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Reference Chemical Potency List (RCPL): A new tool for evaluating the accuracy of skin sensitisation potency measurements by New Approach Methodologies (NAMs)

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ABSTRACT

Considerable progress has been made in the design of New Approach Methodologies (NAMs) for the hazard identification of skin sensitising chemicals. However, effective risk assessment requires accurate measurement of sensitising potency, and this has proven more difficult to achieve without recourse to animal tests.

One important requirement for the development and adoption of novel approaches for this purpose is the availability of reliable databases for determining the accuracy with which sensitising potency can be predicted. Some previous approaches have relied on comparisons with potency estimates based on either human or animal (local lymph node assay) data. In contrast, we here describe the development of a carefully curated Reference Chemical Potency List (RCPL) which is based on consideration of the best available human and animal data.

The RCPL is comprised of 33 readily available chemicals that span a wide range of chemistry and sensitising potency, and contain examples of both direct and indirect (pre- and pro-) haptens. For each chemical a potency value (PV) was derived, and chemicals ranked according to PV without the use of potency categories. It is proposed that the RCPL provides an effective resource for assessment of the accuracy with which NAMs can measure skin sensitising potency.

1. Introduction

Skin sensitisation resulting in allergic contact dermatitis (ACD) is an important health issue. Many hundreds, and possibly thousands, of chemicals have been shown to display the potential to cause skin sensitisation (De Groot, 2008), and there is a need therefore to identify skin sensitisers and to conduct effective risk assessments.

The first step in risk assessment is hazard identification. For the identification of skin sensitisation hazards the earliest methods were based on the use of animal models. Initially these were guinea pig tests; the most widely used being the Guinea Pig Maximization Test (GPMT; Magnusson and Kligman, 1969) and the occluded patch test of Buehler (1965). Subsequently the mouse Local Lymph Node Assay (LLNA) was developed and validated (Kimber and Weisenberger, 1989; Kimber et al., 1989, 2002, 2011). It was found that, in addition to hazard identification, the LLNA could be used for measurement of skin sensitising potency (Kimber and Basketter, 1997; Kimber et al., 2003; Basketter et al., 2000, 2005; Gerberick et al., 2001; Loveless et al., 2010); this being based on an understanding that the dose at which local lymph node responses are induced by exposure to contact allergens correlated with sensitising potency (Kimber and Dearman, 1991; Kimber et al., 2008; Loveless et al., 2010).

More recently attention has turned to the development of non-animal methods/New Approach Methodologies (NAMs) (in vitro, and in silico approaches), and investment in this area has met with some success. A number of in vitro approaches, based upon Key Events (KE) in the Adverse Outcome Pathway (AOP) for skin sensitisation, have been developed, validated and assigned Organisation for Economic Cooperation and Development (OECD) test guideline status.

These tests include the following: Direct Peptide Reactivity Assay (DPRA) (Gerberick et al., 2004), kinetic DPRA (Wareing et al., 2020), Amino acid Derivative Reactivity Assay (ADRA) (Yamamoto et al., 2015), KeratinoSens (Emter et al., 2010), LuSens (Ramirez et al., 2014),

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Abbreviations

ACD	Allergic Contact Dermatitis
ADRA	Amino acid Derivative Reactivity Assay
AEL	Acceptable Exposure Level
AOP	Adverse Outcome Pathway
CMIT	5-chloro-2-methyl-4-isothiazolin-one
DA	Defined Approaches
DNCB	2,4-dinitrochlorobenzene
DPRA	Direct Peptide Reactivity Assay
DSA	Dose per Surface Area
GPMT	Guinea Pig Maximization Test
hCLAT	human Cell Line Activation Test
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
IDEA	International Dialogue for Evaluation of Allergens
KE	Key Events
LLNA	Local Lymph Node Assay
LOEL	Lowest Observable Effect Level
MLLP	Median-Like-Location Parameter
NAMs	New Approach Methodologies
NESIL	No Expected Sensitisation Induction Level
NOEL	No Observable Effect Level
OECD	Organisation for Economic Cooperation and Development
PPD	1,4-Phenylenediamine
PV	Potency Value
RCPL	Reference Chemical Potency List
RIFM	Research Institute for Fragrance Materials
SDS	Sodium Dodecyl Sulphate; WoE, Weight of Evidence

the human Cell Line Activation Test (h-CLAT) (Nukada et al., 2012), Myeloid U937 Skin Sensitisation test (Alepee et al., 2015), and IL-18 Luc assay (Takahashi et al., 2011). In addition to those cited above, other tests, employing either derivative or novel approaches, are in various stages of development or awaiting validation (Saito et al., 2013; Ahmed et al., 2016; Cottrez et al., 2016: Johansson et al., 2017, Galbati et al., 2017; Maeda et al., 2020).

There has, therefore, been some success in the development and validation of alternative test methods for skin sensitisation hazard identification. Nevertheless, there remain concerns that even those tests that have been assigned OECD test guideline status are individually inadequate for the accurate identification of skin sensitisation hazards (Bauch et al., 2012; Reisinger et al., 2015). For this reason, a continuing interest exists in the strategy of using combinations of methods in so-called Defined Approaches (DA) as a means of improving predictive accuracy (Bauch et al., 2012; Ezendam et al., 2016; Kleinstreuer et al., 2018; Casati et al., 2018; Kolle et al., 2020; OECD, 2021a).

Overall, it is clear that considerable progress has been made in the development of NAMs for the identification of skin sensitising chemicals. However, although this represents a significant achievement, it remains the case that effective risk assessment demands an understanding of sensitising potency and the levels of skin exposure that are likely to result in the acquisition of sensitisation (and thereby identification of levels of exposure considered to be safe). Not unexpectedly, the design of NAMs that can provide an accurate assessment of sensitising potency has proven rather more difficult than the development of methods that can identify skin sensitising hazard. Nevertheless, progress is being made and possible strategies are emerging (Natsch et al., 2020; Gradin et al., 2020; Na et al., 2022a).

To determine the ability of NAMs, or combinations of NAMs, to provide an accurate assessment of skin sensitising potency it is necessary to have a sound basis for comparing predicted potency against expected values. In practice, this is achieved by comparing sensitising potency predictions from NAMs with robust potency data of known contact allergens and also chemicals considered to be non-sensitising. The key consideration in pursuing this strategy is how best to develop a curated database of skin sensitisation potency values against which the accuracy of predictions can be judged.

