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Case Study on the Use of Integrated Approaches for Testing and Assessment for skin sensitisation of Diethanolamine: Application of a Next Generation Risk Assessment Framework

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Case Study on the Use of Integrated Approaches for Testing and Assessment for skin sensitisation of Diethanolamine: Application of a Next Generation Risk Assessment Framework

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Foreword

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Cosmetics Europe for illustrating practical use of IATA and submitted to the 2022 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team.

This case study is an illustrative example, and its publication as an OECD monograph does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, this case study should not be interpreted as official regulatory decisions made by the authoring member countries.

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Abbreviations and acronyms

ACD	Allergic contact dermatitis
ANN	Artificial Neural Network
AOP	Adverse outcome pathway
BF	Bayes Factors
BN	Bayesian network
CE	Cosmetics Europe
CEL	Consumer exposure level
DA	Defined approach
DEA	Diethanolamine
DC	Dendritic cells
DIP	Data integration procedure
DPRA	Direct Peptide Reactivity Assay
ECHA	European Chemicals Agency
GHS	Globally Harmonised System
GL	Guideline
HRIPT	Human Repeat Insult Patch Test
h-CLAT	Human Cell Line Activation Test
IATA	Integrated approaches to testing and assessment
ICCS	International Collaboration on Cosmetics Safety
ITS	Integrated Testing Strategy
KE	Key event
LLNA	Local Lymph Node Assay
MoE	Margin of exposure
MDBGN	Methyldibromo glutaronitrile
MIE	Molecular initiating event
MW	Molecular weight
NA	Not applicable
NAM	New approach methodology
NGRA	Next generation risk assessment
NoG	Notes of Guidance
NR	Non-reactive
NS	Non-sensitiser
OECD	Organisation for Economic Cooperation and Development
PoD	Point of departure
QRA	Quantitative risk assessment
SAF	Safety assessment factor
SARA	Skin Allergy Risk Assessment
SCCS	Scientific Committee on Consumer Safety
STS	Sequential testing strategy
TB	Toolbox
TG	Test guideline
UN	United Nations
WoE	Weight of evidence

Executive summary

The induction of skin sensitisation is a key adverse health effect to be addressed in the safety assessment of cosmetic ingredients. Risk assessment of cosmetics and their ingredients has shifted towards the use of new approach methodologies (NAM). Information from NAM, such as in silico predictions and in chemico / in vitro data can be used to provide information on skin sensitisation hazard and potency and Defined Approaches (DA) have been developed to interpret combinations of NAM information. Progress has been made in acceptance of DA for regulatory hazard identification purposes, with three DA now published within an OECD Guideline (GL497). However, the challenge remains as to how DA predictions can be incorporated into a next generation risk assessment (NGRA) to ensure consumer safety. Here we applied our previously published NGRA framework to a hypothetical consumer risk assessment scenario; 0.8% Diethanolamine (DEA) used in a non-spray deodorant (leave-on product). Diethanolamine (DEA) was selected for a case study because the existing NAM information was inconsistent with respect to the outcomes from in silico, in chemico and in vitro assays. Within the NGRA, the following seven DA were applied to obtain skin sensitisation hazard potential and potency predictions: Integrated Testing Strategy (ITSv1, ITSv2), Artificial Neural Network (ANN SS-TIMES and ToxTree), Sequential Testing Strategy (STS), Bayesian Network Integrated Testing Strategy (BN-ITS) and Skin Allergy Risk Assessment (SARA) model. The inconsistent NAM information led to different DA predictions of skin sensitisation hazard and potency. Integration of the DA outcomes within the NGRA led to different risk assessment conclusions (safe vs. unsafe) for the use of 0.8% DEA in the deodorant. The reasons for this were found to be due to the prediction model of the DA (hazard identification vs. potency prediction), the associated uncertainty, as well as the level of conservatism in the derivation of a point of departure (PoD).

1 Introduction

All cosmetic products which are placed onto the market must be safe for their intended use and as such must undergo a human health risk assessment (SCCS, 2021). In Europe, a ban on animal testing for new cosmetic ingredients was implemented within the Cosmetic legislation (Regulation (EC) No 1223/2009) in 2009. Thus, risk assessment of Cosmetics and their ingredients has shifted toward the use of new approach methodologies (NAM). The development of NAM addressing skin sensitisation has been particularly successful, aided by our understanding of the molecular mechanisms of skin sensitisation and documentation of it within "The Adverse Outcome Pathway (AOP) for Skin Sensitisation" (OECD 2014). A number of *in chemico* and *in vitro* NAM aligned to key events (KE) involved in the induction of skin sensitisation, have now been validated and Organisation for Economic Co-operation and Development (OECD) test guidelines adopted. OECD TG 442C describes the direct peptide reactivity assay (DPRA), amino acid derivative reactivity assay (ADRA) and the kinetic DPRA which are aligned to KE-1, the binding of haptens to proteins of the skin (OECD 2021a). OECD TG 442D describes the KeratinoSens™ and LuSens which are aligned to KE-2, the activation of keratinocytes (OECD 2018). OECD TG 442E describes the h-CLAT, U-Sens™ , IL-8 Luc Assay and GARDskin, which are aligned to KE-3, the activation of dendritic cells(OECD, 2022). Currently there are no NAM targeting KE-4, the activation and proliferation of a T cell response, which are sufficiently progressed for implementation in OECD TG or for use in a Next Generation Risk Assessment (NGRA) (van Vliet et al. 2018).

