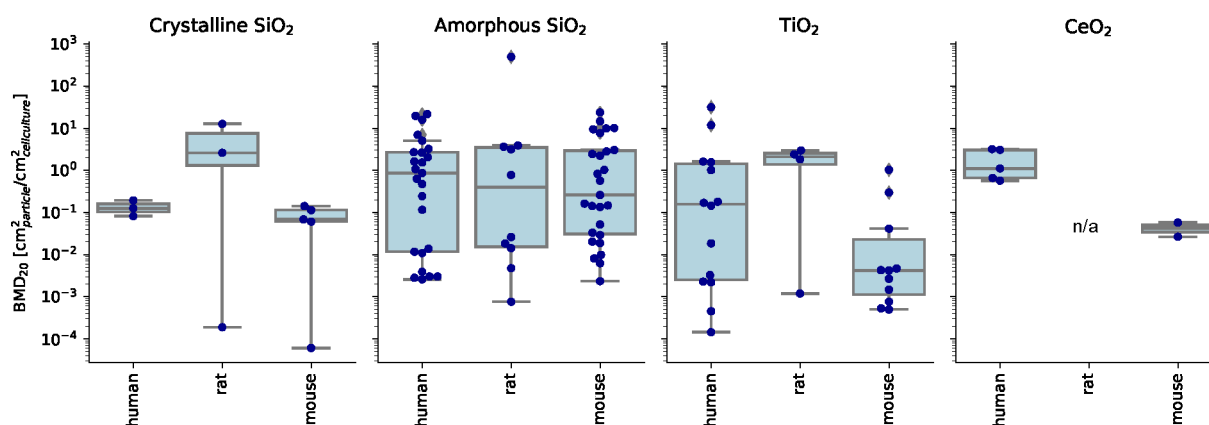
**Figure 2.** Distribution of the $BMD_{20}$ in particle surface area per cell culture area calculated from *in vitro* data for the cytokine release end point, for each particle and cell species. The colored boxes represent the interquartile range, and the whiskers represent 1.5 times the interquartile range.
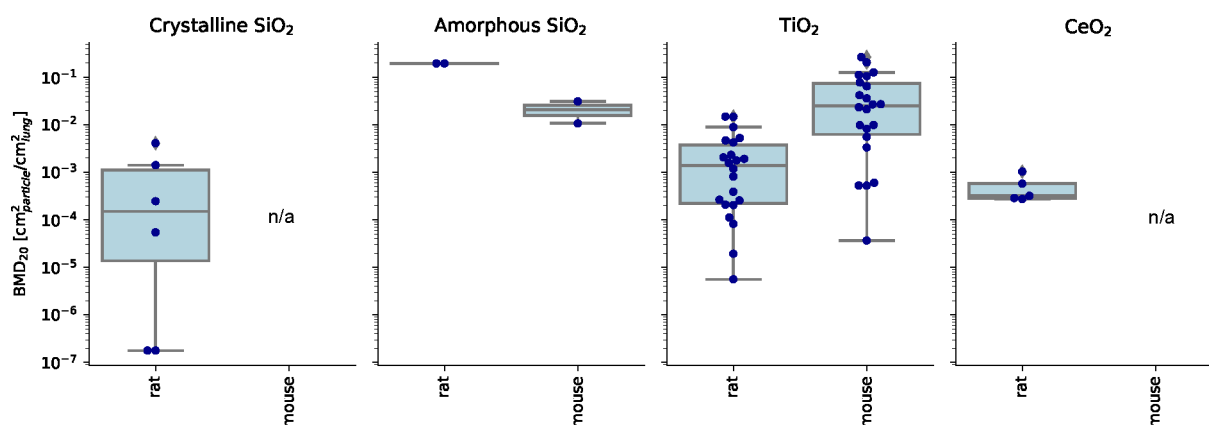


**Figure 3.** Distribution of the $BMD_{20}$ in particle surface area per lung surface area calculated from *in vivo* data for the neutrophil influx end point, for each particle and animal species. The colored boxes represent the interquartile range, and the whiskers represent 1.5 times the interquartile range.

9. Calculate the EF as $0.2$/human $BMD_{20}$ (similar to Pennington et al.,[34] where EF = $0.1/ED_{10}$).

**Calculation of Human Toxicity Effect Factors from Animal Data.** As a comparison, EFs were calculated from the collected animal data:

1. Calculate the median $BMD_{20}$ from animal data via nonparametric bootstrapping;
2. Multiply the median animal $BMD_{20}$ by the animal alveolar surface area to find the total retained dose;
3. Extrapolate to the retained dose in human using the ratio between the human alveoli surface and the animal alveoli surface, as in Fransman et al.[46]

After obtaining the human $BMD_{20}$ as retained dose, the EF was calculated following steps 3 to 8 from the previous section.