Several approaches have been proposed that are based on categorisation of chemicals according to potency based primarily on human data (Basketter et al., 2014), LLNA data (Hoffmann et al., 2018), or by integrating human and animal data with data from in vitro and in silico methods (Na et al., 2022a; b). Acknowledging that these approaches have value, we describe here the development of a curated Reference Chemical Potency List (RCPL) that can be used for evaluation of the performance of novel approaches for measuring skin sensitising potency.

The RCPL was developed by the International Dialogue for Evaluation of Allergens (IDEA) with the purpose of incorporating three important features. The first was to avoid the use of potency categories, such as classifying chemicals as having 'Extreme', 'Strong' or 'Moderate' potency, and to provide a list of chemicals that are ranked according to derived potency values (PV) on a continuous scale. The second was to derive PV based upon consideration of the best available human and animal (almost invariably LLNA) data. The third was to avoid the use of any already available in vitro or in silico data in deriving PVs, so that the future evaluation of NAMs will not be potentially compromised by including in vitro or in silico data in the RCPL. In meeting these objectives an RCPL was created that comprises 33 carefully selected skin sensitising and non-sensitising chemicals that collectively span a wide range of sensitising potency, comprise a broad range of chemistry, and include both direct and indirect (pre- and pro-) haptens.

Described here is the development of the RCPL, the derivation of PVs, and general recommendations how this approach can be applied in practice for evaluation of the accuracy with which NAMs can deliver measurements of skin sensitising potency.

2. Materials and methods

2.1. Consideration of relevant metrics

The purpose of the RCPL is to provide a carefully curated list of skin sensitisers with known potency based on consideration of the best available human and animal data, for assessment of the ability of NAMs to predict accurately skin sensitising potency.

The goal was to create a list of about thirty low-molecular weight chemicals that collectively span a wide spectrum of skin sensitising potency, including the absence of skin sensitising activity. The chemicals incorporated in the RCPL have been ranked according to their relative skin sensitising potency to facilitate assessment of NAMs. Skin sensitising potency is expressed as dose per unit area of skin, or $\mu g/cm^2$.

The aim was to derive an overall Weight of Evidence (WoE) value, where the word 'overall' was intended to indicate that the value would be derived following consideration of both, relevant human data and animal data [Local Lymph Node Assay (LLNA) EC3 values]. Subsequently, the term Potency Value (PV) was adopted as the measure of sensitising potency.

2.2. Potency value

The PV is best defined as an overall measure of skin sensitising potency based upon consideration of relevant human data, LLNA EC3 values, and expert judgement. By definition for inclusion in the RCPL a chemical must have relevant human and/or LLNA data.

In practice skin sensitising potency can be described as the ease with which a chemical is able to induce sensitisation, and this, in turn, is a reflection of the local concentration of the chemical that will be required to initiate the process of sensitisation. The higher the potency of a contact allergen, the lower will be the concentration required for the acquisition of sensitisation. This concentration can be thought of as an inflection point at which, in an experimental setting, sensitisation is first induced.

In summary, the PV describes the lowest concentration of chemical (measured as μg/cm^2 of skin) that is necessary to initiate the development of skin sensitisation under the conditions of the experimental setting used.

2.3. The relationship of PV to other skin sensitisation metrics

It is important to note the differences between the PV and other metrics that are commonly used in skin sensitisation hazard and risk assessment.

- A NESIL (No Expected Sensitisation Induction Level) identifies a concentration of a chemical that will not result in the induction of skin sensitisation in a Human Repeat Insult Patch Test (HRIPT) on 100 subjects (Api et al., 2020). This has value in the risk assessment process but does not necessarily reflect accurately the skin sensitising potency of a chemical. That is, a NESIL differs from a PV in that the former identifies a concentration of a chemical that is expected NOT to induce skin sensitisation, whereas the PV identifies a concentration of chemical that is the inflection point where skin sensitisation is first initiated.
- NOEL (No Observable Effect Level) and LOEL (Lowest Observable Effect Level) also have value in risk assessments, but neither necessarily reflects accurately the skin sensitising potency of a chemical since the NOEL and LOEL, at least for studies in human volunteers, often reflect the only dose tested and not the conclusion of a dose response analysis.

2.4. Derivation of LLNA EC3 values

The main source of high quality curated LLNA data was provided by the OECD database (Supporting document to the OECD Guideline 497 on Defined Approaches for Skin Sensitisation) (OECD, 2021b). In most instances, the needs for the RCPL were met by the OECD LLNA database. However, in instances where that was not the case, data available from the Research Institute for Fragrance Materials (RIFM) database were used. In some cases, such as for very strong sensitisers, the availability of LLNA data was more limited and in such instances additional sources were also accepted.

The LLNA studies included in the OECD database had predefined criteria applied to them which have been adopted for the RCPL (OECD, 2021a; b). In addition, where the EC3 had been extrapolated, further evaluation criteria were applied (partially based on Ryan et al., 2007). The three criteria, previously adopted by the OECD group (OECD, 2021b) and applied in the current exercise to such cases, were that the extrapolated EC3 value is less than 10-fold smaller than the closest tested concentration; the lowest measured SI value is less than 5; and the slope ratio is less than 2. All three criteria had to be met for acceptance of the reported extrapolated EC3 value. Therefore, those studies that did not fulfil these criteria were not used in the PV derivation.

Where a number of individual studies fulfilling the above criteria and providing an individual EC3 were available, the Median-Like-Location Parameter (MLLP) as the averaging metric for EC3s (Hoffmann et al., 2018; OECD, 2021a; b) was adopted. As stated in Hoffmann et al., 2018), "*This parameter was defined as the median for substances with repeat studies with an EC3 in more than 50% of the repeats. For substances with at least 50% negative repeat studies, i.e. no EC3 value was available, the parameter was defined as the modified median. The first step in deriving the modified median was to review the negative studies in detail: when the maximum concentration tested in a given study was lower than the median EC3 of the positive studies for the same chemical, the respective negative study was excluded, because it was considered a limited validity as tested concentrations were too low. From the remaining negative and all positive studies, the median was used as a location parameter (modified median). In the case of 50% of repeat studies being negative and 50% being positive, the highest EC3 value was defined as the modified median.*"

The LLNA data from the RIFM database and their safety assessments were collated and the RIFM LLNA Weighted Mean EC3 Value identified where available. The latter is derived by averaging the EC3 values from the studies with the same vehicle prior to calculating the final mean (Api et al., 2008).

Where the difference between the OECD MLLP EC3 value (in μg/cm^2) and the RIFM weighted mean EC3 value was greater than 2-fold, the RIFM LLNA data were reviewed. If the difference was less than 2-fold, the OECD MLLP EC3 value was selected for the RCPL.