Information from NAM, including those defined with OECD TG, other non-OECD approved methodologies and the predictions from *in silico* tools can be used to provide information on skin sensitisation hazard and potency. Defined Approaches (DA), which follow a specific data interpretation procedure have been developed to interpret combinations of NAM information Ezendam et al. 2016; Gilmour et al. 2020; Hoffmann et al. 2018; Kleinstreuer et al. 2018; Tollefsen et al. 2014; OECD, 2017). Three DA, which provide a hazard or UN GHS classification have now been included within OECD Guideline No. 497: Guideline on Defined Approaches for Skin Sensitisation (OECD 2021b). Despite this progress, there remains the challenge as to how DA can be incorporated into a risk assessment to ensure consumer safety. An NGRA framework has been proposed to allow application of a structured logic to the sensitisation risk assessment (Gilmour et al. 2020; SCCS, 2021) and case studies using only NAM are being increasingly utilised to explore NGRA and application of the different DA in it (Assaf Vandecasteele et al. 2021; Gautier et al. 2020; Gilmour et al. 2020; Gilmour et al. 2022; Natsch et al. 2018; Reynolds et al. 2021; OECD, 2021c).

In this IATA case study, we applied our previously published Next Generation Risk Assessment (NGRA) framework (Figure 2) (Gilmour and Kern et al. 2020) to the hypothetical consumer risk assessment scenario: 0.8% Diethanolamine (DEA) used in a non-spray deodorant. Diethanolamine (DEA) was selected because the existing NAM information were inconsistent with respect to the outcomes from *in silico*, *in chemico* and *in vitro* assays (Hoffmann et al. 2022). The impact of the inconsistent NAM data was analysed with respect to both hazard prediction and the subsequent risk assessment decision. The present case study represents increased complexity compared to previous published case studies, including our IATA case study for Geraniol (OECD, 2021c).

2 Purpose

2.1. Purpose of the case study

The revised Scientific Committee on Consumer Safety (SCCS) Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation (SCCS, 2021) contain a NGRA framework originally published by Gilmour and Kern et al. 2020, to provide guidance for the skin sensitisation safety assessment of cosmetic ingredients. The SCCS accepts NGRA submissions for ingredients to be evaluated on a case-by-case basis (SCCS, 2021).

To date, several hypothetical cosmetic ingredient NGRA case studies have been performed and published, including coumarin, formaldehyde, geraniol, lactic acid, propyl paraben, resorcinol, and the commercial fragrance azurone (Reynolds et al. 2021; Reynolds J and Gilmour N et al. 2022; Gilmour et al. 2022; Assaf Vandecasteele et al. 2021; OECD, 2021c; Gautier, et al. 2020; Natsch et al. 2010, OECD, 2020). The purpose of this IATA case study is to demonstrate the applicability of the NGRA framework by following its tiered workflow to assess the potential risk from consumer exposure to DEA at 0.8% in a non-spray deodorant. The case study builds on previous experience applying the NGRA framework and illustrates how it could be applied for an ingredient with inconsistent NAM information.

The IATA case study does not define a maximum safe use level for DEA.

2.2. Case study chemical selection

Diethanolamine (DEA) (CAS number: 111-42-2) is a cosmetic ingredient that functions as emulsifier or foaming agent and was selected because the existing NAM results were inconsistent with respect to sensitisation potential (Hoffmann et al. 2022). Note that according to the European Union Council Directive 76/768 EEC, the use of DEA in cosmetics is prohibited in the EU, thus, there is no real-life consumer exposure to DEA from cosmetic products.

The aim of this hypothetical case study is to explore the impact of inconsistent NAM information on a hypothetical risk assessment scenario for a leave-on product (0.8% DEA in a non-spray deodorant).

The selected exposure was too high to enable the use of exposure-based waiving. The principle behind exposure-based waiving is that there are situations when human or environmental exposures are so low or infrequent that there is a very low probability that the acquisition of additional effect information may lead to an improvement in the ability to manage risk. It is therefore risk-based and needs thorough knowledge on exposure as well as on effects criteria (Marquart et al. 2012).

Also, the application of read across was excluded, including the use of analogues data which could support setting of a PoD and reduce uncertainly in the risk assessment. The International Collaboration on Cosmetics Safety (ICCS) is currently performing other case studies that will specifically address the application of read across in skin sensitisation NGRA.

2.3. Endpoint

The endpoint of interest is skin sensitisation. The skin sensitisation NAM considered in this case study can be found in Table 1. Other endpoints which could impact the level of DEA used in a product were not considered.

3 Reasoning for performing IATA

3.1. Reasoning

Case study examples applying the NGRA framework for the use of Methyldibromo glutaronitrile (MDBGN) and Geraniol in a cosmetic product were previously described by CE (Gilmour and Kern et al. 2020; OECD, 2021c). These experiences provided a basis for the present DEA case study to illustrate how the NGRA framework can be applied for a more complex scenario, studying the impact of inconsistent NAM information on the risk assessment, without the use of read-across to increase confidence in the risk assessment outcome. It is important to demonstrate that the NGRA may also work without read-across since it is not always possible to apply read-across due to lack of appropriate analogues (especially for new molecules) or due to limited resources.

4 Background information

4.1 Skin sensitisation adverse outcome pathway (AOP)

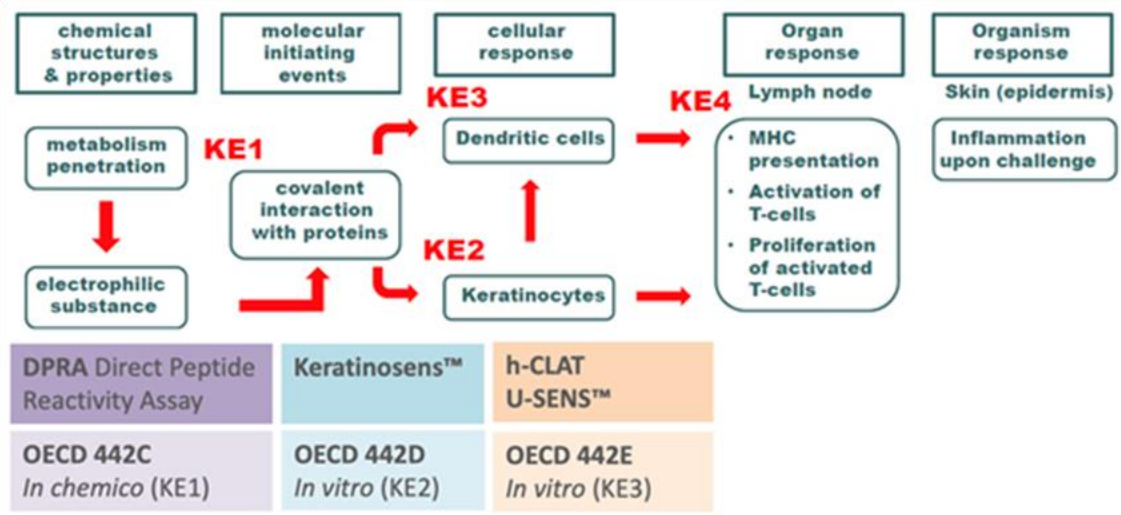
The mechanism behind skin sensitisation and the elicitation of Allergic Contact Dermatitis (ACD) has been documented by the OECD as an Adverse Outcome Pathway (AOP) (OECD, 2014). The AOP for skin sensitisation Initiated by Covalent Binding to Proteins captures the impact of skin exposure to sensitising chemicals as a series of biological and chemical key events (KE).