**Calculation of Uncertainties.** The uncertainty of LCIA extrapolation factors is expressed by the dispersion factor $k$, which indicates how much a factor $x$ might deviate from the median ($M$), with a 95% probability ($P$):[47]

$$P\left\{\frac{M}{k} < x < M \cdot k\right\} = 0.95 \tag{1}$$

For the *in vitro*-to-*in vivo* extrapolation factors, we calculated the dispersion factors from the 95th percentile of the bootstrap distribution, after the removal of outliers, with the formula from Huijbregts et al.,[48] which does not require any assumption on the shape of the data distribution:

$$k = \sqrt{\frac{97.5\text{th percentile}}{2.5\text{th percentile}}} \tag{2}$$

The uncertainty of the final EF was calculated as a combination of the dispersion factors of the extrapolation factors, according to Slob.[47]

## RESULTS AND DISCUSSION

**Benchmark Dose Values.** 109 $BMD_{20}$ values were obtained from the *in vitro* data, most of them regarding human and mouse cells; the values ranged over multiple orders of magnitude, in particular for the larger data sets, i.e., amorphous silica and titanium dioxide (Figure 2 and Table S1). Such differences were due to the collected data rather than the deposition simulations: the particle concentrations used in the studies ranged from $1 \times 10^{-5}$ to 1.7 mg cm$^3$, and the deposited doses ranged from $1 \times 10^{-6}$ to 0.7 mg cm$^2$ or $1.27-8.8 \times 10^3$ cm$^2$/cm$^2$ when using the surface area dose. We did not observe any trend based on the cytokine considered (TNF-$\alpha$, IL-1$\beta$, IL-6, IL-8, MIP-2), supporting our choice of aggregating them in a unique end point called "cytokine release".

For *in vivo* data, we obtained 103 $BMD_{20}$ values, 59 considering the absolute number of neutrophils as end point (Figure 3) and 44 considering the percentage of neutrophils as end point (Figure S1). Also in this case, the $BMD_{20}$ values had a very broad range (Table S2), which is linked to the original

data rather than the deposition calculation, since the deposition rate was set as constant in the case of instilled nanomaterials and only spread over an order of magnitude for the administration via inhalation.

The wide range of both *in vitro* and *in vivo* $BMD_{20}$ can only be explained by the differences in material properties and experimental conditions of the original studies. The impact of such factors on the biological response has been highlighted in multiple publications.[19,41,49,50]

While the nanomaterials we considered are the most studied, our constraints for the inclusion of data are quite stringent. Only studies with a comprehensive characterization of the particle physicochemical properties were included, since this information was necessary for the simulation of the particle behavior in the *in vitro* system. For example, multiple studies had to be discarded because they did not report the diameter of the agglomerated particle in the media. For the calculation of the $BMD_{20}$, at least two doses plus the control were needed to fit a dose−response curve over the data, thus excluding those studies where only one dose was tested (This was often the case for *in vivo* studies.). Last, those data sets without a clear dose−response relationship were discarded as well by the BMD modeling process. This explains why some nanomaterials and species only have a few data points.

**In Vitro-to-In Vivo Extrapolation Factors.** Table 1 reports the *in vitro*-to-*in vivo* extrapolation factors calculated

**Table 1. In Vitro-to-In Vivo Extrapolation Factors Calculated for the Neutrophil Influx End Point and Their Uncertainty Expressed as Dispersion Factors $k$**

| nanomaterial | species | *in vitro*-to-*in vivo* extrapolation factor | $k$ |
|---|---|---|---|
| crystalline $SiO_2$ | rat | $3.24 \times 10^{-4}$ | 8.89 |
| amorphous $SiO_2$ | rat | 4.73 | 14.5 |
| amorphous $SiO_2$ | mouse | $5.32 \times 10^{-2}$ | 2.77 |
| $TiO_2$ | rat | $1.00 \times 10^{-3}$ | 1.61 |
| $TiO_2$ | mouse | 4.55 | 1.78 |

from rat and mouse data and considering the number of PMN as end point. Cerium oxide had to be excluded since it did not have corresponding *in vitro* and *in vivo* data. The extrapolation factors calculated for neutrophil percentage are available in Table S3.