The summary of the LLNA data with the EC3 values can be found with the Supplementary Information (S1).

2.5. Derivation of human NOEL and LOEL (DSA04) values

For characterisation of the skin sensitisation potency of chemicals, high quality human studies provide important and relevant data. For this reason, development of the RCPL has incorporated the results of human studies with well-defined experimental exposure conditions during the induction phase, followed by a well-defined and well-documented elicitation challenge under medical surveillance.

Since only existing studies were evaluated in the development of the RCPL, and no new studies designed for the purpose of potency characterisation were conducted, it was often necessary to rely on an element of expert judgement in the selection of NOEL and LOEL values.

For studies to be considered in the RCPL process, reports had to provide details of the exposure level expressed as the concentration of chemical per unit area of skin (measured as μg/cm^2 of skin) or, at least, to have reported details required for derivation of this value (in particular, size of patch onto which test formulation was applied, amount of test formulation applied per patch, and concentration of substance of interest in test formulation).

In general terms, Human Repeat Insult Patch Tests (HRIPT) fulfil these criteria to a greater extent than do Human Maximization Tests (HMT). The latter are historical data and, for most chemicals, older than any HRIPT.

2.5.1. HMT and HRIPT methods

Most protocols that were used for HMT (Draize et al., 1944; Kimber et al., 2001; Kligman, 1966) included a pre-treatment with sodium dodecyl sulphate (SDS) solution of the skin area to which the test substance is applied later. The SDS pre-treatment is used to induce a local irritation, a hallmark of which is the attraction of cells of the immune system into the treated site. This in turn promotes and augments immune responses induced against the test substance. Therefore, the HMT, in general, has to be considered a more sensitive test than the HRIPT which does not include such pre-treatment. However, it is not possible to determine by what factor an HMT would be more sensitive than an HRIPT because insufficient comparative data are available. Also, a consistent difference in sensitivity between the two protocols is unlikely as known skin sensitisers differ in their inherent skin irritating potential. Many, but not all, potent skin sensitisers are also skin irritants, presumably by virtue of their chemical reactivity causing cytotoxicity. Therefore, skin sensitisers would "profit" differently from a pre-existing skin irritation (by SDS or other causes). Consequently, HMT and HRIPT results cannot be quantitatively correlated with each other, and it was decided therefore not to rely on HMT data for determination of PVs, but to use instead HRIPT results. HMT studies were used merely as supportive evidence in the PV derivation process.

For this reason, HRIPTs conducted according to standardized protocols with approximately 100 subjects or more (Politano and Api, 2008) were given the highest weight of priority for derivation of LOEL values, and where necessary, NOEL values. However, in some instances other protocols were, where necessary, accepted on a case-by-case basis.

It is important to note that for the purpose of developing the RCPL, LOELs have been favoured as an expression of human data because, unlike NOELs, they reflect a signal of sensitisation, rather than the absence of sensitisation.

In the absence of a fully valid study, a HRIPT not fulfilling all criteria (especially with regard to the number of subjects tested) was used if one or more additional human studies provided support for a certain exposure level as a LOEL or NOEL.

In the evaluation of human data, a valid HRIPT reporting one or more cases of induction of skin sensitisation in the panel of subjects tested at a certain exposure level was, in most circumstances, considered the highest weight of evidence, and was therefore the point of departure for derivation of a LOEL. This was the case even though another study performed at the same exposure level, might have reported no cases of sensitisation.

2.5.2. Considerations leading to the selection of the DSA04

For the purposes of the RCPL a DSA (Dose per Surface Area) 04 value (DSA04) (expressed in μg/cm^2) was selected as providing a sound basis for harmonising LOEL values for chemicals that caused different incidences in HRIPT studies. A DSA04 describes the estimated concentration of a chemical that would be required to induce skin sensitisation in 4% of exposed subjects under the conditions of an HRIPT. The mechanism used for deriving DSA04 values is summarised below.

Since the skin sensitising potency of each substance was evaluated in a quantitative way, in order to derive a PV, a method was required to integrate results of independent HRIPT studies reporting different sensitisation incidences for the same or different vehicles.

The approach chosen for quantitative comparisons was to "normalise" HRIPT results to the same incidence, i.e., to estimate from the test results a hypothetical DSA of skin that would cause sensitisation at a 4% incidence (DSA04) (expressed also in μg/cm^2).

For this purpose, a simple linear extrapolation was used because the mathematical form of the dose-response curves for the different chemicals could not be characterised since too few data points were available. For example, if in an HRIPT using a DSA of 1000 μg/cm^2 two out of 100 subjects (incidence 2%) were sensitised, the DSA04 was calculated as DSA04 = DSA(test) x 4%/incidence(test), so 2000 μg/cm^2.

A DSA04 value was selected in preference to any other DSA value (such as DSA02 or DSA05) because in an initial assessment of 20 chemicals the DSA04 value was found to correlate best with LLNA EC3 values. The DSA04 is close to the DSA05 calculated by ICCVAM when evaluating the LLNA for potency assessment (ICCVAM, 2011), and the DSA05 was also found to correlate well to LLNA EC3 values when the ICCVAM data were re-evaluated with multiple mathematical tools by Bil et al. (2017). Based on the initial observation of a good correlation of DSA04 with LLNA data for fragrance ingredients, and because a DSA04 is slightly more conservative in the evaluation of potency in humans than DSA05, the use of DSA04 was selected. Moreover, as in many HRIPT studies the incidence of positive responses is between 2 and 10%, the DSA04 did, usually, not require large extrapolations.

While the 'real' dose-response curve is most likely sigmoid, the dose response curve could not be described mathematically because the number of data points was small for most substances and the observed incidences were usually small. Since the observed incidences were usually low, the linear extrapolation was chosen to calculate the DSA04 value. However, the uncertainty of this extrapolation will increase the more the experimentally observed incidence is different from 4%. Therefore, when the observed incidence differed by more than a factor of 3 from the 4% target incidence, the resulting value was carefully checked against other supporting evidence and modified if expert judgement suggested so.

When none of the available HRIPTs reported cases of sensitisation, the NOEL was established at the highest exposure level reported in a fully valid HRIPT or, if no fully valid study was available, at the highest exposure level that could be supported from an evaluation of the body of available data.

In other documentation noted below, DSA04 values and NOEL values – and PVs that derive from them – are corrected to one decimal place if they are less than 100 (μg/cm^2), and corrected to the nearest whole number if they are 100 or greater (μg/cm^2).

The Supplementary Information (S1) provides a summary of the human data as well as the selected DSA04 or NOEL for each chemical applied through the workflow which are given in italic font.