In the induction phase of skin sensitisation, the chemical or allergen penetrates the outer epidermis of the skin. During this passage, chemicals can either bind directly or after a biotransformation process covalently to skin proteins of the viable cells (key event 1) to form hapten-protein conjugates, which can be immunogenic. In parallel, keratinocytes become activated and release danger signals e.g., pro-inflammatory cytokines as a response to trauma (key event 2). Next, the phenotype of dendritic cells (DC) changes by the concerted recognition of hapten-protein conjugates by MHC (major histocompatibility complex) molecules and of danger signals (key event 3). The activated DCs mobilise and migrate, after maturational changes, from the skin to the draining lymph node. In the lymph nodes, the dendritic cells display major histocompatibility complex molecules, which include part of the hapten-protein complex to naive T-lymphocytes (T-cells). This induces differentiation and proliferation of allergen chemical- specific memory T-cells, some of which re-circulate throughout the body (key event 4).

The elicitation or challenge phase occurs following a subsequent contact with the same allergen. Again, the hapten-protein conjugate is formed and subsequently taken up by epidermal dendritic cells, as well as other antigen-presenting cells. The circulating allergen-specific, activated memory T-cells are triggered to secrete specific cytokines, which induce the release of inflammatory cytokines and mobilization of cytotoxic T-cells, as well as other inflammatory cells leading to the eventual adverse outcome ACD.

The mechanistic understanding of skin sensitisation and description of the AOP has enabled the development and regulatory acceptance of a multitude of NAM that each aim to measure the impact of chemical on one or more of the AOP KE to distinguish sensitising from non-sensitising chemicals or to generate information on skin sensitisation potency (Figure 1).

Figure 1. Overview of biological and chemical key events (KE) as described in the skin sensitisation AOP and used NAM in this case study covering these events.



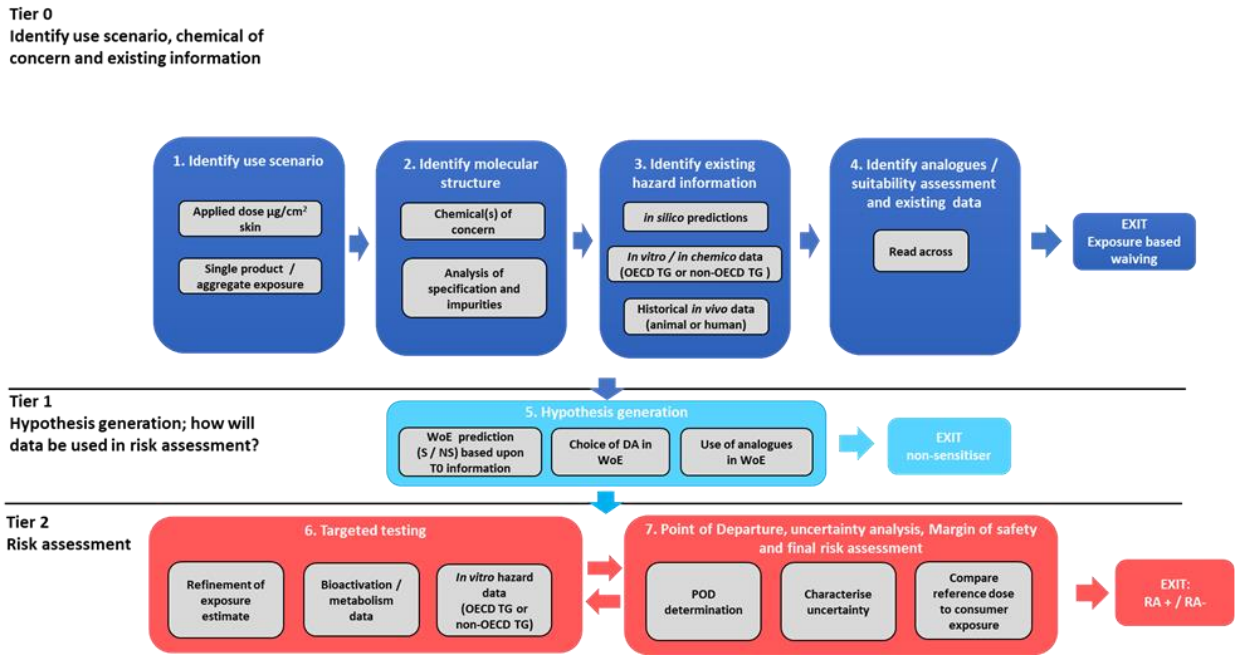
Please note that the NAM listed in the figure are not exhaustive and OECD test guidelines 442C, D and E include additional NAM which have not been considered in this case study.

4.2. NGRA framework

The experience gained how to conduct NAM-based risk assessments has allowed the cosmetic industry to develop a non-animal, next generation risk assessment (NGRA) framework for the assessment of skin sensitisers (Gilmour and Kern et al. 2020). The framework is based upon the principles published by the International Cooperation on Cosmetic Regulation (ICCR) and is human relevant, exposure led, hypothesis driven and designed to prevent harm. It is structured into three tiers, integrating all relevant information using a weight of evidence approach that can be iterated when new information becomes available (Figure 2).