The extrapolation factors obtained from the ratios of *in vivo* and *in vitro* $BMD_{20}$ values (Figures S2 and S3) via bootstrapping are different for each particle and show a level of uncertainty dependent on the number and variability of the $BMD_{20}$ values. Depending on the species considered,

amorphous silica and titanium dioxide follow opposite trends. Such a difference does not support the hypothesis that a unique extrapolation factor might be valid for low-toxicity low-solubility particles; however, given the wide spread of $BMD_{20}$ values, some data sets (e.g., for amorphous silica) are so small that it is questionable whether they correctly represent the distribution of the $BMD_{20}$. A more reliable approach to test this hypothesis would be to have triads of *in vivo* data, *in vitro* data using mouse cells, and *in vitro* data using human cells obtained by testing in (as much as possible) the same exposure conditions and using the same nanomaterial. In this case, the comparison of the ratios of multiple nanomaterials would not suffer from the large variability of the $BMD_{20}$ values. Unfortunately, the current lack of such fit-for-purpose data prevents us from applying this approach today.

**Human Toxicity Effect Factors from In Vitro Data.** The EFs have been calculated from *in vitro* human data as ranges (Table 2), to account for the effect that the aerodynamic particle size (which in our case was unknown) has on the retention of the particles in the human lung. Considering particles with an aerodynamic diameter between 10 nm and 1 $\mu$m, the retention rate ranged between 6% and 24% of the intake dose considering 7 days of continuous exposure.

The calculated EFs significantly differ depending on which species was used to calculate the *in vitro*-to-*in vivo* extrapolation factors. In the case of rat, the calculated EFs correctly represent the higher toxicity of crystalline silica, but amorphous silica and titanium dioxide, both considered low-toxicity materials, show a great difference in potency, with the latter multiple orders of magnitude more toxic. Looking at the EFs using mouse data, both particles show a similar low toxicity, though no data is available to compare it with crystalline silica. This difference is explained by the fact that titanium dioxide is reported in the data we collected as very inflammogenic for rats *in vivo*, while the same effect was not observed for amorphous silica or for mice.

The higher susceptibility to inhaled nanomaterials of rats compared to mice due to a faster lung overload and a stronger inflammatory response is well-known.[51,52] This suggests that, despite being frequently used in animal studies, the rat might be a precautionary choice rather than a representative one for the effects of particles on human lungs.

**Comparison between In Vitro- and In Vivo-Based Human Toxicity Effect Factors.** Table 3 and Figure 4 show the comparison between the *in vitro*-based EFs, the *in vivo*-based EFs calculated from the same animal data used for the *in vitro*-to-*in vivo* extrapolation factors (see also Table S4), and the EFs available from the literature and obtained from chronic or subchronic animal studies.

**Table 2. Human Toxicity EFs Calculated from In Vitro Data, Expressed as cases/(m²/g·kg intake), Based on the In Vitro-to-In Vivo Extrapolation Factors Respectively Obtained from Rat and Mouse Data[a]**

| nanomaterial | type | no. of data points | median *in vitro* human $BMD_{20}$ | EF extrapolated from rat data | $k_{rat}$ EF | EF extrapolated from mouse data | $k_{mouse}$ EF |
|---|---|---|---|---|---|---|---|
| crystalline $SiO_2$ | | 3 | 0.132 | 4.85−19.4 | 27.3 | | |
| amorphous $SiO_2$ | | 25 | 0.954 | $4.60 \times 10^{-5}$−$1.84 \times 10^{-4}$ | 38.5 | $4.07 \times 10^{-3}$−$1.63 \times 10^{-2}$ | 14.7 |
| $TiO_2$ | anatase + rutile | 13 | 0.160 | 1.30−5.18 | 12.6 | $2.76 \times 10^{-4}$−$1.10 \times 10^{-3}$ | 12.8 |
| $TiO_2$ | anatase | 7 | 0.361 | 0.58−2.30 | 13 | $1.40 \times 10^{-4}$−$5.61 \times 10^{-4}$ | 12.8 |
| $TiO_2$ | rutile | 6 | 0.430 | 0.48−1.93 | 12.6 | $1.09 \times 10^{-4}$−$4.35 \times 10^{-4}$ | 12.8 |
| $TiO_2$ | P25 | 1 | 0.0185 | 11.2−44.9 | 12.6 | $2.47 \times 10^{-3}$−$9.89 \times 10^{-3}$ | 12.8 |

[a]The uncertainties $k$ of the EF represent the combination of the uncertainties of all extrapolation factors used to calculate the EF.