2.6. Workflow for the derivation of PV

A weight of evidence workflow was developed to guide the data analysis in the derivation of the PV. The workflow follows the available LLNA and human data fulfilling the criteria described in Sections 2.4 and 2.5, respectively. It applies different weights to each data source, while the adopted criteria allow decision making at each point. Beginning with Fig. 1, the first decision is based on whether human data are available.

When human data are available the decision point moves on to Fig. 2 where the availability of the human threshold DSA04 is determined as the next step in the decision tree. If this is available and the difference between the LLNA EC3 and the DSA04 is greater than 2-fold, the final PV will be derived depending on the relation between these two values, and on whether the DSA04 would lead to an underestimation of human potency.

If the DSA04 cannot be calculated then the workflow moves to Fig. 3, where the human value relied on is the NOEL. Similarly to Fig. 2, the availability of the LLNA and the difference between the EC3 and NOEL determines the final PV selected.

3. Results

The selection of the chemicals included in the RCPL was obtained by considering the full RIFM database and the already highly curated OECD

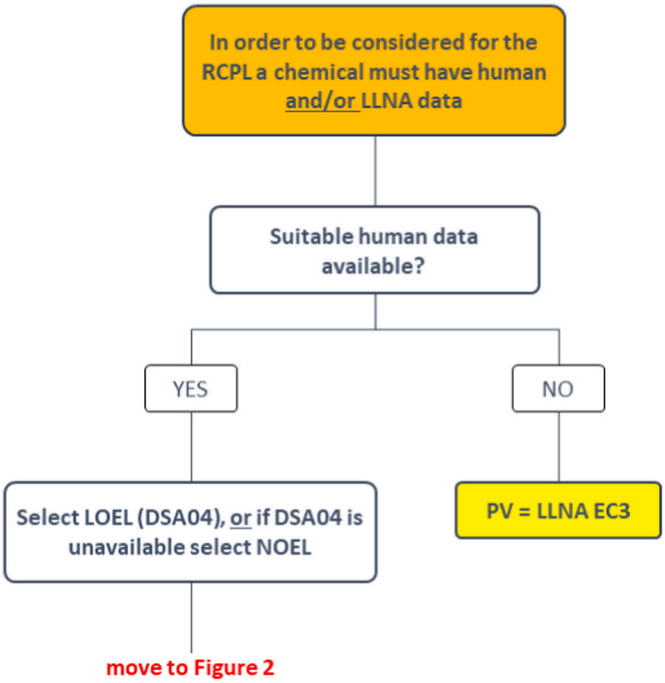

Fig. 1. Workflow #1 for derivation of PV.

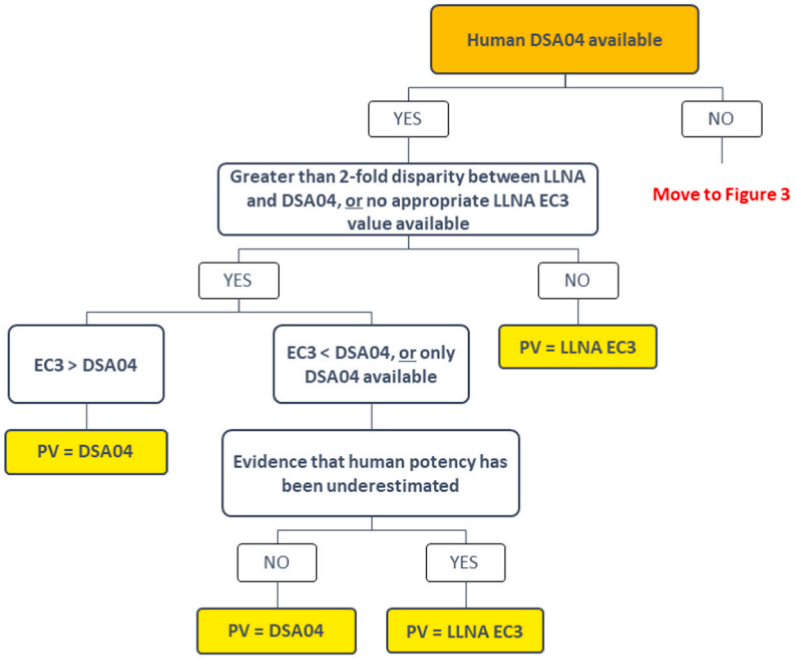

Fig. 2. Workflow #2 for the derivation of PV.

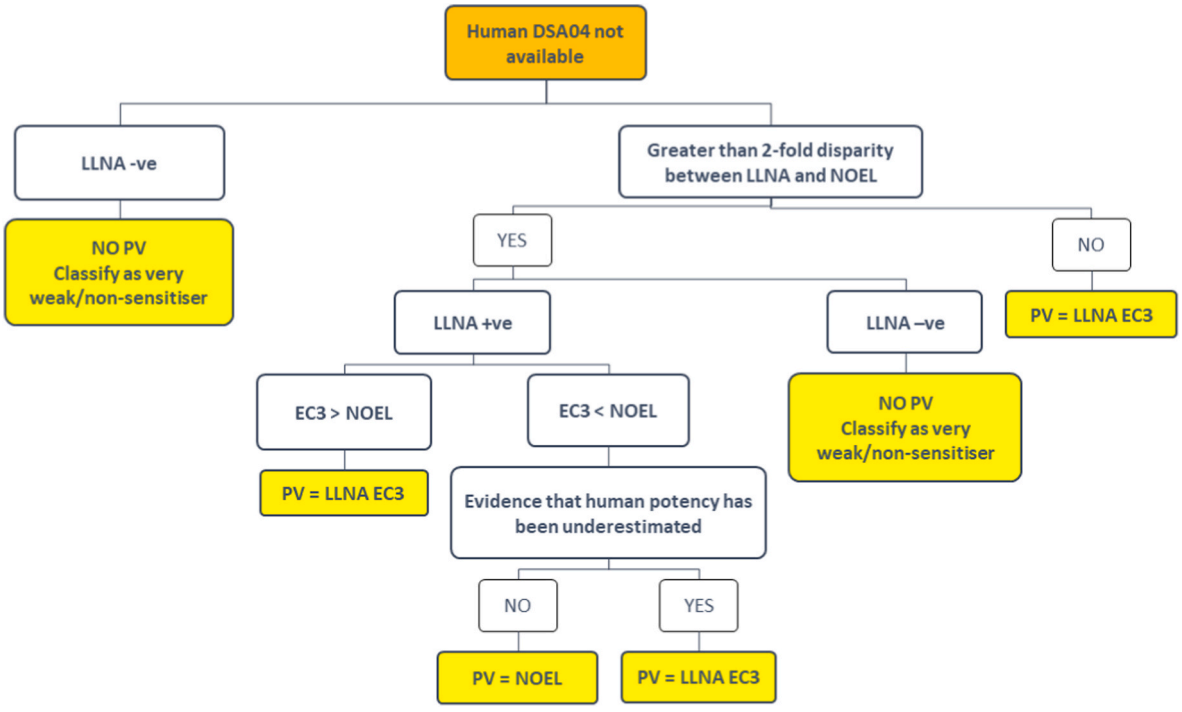

Fig. 3. Workflow #3 for the derivation of PV.