The initial tier (Tier 0) involves a thorough review of the existing information including identification of the use scenario/consumer exposure; characterisation of the chemical purity and structure; *in silico* predictions; existing data pertaining to skin sensitisation hazard (historical or non-animal); the identification of suitable read-across candidates with supporting hazard identification/characterisation information and application of exposure-based waiving. Considering all information identified in Tier 0, the next step is the generation of a hypothesis (Tier 1). All data are considered in an exposure-led weight of evidence (WoE) approach, taking an initial view on whether a chemical is likely to be a skin sensitiser or not, choice of defined approach and availability of read-across candidates (note: in this IATA case study read across was not applied). If existing information is insufficient for concluding the risk assessment, the generation of additional information may be required to proceed (Tier 2). Such targeted testing could involve refinement of the exposure estimation or generation of data from *in vitro* or *in chemico* NAM. Once sufficient information is available, the final stage of the NGRA framework is the determination of a point of departure (PoD), characterising uncertainty and comparing to the consumer exposure in a WoE. Thorough evaluation of the sources of uncertainty is essential to obtain the required level of confidence in the risk assessment decision, ensure transparency and build trust in NGRA approaches.

Figure 2. Next generation risk assessment (NGRA) framework for skin sensitisation. Adopted from Gilmour and Kern et al. 2020.



5 Application of the NGRA framework

TIER 0

Identify use scenario and consumer exposure

The hypothetical use scenario for this illustrative case study was identified as 0.8% DEA in a non-spray deodorant (leave-on product) applied to the axilla. Based on an estimated daily amount of 1.5 g deodorant non-spray/day x 0.8% use concentration x 1 skin retention factor x 200 cm^2 skin surface area), the consumer exposure was determined to be 60 µg/cm^2 (SCCS, 2021).

The consumer exposure level (CEL) in dose per unit area, expressed as micro-grams applied per day (µg/cm^2) was calculated based upon 90th percentile consumer use provided in the SCCS Notes of Guidance (SCCS, 2021).

Identify chemical of interest and molecular structure

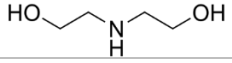
Molecular structure and physicochemical properties for DEA were identified (Table 1).

Identify existing hazard information.

The existing *in silico*, *in vitro* and *in chemico* information regarding the skin sensitisation hazard and potency for DEA is presented in Table 1.

For this case study, historical *in vivo* toxicity data, human clinical evidence and read across analogues were not considered.

Table 1. Existing information collected for DEA in Tier 0 of the NGRA framework.

Name	Diethanolamine	AOP KE addressed
CAS number	111-42-2	
SMILES	C(CO)NCCO	
Structural formula		
Physicochemical properties	Molecular weight: 105.14 Da LogP: -1.43 LogS: 0.98 LogVP: -3.55 Boiling pt. [°C]: 268.8 Melting pt. [°C]: 28 Volatility[1]: semi-volatile pH: 10.3	

	LogD @ pH 7: -3.38 H2O solubility @ pH 7: 3 g/L Plasma protein binding (% bound): 11.3	
Mechanistic domain based on expert review	Pro-Schiff base	KE-1
TIMES-SS (v2.30.1.11)	Parent: Non-sensitiser Metabolite: Non-sensitiser	KE-1
TOXTREE (v2.6.13) Skin sensitisation reactivity domains Protein binding alerts	No alert Schiff base formation	KE-1
OECD Toolbox (TB) (v4.4): https://qsartoolbox.org OASIS protein binding alerts for skin sensitisation Skin sensitisation automated workflow for DASS	No alert Negative (no analogues identified)	KE-1
DEREK 6.01 (Nexus 2.2.2)	Positive (Equivocal) [2]	KE-1
DPRA	Negative/minimal (Cys depl: 5.9% and Lys depl: 2.2%)	KE-1
KeratinoSens™	Negative (EC1.5: >2000 µM, EC3: >2000 µM, Imax: 1, IC50%: >2000 µM)	KE-2
U-SENS™	Positive (CD86 EC150: 26.9 µg/mL, CV70: >200 µg/ml)	KE-3
h-CLAT	Positive (CD86 EC150: 1242.5 µg/mL, CD54 EC200: 1280.9 µg/mL, CV75: 2277 µg/mL)	KE-3
Dermal penetration rate (from Brain et al. 2005; Kraeling et al. 2004)	Minimal <3%	

Information was obtained from the Cosmetics Europe database (Hoffmann et al. 2018; 2022),

[1] Volatility class was calculated using a method by Spicer, 2002.

[2] The prediction in Derek Nexus is equivocal due to inconsistent in vivo data for analogues and is interpreted as sensitising based upon the LHASA expert rule-base system and OECD GL497. For more information on the individual in silico and in vitro information sources see Annex I: Individual information sources used.

Identify analogues/suitability assessment and existing data

Not applicable. We purposely decided to restrict decision making solely on available NAM data and read across was beyond the scope for this IATA case study. ICCS is currently performing other case studies that will specifically address the application of read across in skin sensitisation NGRA.

TIER 1

Hypothesis generation

DEA is a novel ingredient which is proposed for use at 0.8% in a non-spray deodorant product.

- Two of the four *in silico* tools applied predicted no reactivity or skin sensitisation potential (TIMES-SS and OECD TB). Derek Nexus predicted that DEA being a skin sensitiser and ToxTree reported DEA could form a Schiff base after activation.

- The available NAM data (Tier 0) demonstrate inconsistent outcomes with respect to sensitisation potential of DEA. DPRA and KeratinoSens™ gave negative results while U-SENS™ and h-CLAT were positive, all according to the prediction models specified in the respective OECD TG. Due to the possibility that DEA could be a pro-hapten, the DPRA and KeratinoSens™ data need to be considered with caution, as the lack of metabolic activation is a recognized limitation of these test systems (OECD 2018; OECD 2021a).