**Table 3. Comparison between the *In Vitro*-Based EFs (Highlighted in Light Blue), the *In Vivo*-Based EFs Calculated from Our Data Set, and the EFs Available from the Literature[a]**

| | $TiO_2$ | Amorphous $SiO_2$ | Crystalline $SiO_2$ | $CeO_2$ |
|---|---|---|---|---|
| *In vitro*-based EF extrapolated from rat data | 1.30 - 5.18 | $4.60 \cdot 10^{-5}$ - $1.84 \cdot 10^{-4}$ | 4.85 - 19.4 | - |
| *In vitro*-based EF extrapolated from mouse data | $2.76 \cdot 10^{-4}$ - $1.10 \cdot 10^{-3}$ | $4.07 \cdot 10^{-3}$ - $1.63 \cdot 10^{-2}$ | - | - |
| *In vivo*-based EF from rat data | 0.181 - 0.723 | $1.05 \cdot 10^{-3}$ - $4.22 \cdot 10^{-3}$ | 0.630 - 2.52 | 0.646 - 2.58 |
| *In vivo*-based EF from mouse data | $8.87 \cdot 10^{-3}$ - $3.55 \cdot 10^{-2}$ | $9.88 \cdot 10^{-3}$ - $3.95 \cdot 10^{-2}$ | - | - |
| EF from published studies | $5.6 \cdot 10^{-2}$ from [53] $1.51 \cdot 10^{-4}$ from [54] $2.40 \cdot 10^{-2}$ from [55] | - | - | - |

[a]All EFs are reported as cases/($m^2$/g·kg intake). When the EF was not reported with respect to the particle surface area, a default specific surface area of 48 $m^2$/g was used, as in Buist et al.[53]
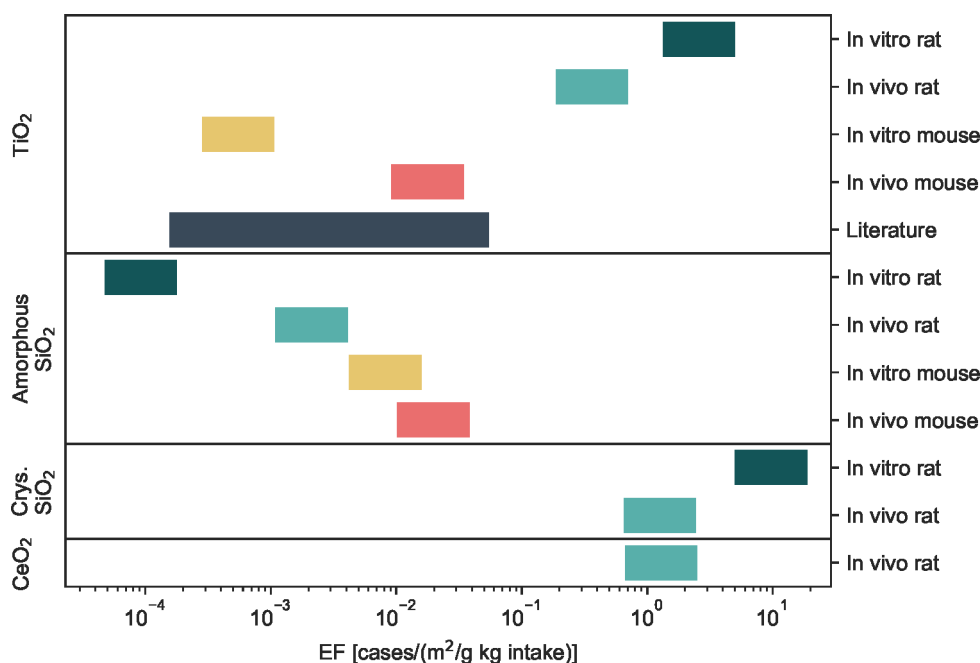


**Figure 4.** Comparison between the ranges of EFs (on a log scale) calculated from *in vitro* rat and mouse data, *in vivo* rat and mouse data (our data sets), and the EFs available from the literature. When the EF was not reported with respect to the particle surface area, a default specific surface area of 48 $m^2$/g was used, as in Buist et al.[53] Crys. $SiO_2$ = crystalline silica.

The results show a good agreement between the *in vitro* EFs and the *in vivo* EFs we calculated; while these values are partially correlated, since the *in vivo* $BMD_{20}$ values are used to calculate the *in vitro*-to-*in vivo* extrapolation factors, it is also true that the extrapolation factor depends also on the *in vitro* animal $BMD_{20}$ values and that different extrapolation procedures are used for the two data sources to calculate the EFs. When comparing also with the published EFs for titanium dioxide, both our *in vitro* and *in vivo* EFs fall in the same range when mouse data is used, while the EFs based on rat data confirm the strong response this species has to this nanomaterial. No EFs have been published for the other materials, but the good correspondence between our calculated

*in vitro* and *in vivo* EFs for amorphous silica and titanium dioxide from mouse data suggests this might be a representative result as well.