database (OECD, 2021b). The prerequisite was the requirement to include a wide range of sensitising potencies. From an initial proposed list of >40 chemicals and proceeding through an iterative process where the available human and LLNA data were reviewed to ensure they fulfilled the criteria set for the determination of a robust PV, a final RCPL was derived according to the workflow (Figs. 1–3).

Table 1 presents the final selection of individual human and animal data for each chemical as well as the PV derived. Table 2 outlines the final RCPL PV including the identification of those that are pre and/or pro-haptens (Patlewicz et al., 2016; Casati et al., 2016). The detailed data from both LLNA and human studies are given for each chemical in separate sheets of the associated supplemental data (Supplementary Information S1). In addition, for six chemicals a graphical representation of all the individual data with the derived DSA04, LLNA MLLP and PV is provided in Supplementary Information S2. These graphs show that for some chemicals the WoE derives from a large number of studies (e.g. cinnamic aldehyde and hydroxycitronellal), whereas in other instances fewer studies were available. The data available for some

Table 1
Weight of evidence applied for derivation of Potency Values.

Chemical	CAS No	Human LOEL/DSA04 [μg/cm^2]	Human NOEL [μg/cm^2]	LLNA EC3 [μg/cm^2]	Workflow number and Rationale	Potency Value [μg/cm^2]
5-Chloro-2-methyl-4-isothiazolin-one (CMIT)	26172-55-4	None	None	2.3	1	2.3
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	LOEL (HRIPT) of 7 giving DSA04 of 3.4	NA	13.5	2 >2-fold difference between LLNA and DSA04 EC3 > DSA04	3.4
1,4-Phenylenediamine (PPD)	106-50-3	LOEL (HRIPT) of 7 giving DSA04 of 3.9	NA	27.5	2 >2-fold difference between LLNA and DSA04 EC3 > DSA04	3.9
Glutaraldehyde	111-30-8	LOEL inadequate[a]	None	20.0	1	20.0
trans-2-Hexenal	6728-26-3	LOEL (HRIPT) of 236 with higher uncertainty in extrapolation to DSA04 since HRIPT incidence was > 3 × 4% incidence. Extrapolated DSA04 of 39.3 was used in absence of conflicting human data	NA	1013	2 >2-fold disparity between LLNA EC3 and DSA04 EC3 > DSA04	39.9
1,4-Dihydroquinone	123-31-9	None	None	47.5	1	47.5
Benzyl bromide	100-39-0	None	None	50.0	1	50.0
1,1,3-Trimethyl-2-formylcyclohexa-2,4-diene (Safranal)	116-26-7	LOEL (HRIPT) of 250 giving DSA04 of 106 was used although the subject number was just 53 because another HRIPT using 99 subjects reported an incidence <1/3x of 4% at 59.1 resulting in a higher uncertainty of the extrapolated DSA04 (234)	NA	1875	2 >2-fold disparity between LLNA and DSA04 EC3 > DSA04	106
Methyl 2-nonynoate (Methyl octine carbonate)	111-80-8	LOEL (HRIPT) of 118 giving DSA04 of 109	NA	None[b]	2 Only DSA04 available	109
Methyl 2-octynoate (Methyl heptine carbonate)	111-12-6	LOEL (HRIPT) = 194 giving DSA04 of 159	NA	125	2 <2-fold disparity between LLNA and DSA04	125
Isoeugenol	97-54-1	LOEL (HRIPT) of 775 giving DSA04 of 589	NA	325	2 <2-fold disparity between LLNA and DSA04	325
Phenylacetaldehyde	122-78-1	LOEL (HRIPT) = 1181 with higher uncertainty in extrapolation to DSA04 since HRIPT incidence was > 3 × 4% incidence. Extrapolated DSA04 of 182 was lower than level of valid negative HRIPT (591) and the latter was therefore used	NOEL (HRIPT) = 591	750	3 <2-fold disparity between LLNA and NOEL	750
Allyl phenoxyacetate	7493-74-5	None[c]	NOEL (HRIPT) = 709	775	3 <2-fold disparity between LLNA and NOEL	775
Cinnamic aldehyde	104-55-2	LOEL (HRIPT) of 620 giving DSA04 of 885[d]	NA	250	2 >2-fold disparity between LLNA and DSA04 EC3 < DSA04	885
3-Propylidenephthalide	17369-59-4	LOEL (HMT) = 2760 not suitable for DSA04 extrapolation	NOEL (HRIPT) = 945 [e]	925[f]	3 <2-fold disparity between LLNA and NOEL	925
4-Hydroxy-2,5-dimethyl-furanone (Furaneol)	3658-77-3	None[g]	NOEL (HRIPT) = 1181	450	3 >2-fold disparity between LLNA and NOEL EC3 < NOEL No evidence that human potency is underestimated	1181
Citral	5392-40-5	LOEL (HMT) of 2760 not suitable for DSA04 extrapolation	NOEL (HRIPT) 1417 [h]	1450	3 <2-fold disparity between LLNA and NOEL	1450
p-Mentha-1,8-dien-7-al (Perillaldehyde)	2111-75-3	LOEL (HMT) of 2760 not suitable for DSA04 extrapolation	NOEL (HRIPT) = 709 [i]	2175[j]	3 >2-fold disparity between LLNA and NOEL EC3 > NOEL	2175
Benzaldehyde	100-52-7	LOEL (HRIPT) of 5905 giving DSA04 of 4094	NA	>6250 (regard as not available)	2 In the absence of LLNA EC3 use DSA04	4094
Lyral (HICC)		None[k]		4275		4275

(*continued on next page*)

Table 1 (*continued*)