Based upon the above information, a weight of evidence assessment demonstrated that it is not possible to reach the conclusion with high certainty that DEA is a non-sensitiser. This is because DEA cannot be excluded to be a pro-hapten and due to the positive results observed in U-SENS™ and h-CLAT methods investigating KE-3.

In addition, the deodorant is a leave-on product, which may be associated with a relatively high consumer exposure, under occlusion. A definitive non-sensitiser conclusion, for the purposes of cosmetic risk assessment, would result in concluding that there is no maximum limit to use.

Thus, the NGRA framework cannot be exited at Tier 1 and should be progressed to Tier 2, (Figure 2). The next step was the application of DA to generate skin sensitisation potential and potency predictions.

Choice of DA

Seven DA were considered as suitable, useful and accessible. The DA have been submitted as case studies to OECD (OECD, 2016; 2017) and supported previous case studies on MDBGN (Gilmour and Kern et al. 2020) and Geraniol (OECD, 2021c) demonstrating the application of individual DA predictions in individual WoE-based POD derivations for the risk assessment. The choice for selecting these seven DA was based on a number of factors, as outlined in the case study document and in Gilmour and Kern, et al. 2020 and below for this specific case study:

- Coverage of various key events of the skin sensitisation AOP.
- DA provide information on skin sensitisation potency to support the derivation of a PoD.
- All input data for the DA were available.
- DEA falls within the applicability domains of the selected DA.
- DA were all accessible to CE, including licences for *in silico* NAM inputs.

The intent of this case study is to show that all of these DA can be used in an IATA and to evaluate if the final risk assessment decision is the same despite the inconsistent NAM data. In practise, however, there is usually no need to use more than one DA. The risk assessor would choose one (or more DA) depending on the risk assessment question to be answered.

The individual DA are described in the sections below, more detailed information about their development and construction can be found in Annex II: Defined Approaches, the OECD case study document (OECD, 2017) and cited references. The individual data sources used in the DA have been described in detail in Annex I: Individual information sources used.

6 Defined Approaches

6.1: Sensitiser potency categorization based on test methods addressing KE 1+3 and *in silico* prediction (ITSv1 and ITSv2 DA)

Summary

The DA is constructed as ITS version 1 (ITSv1) and ITS version 2 (ITSv2) for the prediction of the skin sensitisation potential and potency of a substance (Takenouchi et al. 2015). Both DA were recently adopted as an OECD guideline No. 497 (OECD 2021b). The DA include an *in silico* prediction (Derek Nexus in ITSv1 and the OECD automated workflow for DASS in ITSv2) and uses test methods that address two key events (KE) 1 and 3 as defined in OECD Adverse Outcome Pathway (AOP) of skin sensitisation: KE1 of protein binding is evaluated using the Direct Peptide Reactivity Assay (DPRA; OECD TG 442C); KE3 of dendritic cell activation is evaluated using the human cell line activation test (h-CLAT; OECD TG 442E). Derek Nexus in ITSv1 predicts the probability that a substance will be a Sensitisers / Non-Sensitisers (S/NS) by an alert. The OECD automated workflow for DASS in ITSv2 is an open access software application, which predicts protein binding alerts and skin sensitisation hazard (https://qsartoolbox.org). The alert in Derek Nexus or OECD automated workflow and the quantitative outcomes in the DPRA and h-CLAT are converted to a score of 0 to 3. The summed score of three information sources can be used to predict the skin sensitising potential (hazard identification; S/NS) and potency of a substance. The potency prediction is given as sub-categorisation according to the UN GHS: UN GHS Cat. 1A (EC3<=2% in LLNA), UN GHS Cat. 1B (EC3>2%), Not Classified (Non-Sensitisers (NS)).

Prediction for DEA

DEA was predicted to be a UN GHS Cat. 1B (score of 2) by the ITSv1 DA and as inconclusive by the ITSv2 DA. The ITSv2 predicts an overall score of 1, however the DA outcome is considered as inconclusive based upon DEA predicted to be a pro-hapten and thus out of domain for the DPRA (OECD 2021a). The difference in scores is due to the inconsistent *in silico* predictions in the Derek Nexus (sensitiser) and OECD toolbox (non-sensitiser).

6.2 Sensitiser potency prediction based on KE 1+2+3: The artificial neural network model for predicting LLNA EC3 (ANN)

Summary

The artificial neural network (ANN) DA is an integrated testing strategy for prediction of the skin sensitisation potential and potency (predicted LLNA value) of a substance (Hirota et al. 2018). The combination of test methods used covers the first three KE of the AOP leading to skin sensitisation as formally described by the OECD: KE 1: protein binding (e.g. via the DPRA; OECD TG 442 C); KE

2: keratinocyte activation (e.g. via the KeratinoSens™; OECD TG 442D); and KE3: dendritic cell activation (e.g. via the h-CLAT; OECD TG 442E). As well an *in silico* prediction from TIMES-SS or Toxtree is used as an input parameter in the model. The ANN is based on a non-linear statistical data-modelling tool which consists of an input layer, hidden layer and output layer and can be used to predict an EC3 value (pEC3) useful to derive a point of departure for risk assessment. The EC3 value is defined as the amount of chemical required to induce in the LLNA a three-fold increase in lymph node cell proliferation compared with vehicle control values (ECETOC, 2008).

Prediction for DEA

DEA was predicted to be a weak sensitiser by the ANN DA with an EC3 value of 59.1% using Toxtree as *in silico* input parameter and with an EC3 value of 81.5% using TIMES-SS as *in silico* input parameter.