Another interesting point is that while our data was restricted to short-term inflammation, published data referred to longer term studies looking at a variety of effects such as alveolar epithelial cell hypertrophy, cell necrosis, histopathological findings, and neutrophil levels in BALF and often considered the No Observed Adverse Effect Level (NOAEL) or the Lowest Observed Adverse Effect Level (LOAEL) rather than an $ED_{50}$. This seems to suggest that short-term effects might be predictive of more chronic effects (for which they are suggested to be necessary but not sufficient precursors, see,

e.g., the proposed Adverse Outcome Pathway for lung fibrosis[56]), even though more analyses are needed to confirm this hypothesis.

**Implications for Further Research.** Published experimental studies suggest that inflammation might be a promising predictive end point to be tested *in vitro*.[22,57−59] Other studies point out that the surface area better correlates with the lung effect of nanomaterials compared to mass doses.[24,36,60,61] Multiple studies address the importance of considering the deposited dose *in vitro* instead of the nanomaterial concentration for a better characterization of the dose−response relationship.[62−64] We put together these pieces of information in developing our EF calculation strategy and tested it with data collected from the literature. The goal was to calculate *in vitro*-to-*in vivo* extrapolation factors, which can then be used similarly to any other extrapolation factor for the estimate of human toxicity EFs. Ideally, once an *in vitro*-to-*in vivo* extrapolation factor has been estimated and its predictive power confirmed for multiple nanomaterials, there would be no need for animal and *in vitro* animal data but only for *in vitro* human data.

The comparison with published EFs can be used as a benchmark for the *in vitro*-based EFs, to understand whether this new data pool provides comparable results. This was the case for titanium dioxide when using the *in vitro*-to-*in vivo* extrapolation factor based on mouse data, where the EF is in the same range as published values. The good coverage of the *in vivo* and *in vitro* data used to calculate the extrapolation factor for titanium dioxide makes the factor more robust, since the real distribution of the $BMD_{20}$ values is better approximated by our samples.

Despite this promising result, the difficulty in calculating the extrapolation factors for the other nanomaterials shows the limitations of applying our approach with the currently available data. The main challenge we face is the quality and consistency of the toxicological data. For example, only a few $BMD_{20}$ values were available for amorphous silica *in vivo*, which questions the reliability of the extrapolation factor; even worse, for cerium oxide there were no corresponding *in vivo* and *in vitro* data, preventing the calculation of any factor. The wide toxicity range of *in vitro* and *in vivo* data confirms that the particle properties and the experimental conditions can have a huge impact on the results, hindering their comparison. Using a median $BMD_{20}$ obtained from a large data set is a better choice than using a single value from a specific study and allows keeping track of the $BMD_{20}$ uncertainty, but it can produce skewed results when the data is scarce and is not a representative sample of the $BMD_{20}$ distribution.

For the *in vitro*-to-*in vivo* extrapolation factors, coupled *in vitro* and *in vivo* data (i.e., obtained using similar particles and experimental conditions) for animals, animal cells, and human cells are needed to verify the parallelogram approach and investigate whether a single extrapolation factor might be valid for multiple particles. Moreover, removing the variability connected to the differences in experimental conditions would reduce the amount of data required to describe the distribution of the $BMD_{20}$ values, as we would expect the values to be more precise. However, on a provisional level and keeping in mind the factor 10 uncertainty, an *in vitro*-to-*in vivo* extrapolation factor of ca. $5 \times 10^{-1}$ (halfway between the $TiO_2$ and amorphous silica factors) might be used to compare non-soluble particles, especially if the *in vitro* human data has a high level of comparability (e.g., same experimental conditions).

In conclusion, we are not yet there for a consistent and systematic calculation of *in vitro*-based EFs. However, we showed a promising method to calculate these factors and identified which further steps are needed to reduce the uncertainty and improve and expand the results. One example above all, the conduction of fit-for-purpose *in vitro* experiments on human and animal cells, could be done systematically for multiple particles, thus providing a way to refine and test further our procedure without the need for animal testing.

We believe that our work not only can help direct future interdisciplinary efforts to tackle the critical aspects of the use of *in vitro* data in LCIA but also be of interest for the Risk Assessment community, which is facing similar challenges in extrapolating human responses without the use of animal data.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01816.

> Additional methodological details and formulas, figures, and tables reporting result details (PDF)

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### Notes

The authors declare no competing financial interest.

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