Chemical	CAS No	Human LOEL/DSA04 [μg/cm^2]	Human NOEL [μg/cm^2]	LLNA EC3 [μg/cm^2]	Workflow number and Rationale	Potency Value [μg/cm^2]
	31906-04-4		NOEL (HRIPT) = 8264		3 <2-fold disparity between LLNA and NOEL	
Hydroxycitronellal	107-75-5	LOEL (HRIPT) of 4500 with higher uncertainty in extrapolation to DSA04 since HRIPT incidence was <1/3 × 4% incidence. Extrapolated DSA04 of 27000 is considered high. Considering the sensitisation occurring at concentrations tested in other HRIPTs and HMTs, the HRIPT NOEL was considered a suitable replacement for the DSA04 in this case	NOEL (HRIPT) = 4960	5275	3 <2-fold disparity between LLNA and NOEL	5275
Cinnamic alcohol	104-54-1	LOEL (HRIPT) of 4000 giving DSA04 of 6000	NA	5775	2 <2-fold disparity between LLNA and DSA04	5775
Eugenol	97-53-0	LOEL (HRIPT) of 5039 giving DSA04 of 7357	NA	2900	2 >2-fold disparity between LLNA and DSA04 EC3 < DSA04 No evidence that human potency has been underestimated	7357
Geraniol	106-24-1	LOEL (HRIPT) of 6299 giving DSA04 of 9197	NA	4025	2 >2-fold disparity between LLNA and DSA04 No evidence that human potency has been underestimated	9197
Coumarin	91-64-5	LOEL (HRIPT) of 5669 giving DSA04 of 11792	NA	>12500 (regard as not available)	2 In the absence of LLNA EC3 use DSA04	11792
Carvone	6485-40-1	LOEL (HRIPT) of 18896 giving a DSA04 of 17573	NA	3250	2 >2-fold disparity between LLNA and DSA04 EC3 < DSA04 No evidence that human potency has been underestimated	17573
Benzyl salicylate	118-58-1	LOEL (HMT) = 13500 not suitable for DSA04 extrapolation	NOEL (HRIPT) = 17715	725	3 >2-fold disparity between LLNA and NOEL EC3 < NOEL No evidence that human potency has been underestimated, but HMT, having a higher sensitivity due to SDS pre-treatment, indicates that HRIPT NOEL is close to inflection point	17715
Hexyl cinnamic aldehyde	101-86-0	None[l]	NOEL (HRIPT) = 23620	2700	3 >2-fold disparity between LLNA and NOEL EC3 < NOEL No evidence that human potency has been underestimated	23620
Benzyl alcohol	100-51-6	LOEL (HRIPT) of 8858 with higher uncertainty in extrapolation to DSA04 since HRIPT incidence was <1/3 × 4% incidence. Extrapolated DSA04 is 38975. Two other HRIPTs tested a lower number of subjects and would have resulted in DSA04 values of 52913 and 8150, respectively	NA	>12500 (regard as not available)	2 In the absence of LLNA EC3 use DSA04	>25000
Benzyl benzoate	120-51-4	None[m]	NOEL (HRIPT) = 59050	4250	3 EC3 < NOEL No evidence that human potency has been underestimated	>25000
α-iso-Methylionone	127-51-5	None[n]	NOEL (HRIPT) = 70866	5450	3 >2-fold disparity between LLNA and NOEL EC3 < NOEL No evidence that human potency has been underestimated	>25000
Methyl salicylate	119-36-8	None[o]	NOEL (HMT) = 5520	5000[p]	3 No PV	very weak/non-sensitiser
Vanillin	121-33-5	None[q]		>12500 (regard as		

(*continued on next page*)

Table 1 (*continued*)

Chemical	CAS No	Human LOEL/DSA04 [μg/cm^2]	Human NOEL [μg/cm^2]	LLNA EC3 [μg/cm^2]	Workflow number and Rationale	Potency Value [μg/cm^2]
			NOEL (HRIPT) = 5314	not available)	3 The NOEL overestimates human potency No PV	very weak/ non-sensitiser

None: Not available; NA: Not applicable.

[a] Not derived according to established criteria - HRIPT with low number of subjects, no backup by other human studies.

[b] EC3 was extrapolated and it does not fulfil at least one of the criteria.

[c] Negative human studies at all dose levels tested.

[d] In this study a total of 107 subjects were induced with either 95% EtOH as the vehicle or Petrolatum as the vehicle. Elicitation was performed on all subjects with both vehicles. Positive reactions were found (3/107) when elicitation was performed using EtOH as a vehicle. This is regarded as a sound study yielding a result of 3/107 positives.

[e] Considering the sensitisation occurring at the concentration tested in the HMT, the HRIPT NOEL was considered a suitable replacement for the DSA04 in this case.

[f] Extrapolated fulfilling the Ryan et al. (2007) criteria.

[g] Not derived according to established criteria - RIFM #68926: 1 positive likely irritation not sensitisation as only present at 48h reading, not at 72 and 96h. RIFM #60392: positive HRIPT questionable as all reactions occurred during induction and these subjects were not challenged. Reactions tended to occur late in induction phase, but e.g. one counted positive subject showed grade 2 to 5th patch but not reaction to following 4 patches (Supplementary Information S1).

[h] A HRIPT at 3876 reporting a 62.5% incidence was not used for DSA04 calculation as the uncertainty in extrapolation was deemed too high. The HRIPT NOEL was considered a suitable replacement for the DSA04 in this case.

[i] Considering the sensitisation occurring at the concentration tested in the HMT, the HRIPT NOEL was considered a suitable replacement for the DSA04 in this case.

[j] There is more that 2-fold difference between the OECD EC3 and RIFM EC3. Further investigation has concluded that there is an error in the OECD value - They refer to Patlewicz et al. (2002) as primary reference and Gerberick et al. (2005) as secondary. Both of them give an EC3 of 8.1%, and not 4.04% as outlined. Therefore, the RIFM EC3 is taken forward.

[k] Negative human studies at all dose levels tested.

[l] Negative human studies at all dose levels tested.

[m] Negative human studies at all dose levels tested.

[n] Negative human studies at all dose levels tested.

[o] Negative human studies at all dose levels tested.

[p] It is noted that 16 out of 18 LLNA studies were negative, also the study for the NOEL was tested at relatively low dose. Consequently it is agreed that no PV can be derived and instead it is regarded as very weak/non sensitiser.

[q] Negative human studies at all dose levels tested.

chemicals, such as geraniol, illustrates that in some instances there was variability in the available in vivo data.

4. Discussion

There is a growing and sustained commitment to the development of non-animal methods for evaluation of the skin sensitising potential of chemicals, and progress has been substantial. There are now available a variety of validated methods for the identification of skin sensitising hazards that have been assigned OECD test guideline status (Gerberick et al., 2004; Emter et al., 2010; Takahashi et al., 2011; Nukada et al., 2012; Ramirez et al., 2014; Alepee et al., 2015), and there are other promising approaches in the pipeline (Saito et al., 2013; Ahmed et al., 2016; Cottrez et al., 2016; Johansson et al., 2017; Galbati et al., 2017; Maeda et al., 2020).