6.3: Sequential testing strategy for hazard identification (Tier 1) and potency (UN GHS cat. 1A / 1B) categorization (Tier 2) of skin sensitisation (STS)

Summary

In this sequential testing strategy DA, hazard identification and potency (UN GHS Cat. 1A/1B/no Cat.) categorization is based on the combination of multiple *in vitro* and *in silico* parameters covering the Adverse Outcome Pathway (AOP's) key events 1 to 3 leading to skin sensitisation. The DA is constructed as a tiered approach with a decision point at the end of each tier, allowing stepwise and efficient information gathering. The first tier, which combines protein reactivity and skin sensitisation predictions (TIMES-SS, Toxtree), Direct Peptide Reactivity Assay (DPRA), U-SENS™ and KeratinoSens™ data as well as physicochemical parameters (pH, volatility), was built on 219 chemicals having a LLNA-based No Cat./Cat.1 classification (Del Bufalo et al. 2018; Tourneix et al. 2019). The second tier, which combines physicochemical parameters (Molecular Weight, volatility and clogP) as well as DPRA, U-SENS™, KeratinoSens™ and optionally SENS-IS data, was built on 100 chemicals having a LLNA-based UN GHS Cat. 1A/1B classification. For each of those tiers, the combination of the different input parameters was achieved using a meta-model stacking five different statistical methods (Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring), providing a probability to belong to the group of interest ("to be a sensitiser" Tier 1, "to be a Cat. 1A" Tier 2).

Prediction for DEA

The Tier 1 of the STS DA predicted DEA as a No Cat. / Non-sensitiser, with a probability "to be a sensitiser" of 13%, which was well below the ≤ 30% cut-off. Based on this prediction the probability DEA is a non-sensitiser is considered to be high (87%).

6.4. Sensitiser potency prediction based on Key event 1+2+3: Bayesian Network ITS/DS for hazard and potency identification of skin sensitisers (BN-ITS)

Summary

This DA is based on Bayesian Network (BN) methodology. The skin sensitisation AOP structure (i.e., sequence of events, MIE) as well as data related to KE 1 (DPRA), 2 (KeratinoSens™), 3 (h-CLAT) are encoded in the BN-ITS. Cysteine and Lysine reactivity are treated as two separate, independent

molecular initiating events (MIE). BN ITS uses information on metabolic transformation and auto-oxidation from TIMES-SS in the prediction process. Bioavailability considerations are applied via physicochemical properties, to represent an estimate of the potential to penetrate the stratum corneum and the free concentration respectively. Since the BN-ITS can reason based on partial information, only relevant or available data are used for predictions. This allows explicit consideration of the applicability domains of individual assays. Data outside of domains can be excluded in the integrated prediction or treated with caution according to the prediction process. The prediction is given as potency probability distribution, the pEC3, in 4 potency classes: non-sensitisers (NS), weak (W), moderate (M), strong/extreme (S). Expressing prediction as a probability distribution naturally quantifies prediction uncertainty. In turn, it allows conversion of the prediction into a decision based on the strength of the evidence which is done using Bayes factors. Since the process of adding *in vitro* assay data to the BN ITS can be cumulative, it can also be used to guide and optimize testing strategies before testing is commenced.

Prediction for DEA

DEA was predicted as a non-sensitiser in the BN-ITS with a high probability (> 99%) and a high Bayes Factor (>30), which provides strong evidence for the prediction.

6.5. DIP for Skin Allergy Risk Assessment (SARA)

Summary

The SARA model utilises a Bayesian statistical approach to infer a human-relevant metric of sensitiser potency and a measure of consumer risk for any given consumer exposure to a chemical of interest (Reynolds, 2022). It can utilise any combination of data from (historical) HRIPT (Politano and Api 2008), (historical) LLNA (OECD, 2010), DPRA, KeratinoSens™, h-CLAT or U-SENS™ to derive sensitiser potency, which is expressed as the ED01 (the dose in µg/cm^2 which is predicted to sensitise 1% of a HRIPT population), with explicit quantification of the uncertainty in the prediction. In addition to potency assessment the SARA model also provides the probability of whether a given exposure is low risk (SARA risk metric). The margin of exposure (MoE) is calculated for the given exposure within the SARA model, by dividing the ED01 by the consumer exposure (in µg/cm^2). This MoE is then regressed against the MoE derived for benchmark consumer exposures. The benchmark exposures are historical/current exposures to consumer products which been designated as high or low risk for induction of skin sensitisation based upon the publicly available clinical/patch test data.

Prediction for DEA

The SARA model predicted an ED_{01} of 13000 µg/cm^2 for DEA (95th % confidence interval 530 – 370000 µg/cm^2). This outcome is consistent with a prediction of a weak/moderate skin sensitiser potency. The SARA risk metric, which is the probability that the given exposure in low risk is 0.5.

Individual data sources used in the selected DA for the DEA case study

Table 2. Overview of DA input data for DEA.

NAM	NAM information	ITSv1	ITSv2	ANN	STS		BN -ITS	SARA
					Tier 1	Tier 2		
PC properties	MW: 104.14 Da					√		
	LogP: -1.46					√		
	Fraction ionised: 0						√	
	LogD @ pH 7: -3.38						√	
	Volatility[1]: semi-volatile				√	√		
	pH: 10.3				√			
	H_2O solubility @ pH 7: 3.0						√	
	Plasma protein binding (% bound): 11.3						√	
TIMES-SS	(P/M) (non-sensitiser/non-sensitiser)			√	√		√	
TOXTREE	Schiff Base			√	√			
OECD Toolbox (4.5)	OASIS protein binding alert: None Skin sensitisation automated workflow for DASS: Negative		√					
DEREK Nexus	Positive equivocal) (not in training set)	√						
DPRA	Negative/minimal				√			
	Cys depl: 5.9%	√	√	√		√	√	√
	Lys depl: 2.2%	√	√	√		√	√	√
KeratinoSens™	Negative				√			
	EC1.5 : >2000 µM			√		√	√	√
	EC3 : >2000 µM						√	
	IC50% : >2000 µM						√	
U-SENS™	Positive				√			
	CD86 EC150: 26.9 µg/mL					√		√
h-CLAT	Positive							
	CD86 EC150: 1242.5 µg/ml	√	√	√			√	√
	CD54 EC200: 1280.9 µg/ml	√	√	√			√	√
	CV75: 2277 µg/ml			√			√	

TIER 2

Targeted testing

Although additional targeted testing could attempt to address some of the inconsistencies in the NAM information, for the purpose of this case study we did not consider targeted testing to avoid adding further complexity.

DA risk predictions for DEA

The skin sensitisation hazard and potency predictions (and for the SARA model the risk prediction) for DEA, generated by the seven DA can be found in Table 3.

Table 3. Predictions for DEA by the DA used.

Defined Approach	DA prediction for DEA	Comment
ITSv1 DA	GHS Cat. 1B skin sensitiser (ITS score of 2).	The ITS score of 2 was within the 2-5 GHS Cat. 1B range.
ITSv2 DA	Inconclusive	The ITS score of 1 was within the 0-1 GHS No Cat. range, however the DA outcome is considered as inconclusive because DEA was predicted to be a pro-hapten and is therefore out of domain for the DPRA.
ANN (TIMES-SS)	Weak sensitiser (EC3 value: 81.5%).	The use of different *in silico* input parameters both lead to a weak sensitiser prediction with different predicted EC3 values.
ANN (Toxtree)	Weak sensitiser (EC3 value: 59.1%).	The use of different *in silico* input parameters both lead to a weak sensitiser prediction with different predicted EC3 values.
Sequential testing strategy (STS)	Tier 1: Non-sensitiser (13% probability to be a sensitiser) Tier 2: Due to non-sensitiser in Tier 1 potency prediction in Tier 2 not applicable.	As the probability in tier 1 (13%) was well below the = 30% prediction model cut-off for sensitiser, there was a high confidence in the DA prediction.
BN ITS	High probability (> 99%) to be a non-sensitiser (Bayes Factor: >30, strong evidence).	The conversion of the probability value to BF to assess the strength of the evidence showed “strong evidence”.
SARA	Human sensitiser potency ED01 = 13000 µg/cm^2 (95th % confidence interval 530 – 370000 µg/cm^2) SARA risk metric (Probability exposure is low risk) = 0.5.	The SARA ED_{01} ranks DEA with other weak/moderate skin sensitisers.

Use of DA to derive a Point of Departure (PoD) and calculate Margin of Exposure (MoE)

The predictions from the DA were converted to PoD, based on each unique DA approach. Based on the derived PoD values, the MoE values were calculated for use of 0.8% DEA in a deodorant product using the equation MoE= PoD/CEL.

Conversion of skin sensitisation DA predictions (e.g., EC3 value, ED01) into a PoD:

A number of proposals have been published on how to convert LLNA EC3 values into sensitisation potency categories or PoD values for risk assessment (Griem et al. 2003). For this case study we applied a unified approach, by converting the DA predicted LLNA threshold values (EC3%) into a PoD [µg/cm²] as dose per unit area by using a factor of 250 (Robinson et al. 2000). This is based on the standard LLNA protocol where 25 µL test solution are distributed over a surface of 1 cm^2 per mouse ear (Griem et al. 2003). The SARA ED01 prediction can be directly used as a PoD.

This was applied to the DA predictions for DEA as follows:

- The ITSv1 predicts DEA to be a UN GHS Cat. 1B based upon an overall score of 2. This equates to a default LLNA value of >2% which when converted to dose per unit area provides a PoD of >500 µg/cm^2.

- The ITSv2 predicts an overall score of 1, however the DA outcome is considered as inconclusive based upon DEA predicted to be a pro-hapten and thus out of domain for the DPRA (OECD 2021a). Based upon the weight of evidence regarding the requirement for DEA to undergo metabolism within the cells and only weak responses being reported in the h-CLAT and U-SENS™ (Table 1) expert judgement was applied, and it was considered that DEA is more likely to be a weak/moderate skin sensitiser than a very strong skin sensitiser. Thus, in this case DEA is treated as a UN GHS Cat. 1B. This equates to a default LLNA value of >2% which when converted to dose per unit area provides a PoD of >500 µg/cm^2.

Note that the different outcomes of the two versions of the ITS were attributable to the different *in silico* tools applied, i.e., Derek Nexus (ITSv1) predicted sensitiser and OECD automated workflow (ITSv2) and the use of an out of input when from an out of domain test method having greater impact and decreasing confidence when the overall DA outcome is non-sensitising compared to when outcome is sensitising.

- The ANN with TIMES-SS predicts an EC3 value of 81.5%, which when converted to dose per unit area to provide a PoD 20375 µg/cm^2 and ANN with ToxTree predicts an EC3 value of 59.1%, which when converted to dose per unit area to provide a PoD of 14775 µg/cm^2.

- The STS predicts DEA to be a non-sensitiser (pCat1 = 13%). The high probability of 87% to be a non-sensitiser, equates to a default LLNA value of 100%, which converted to dose per unit area provides a PoD of 25000 µg/cm^2.

- The BN-ITS predicts DEA to be a non-sensitiser with a high (> 99%) and a high Bayes Factor (>30), which again equates to a default LLNA value of 100%, which converted to dose per unit area provides a PoD of 25000 µg/cm^2.

- The SARA model predicts an expected ED01 of 13000 µg/cm^2 (95th % confidence interval of 530 – 370000 µg/cm²), which is consistent with a prediction of a weak/moderate skin sensitiser potency.

Although the STS and BN-ITS DA predicted DEA to be non-sensitising. A definitive non-sensitiser conclusion, for the purposes of a cosmetic risk assessment, would allow unlimited use (here DEA) in a product. Taken together high exposure and inconsistent NAM data used in the DA, a remaining level of uncertainty was identified, precluding to exit at Tier 1 with high confidence as a non-sensitiser based upon solely the DA outcome. Whilst not necessarily required for all purposes, in this case study the non-sensitiser DA predictions were converted into a PoD (LLNA EC3 of 100%) with the aim to increase confidence in the risk assessment.

In summary, application of these seven DA, resulted in PoD values ranging from >500 to 25000 µg/cm^2. For the use of 0.8% DEA in a deodorant product the exposure was calculated to be 60 µg/cm^2 and the MoE values obtained from the seven DA ranged from >8 to 416 (Table 4).