Importantly, despite the progress that has been achieved in developing NAMs for hazard identification, it must be appreciated that the identification of skin sensitising potential per se is insufficient for the development of effective risk assessments. For that purpose there is a requirement for information regarding sensitisation potency. This is of particular relevance for skin sensitisation because it is clear that contact allergens vary significantly in terms of their relative potency which spans up to 4 orders of magnitude. In this respect it will be noted that the derived PVs for chemicals in the RCPL vary from 2.3 to 23,000 μg/cm^2.

However, the assessment of skin sensitising potency is challenging, not least because suitable methods must provide readout(s) that correlate quantitatively with the inherent skin sensitising potency of a chemical. Those challenges are being addressed, progress is being made (Natsch et al., 2020; Gradin et al., 2020; Na et al., 2022a), and it is anticipated that there will emerge other approaches (individual methods, or combinations of methods) that will provide a sound basis for characterising sensitising potency and thereby permitting effective risk assessments without recourse to animal studies.

It is important, therefore, that NAM strategies are developed that are able to measure skin sensitising potency, and that there is confidence among the scientific and regulatory communities that at least some such approaches provide a sound and accurate basis for risk assessment.

The purpose of the initiative described in this report was to provide a template for assessment of the accuracy with which candidate methods are able to measure the skin sensitising potency of chemicals.

It is, of course, acknowledged that the RCPL described here is not the only tool available for this purpose. Other approaches using human data, animal (LLNA) data, or a mixture of both combined with NAM data, have been described and undoubtedly have value (Basketter et al., 2014; Hoffmann et al., 2018; OECD, 2021a; Na et al., 2022a; b).

Construction of the RCPL described here adopted a somewhat different approach, namely the strategy being to make use of the best available human and animal data to derive PVs that would provide an index of sensitising potency. This RCPL comprises a list of 33 readily available chemicals that together span the full spectrum of skin sensitising potency (and including those that lack sensitising activity), represent a range of chemistry, incorporate both fragrance and non-fragrance materials, and include both direct and indirect (pre- and pro-) haptens.

There are several features of the RCPL that warrant some discussion.

Firstly, the RCPL makes use of a derived PV to rank chemicals according to skin sensitising potency. It is important to distinguish between this metric and the other measure of skin sensitisation in humans that is used for the purposes of risk assessment: the NESIL. The latter is an estimated level of exposure (in dose per unit area of skin) at which sensitisation is expected not to develop under the conditions of an HRIPT. The NESIL is based upon a NOEL value and used to derive an Acceptable Exposure Level (AEL) by the incorporation of safety or uncertainty factors. In contrast, the PV is a concentration (again measured in dose per unit area of skin) derived from interrogation of available experimental human and animal data, at which it is estimated that skin

Table 2
Hypothetical protein reactivity, pre-/pro-hapten status and Potency Values of RCPL chemicals.

Name	Protein Reactivity[a]	Pre/Pro - Hapten[b]	Potency Value [μg/cm^2]
5-Chloro-2-methyl-4-isothiazolin-one (CMIT)	Nucleophilic substitution (SN2)		2.3
2,4-Dinitrochlorobenzene (DCNB)	Nucleophilic aromatic substitution		3.4
1,4-Phenylenediamine (PPD)	Michael addition to quinoid type structures	Pre	3.9
Glutaraldehyde	Schiff base formation (bifunctional)		20.0
trans-2-Hexenal	Michael addition to alpha, beta unsaturated carbonyl		39.3
1,4-Dihydroquinone	Michael addition to quinoid type structures	Pre	47.5
Benzyl bromide	Nucleophilic substitution (SN2)		50.0
1,1,3-Trimethyl-2-formylcyclohexa-2,4-diene (Safranal)	Schiff base formation, Michael addition to alpha, beta unsaturated carbonyl		106
Methyl 2-nonynoate (Methyl octine carbonate)	Michael addition to alpha, beta unsaturated ester		109
Methyl 2-octynoate (Methyl heptine carbonate)	Michael addition to alpha, beta unsaturated ester		125
Isoeugenol	Michael addition to quinoid (or quinone methide) type structures	Pre	325
Phenylacetaldehyde	Schiff base formation		750
Allyl phenoxyacetate	Nucleophilic substitution (SN2)		775
Cinnamic aldehyde	Michael addition to alpha, beta unsaturated carbonyl		885
3-Propylidenephthalide	Acylation, Autoxidation to hydroperoxide	Pre	925
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol)	Nucleophilic substitution (SN2)		1181
Citral	Michael addition to alpha, beta unsaturated carbonyl, Schiff base formation		1450
p-Mentha-1,8-dien-7-al (Perillaldehyde)	Michael addition to alpha, beta unsaturated carbonyl		2175
Benzaldehyde	Schiff base formation		4094
Lyral (HICC)	Schiff base formation		4275
Hydroxycitronellal	Schiff base formation		5275
Cinnamic alcohol	Michael addition to alpha, beta unsaturated carbonyl, Schiff base formation, SULT-mediated cation formation	Pre/Pro	5775
Eugenol	Michael addition to quinoid type structures	Pre/Pro	7357
Geraniol	Michael addition to alpha, beta unsaturated carbonyl, Autoxidation to hydroperoxide	Pre/Pro	9197
Coumarin	Michael addition to alpha, beta unsaturated ester, Acylation		11792
Carvone	Michael addition to alpha, beta unsaturated carbonyl		17573
Benzyl salicylate	Nucleophilic substitution (SN2), Acylation		17715
Hexyl cinnamic aldehyde	Michael addition to alpha, beta unsaturated carbonyl		23620
Benzyl Alcohol	Schiff base formation, SULT-mediated cation formation	Pro	>25000
Benzyl benzoate	Nucleophilic substitution (SN2), Acylation, also predicted as non-reactive		>25000
Isomethylionone (α-)	Michael addition to alpha, beta unsaturated carbonyl, also predicted as non-reactive		>25000
Methyl salicylate	Acylation, also predicted as non-reactive		No PV derived- very weak/non-sensitiser
Vanillin	Schiff base formation, also predicted as non-reactive		No PV derived - very weak/non-sensitiser

[a] Based on OECD QSAR Toolbox version 4.4.1, Casati et al. (2016), and expert judgement.
[b] Based on Patlewicz et al., 2016 and Casati et al. (2016).

sensitisation will first be induced. This can also be described as the inflection point at which skin sensitisation is initiated for that particular chemical. Naturally, the expectation would be that in most instances the derived PV for a chemical would be higher than the NESIL. However, it must be recognised that the two values are determined using different data sets (and for different purposes) and there is no set relationship between these metrics, although as expected in most cases the previously reported NESIL was lower than the PV.

The second is that the RCPL comprises 33 chemicals, and it is legitimate to question why this number was deemed to be appropriate for this purpose. It was considered that any fewer than 30 chemicals would be insufficient to represent a required range of chemistry and sensitising potency, and to include examples of both direct and indirect haptens. It could be argued that an even larger number of chemicals could have been included in the final list. It will be apparent, however, that a considerable amount of work was required to collate and review all of the available evidence, and to curate a final list of 33 chemicals. To have expanded significantly the number of chemicals in the RCPL would have required a significantly larger investment that, in the view of the authors, was unnecessary. In practice, if a candidate method were found to rank the 33 chemicals in the RCPL in the same or closely similar order according to potency then that would provide confidence that the approach had real merit in measuring sensitising potency.

Thirdly, it is important to emphasise that the 33 chemicals that comprise the RCPL should not be used as a training set for the calibration of new methods designed to measure skin sensitising potency. This would lead to the development of 'over-fitted' models. Use of the RCPL should therefore be limited to the evaluation of approaches that have been 'trained' using other datasets.

The fourth is that the decision was reached to use the best available human and animal data to derive an overall PV for each of the chemicals in the list. This is in contrast to other approaches that have relied largely or exclusively on animal data (Hoffmann et al., 2018), or on human data (Basketter et al., 2014). The justification for adopting this approach was

a desire to make use of robust LLNA EC3 values when available because these data have been derived specifically for the purposes of potency assessment based upon dose-response studies in the mouse, and have been shown to correlate with human skin sensitisation thresholds (Basketter et al., 2005; Schneider and Akkam, 2004). However, the strategy also called for consideration of human data when available in recognition of the fact that, although LOEL and NOEL values do not necessarily provide a direct measure of potency, they do reflect sensitising activity in the species of interest. The use of human data was particularly valuable when it appeared stronger than LLNA data, or in circumstances where LLNA data were equivocal, were judged unreliable, or were unavailable. In addition, as described previously, decisions regarding overall PVs were made without recourse to consideration of any available in vitro or in silico data. The rationale for this was discussed above.

The fifth feature that warrants mention is the fact that the RCPL avoids grouping chemicals of similar potency into categories described as being, for instance, extreme, strong, moderate and weak sensitisers, or non-sensitisers. The value in having a continuous scale for potency without such divisions avoids uncertainty of interpretations when a chemical of interest is found to show activity that falls at the border between two categories.

It will be noted that in an attempt to harmonise and rationalise the available human data from LOEL values the decision was reached to derive a DSA; the DSA being an estimate, based on HRIPT studies, of the concentration of chemical per unit area of skin ($\mu g/cm^2$) required to induce skin sensitisation in a certain percentage of the exposed subjects. Previously a DSA05 value has been used for comparing LLNA EC3 values with human data (ICCVAM, 2011; Bil et al., 2017); this being the concentration estimated to induce sensitisation in 5% of exposed subjects. For the purpose of the RCPL the decision was made to use a DSA04. This choice was based on an initial preliminary assessment which indicated that the DSA04 correlated closely with LLNA EC3 values for the range of chemicals included in the RCPL.

The limitation of DSA values is of course that they do not necessarily reflect the true relationship between the level of skin exposure and the incidence of sensitisation among the exposed population. In derivation of a DSA04 value (for instance) an arithmetic linear extrapolation was applied to the observed incidence of skin sensitisation with a given concentration of the test chemical in order to estimate the concentration that is estimated to be necessary to induce sensitisation in 4% of exposed subjects. That is, if a concentration X of the test chemical was found to induce sensitisation among 2% of exposed subjects, then the DSA04 would be calculated as being 2 times higher. However, there is evidence from experimental human studies that in fact the proportion of subjects that become sensitised to a skin sensitiser displays a sigmoidal relationship with the log of the sensitising dose (Friedmann and Moss, 1985).

The above illustrates one source of uncertainty regarding the derivation of PVs, but not unexpectedly there are undoubtedly others also. It is inevitable that the use of human experimental data, in concert with LLNA data, to derive estimates of the inflection point at which skin exposure to a chemical will initiate the process of sensitisation will be subject to some uncertainty. Nevertheless, these are the best available data on which to base the sensitisation inflection point, and it is proposed that the PV provides a robust basis for evaluating the ability of new methodologies to predict accurately skin sensitising potency.

It is suggested that there should be some flexibility in how the RCPL can be used for assessment of the accuracy with which NAMs are able to measure sensitising potency. It could be argued that a gold standard would be a complete alignment between the RCPL list and predictions of potency generated using a new approach. However, it would be appropriate to take a pragmatic approach, and if in the first instance a new method displayed some concordance with the rank order of chemicals in the RCPL this should perhaps be interpreted as indicating that method is worthy of continued development.

It is a sobering thought that if it were to prove impossible to develop NAM approaches that cannot be used with confidence as a basis for effective risk assessments, combined with an unwillingness to rely on animal studies, then there would be the prospect of regulation on the basis of hazard rather than risk, and that would clearly be a retrograde step. It is our hope, therefore, that availability of the RCPL as described here can play a part in facilitating the development, regulatory approval and adoption of NAMs that have a proven ability to measure skin sensitising potency and provide a basis for effective risk assessments without the need for tests using animals.

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CRediT authorship contribution statement

Amaia Irizar: Conceptualization, Methodology, Data curation, Writing – original draft, Visualization. **Hans Bender:** Conceptualization, Methodology, Writing – review & editing. **Peter Griem:** Conceptualization, Methodology, Writing – review & editing, Visualization. **Andreas Natsch:** Conceptualization, Methodology, Writing – review & editing, Visualization. **Matthias Vey:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Ian Kimber:** Conceptualization, Methodology, Writing – original draft, Visualization, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amaia Irizar reports financial support was provided by The International Fragrance Association. Ian Kimber reports financial support was provided by The International Fragrance Association. Hans Bender reports financial support was provided by The International Fragrance Association. Andreas Natsch reports a relationship with Givaudan Schweiz AG that includes: employment. Peter Griem reports a relationship with Symrise AG that includes: employment. Matthias Vey reports a relationship with The International Fragrance Association that includes: employment.

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Appendix A. Supplementary data

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