Weight of Evidence (WoE) and Risk Assessment

The overall risk assessment outcome is evaluated as a weight of evidence considering the calculated MoE [and in the case of SARA the corresponding P(low risk)], the confidence in use of NAM input data within the DA, and the relative conservatism in the transformation of the DA outcome to a PoD.

- The value associated with an acceptable MoE for risk assessments based upon NAM is yet to be defined. For the purposes of this case study the MoE was considered as high if > 100 considered as low if < 100 in order to illustrate the decision-making process. Note, this does not apply to SARA, which incorporates clinical benchmarks to provide empirical support for the size of the MoE.
- Confidence in use of the NAM information informing each DA was defined by the predicted reactivity domain of the chemical, which indicates whether the test method should be considered as in or out of domain and whether the *in silico* prediction was in domain. For the purposes of this case study this is considered as high if all NAM utilised in the DA are within domain and low if all NAM utilised in the DA are out of domain.
- Relative conservatism in transformation of DA outcome to PoD was considered as high when the PoD is derived from a non-sensitiser outcome from the DA. This was considered as low when the PoD is provided as a quantitative output of the DA and considered as unknown when the PoD is derived from CLP Cat. 1B or 1A DA outputs.

The risk assessment outcomes based upon the seven DA for the use of 0.8% in a non-spray deodorant are described below and summarised in Table 4.

ITSv1

- As DEA might be a pro-Schiff base, the result of the DPRA is associated with a moderate confidence.
- Conservatism in transformation of DA outcome to PoD: Unknown based upon the PoD being derived based upon the use of GHS Cat. 1B, categorising DEA as a weak / moderate sensitiser with an EC3 value of >2% which is converted to a PoD of >500 µg/cm ^{2}. The exact PoD value is undetermined, and could be anywhere >500 µg/cm ^{2}. Using the 500 µg/cm^2 as a low-end PoD value, the MoE for use of 0.8% DEA in a deodorant was >8. This was considered as low (<100).
- The overall conclusion was that this exposure was un-safe.

ITSv2

- As DEA might be a pro-Schiff base, the result of the DPRA is associated with a moderate confidence.
- Conservatism in transformation of DA outcome to PoD: Unknown, as the PoD was derived by expert judgement and in addition since it was based upon the use of GHS Cat. 1B, as for the ITSv1, the exact PoD value is undetermined and could be anywhere >500 µg/cm^2.
- Using the 500 µg/cm^2 as a low-end PoD value, the MoE for use of 0.8% DEA in a deodorant was >8. This was considered as low (<100).
- The overall conclusion was that this exposure was un-safe.

***ANN* (TIMES-SS and ToxTree)**

- As DEA might be a pro-Schiff base, the result of the DPRA and KeratinoSens^{TM} is associated with a moderate confidence.
- ANN DA predictions of pEC3= 59.1% and 81.5% were used directly as PoD. Conservatism in the PoD derivation was considered as low as the point estimate p(EC3) is used directly to derive PoD, without considering any uncertainty associated with the estimate.

- The MoE for use of 0.8% DEA in a deodorant was 246 (ANN with TIMES-SS) or 340 (ANN with ToxTree), this was considered as high (>100).
- The overall conclusion was that this exposure was safe.

STS

- As DEA might be a pro-Schiff base, the result of the DPRA and KeratinoSens™ is associated with a moderate confidence.
- Conservatism in transformation of DA outcome to PoD: high as the DA outcome was non-sensitiser.
- The PoD was calculated based upon a LLNA EC3 value of 100%.
- The MoE for use of 0.8% DEA in a deodorant was 416 this was considered as high (>100).
- The overall conclusion was that this exposure was safe.

BN-ITS

- As DEA might be a pro-Schiff base, the result of the DPRA and KeratinoSens™ is associated with a moderate confidence.
- Conservatism in transformation of DA outcome to PoD: High as the DA outcome was non-sensitiser.
- The PoD was calculated based upon a LLNA EC3 value of 100%.
- The MoE for use of 0.8% DEA in a deodorant was 416, which was considered as high (>100).
- The overall conclusion was that this exposure was safe.

SARA

- As DEA might be a pro-Schiff base, the result of the DPRA and KeratinoSens™ is associated with a moderate confidence.
- Conservatism in transformation of DA outcome to PoD: Low as the DA outcome is a quantitative measure of potency (ED01) which is converted top (low risk) for a given exposure.
- The MoE for use of 0.8% DEA in a deodorant was 217 and the p(low) risk is 0.5, i.e., it is highly uncertain to whether the exposure is high or low risk.
- The overall conclusion was that this exposure was unsafe.

Table 4. Summary of the output of the seven DA, PoD and MoE determination and risk assessment outcomes for the use of DEA at 0.8% in a non-spray deodorant product.

DA	ITSv1	ITSv2	ANN (TIMES)	ANN (Toxtree)	STS	BN-ITS	SARA
DA output							
	Cat. 1B	Inconclusive	EC3=81.5 %	EC3=59.1 %	NS P(S)= 13%	NS P(NS)=99% BF (>30%)	ED_{01}=13000 µg/cm^2 (530–370000) µg/cm^2
PoD (ug/cm^2)							
	>500	>500	14775	20375	25000	25000	13000
Calculate MoE for 0.8% in non-spray deodorant							
Consumer exposure level (ug/cm^2)	60	60	60	60	60	60	60
MoE (PoD/CEL) P(low risk)*SARA ONLY	>8	>8	246	340	416	416	217 (8.8-617) P(low risk) = 0.5
Weight of evidence assessment / Characterise uncertainty							
Confidence in NAM input	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Conservatism in transformation of DA outcome to PoD	Unknown	Unknown	Low	Low	High	High	Low
MoE	Low	Low	High	High	High	High	Low

P(low risk)*SARA ONLY							P (low risk) = 0.5
Risk assessment							
Risk assessment outcome	Un-safe	Un-safe	Safe	Safe	Safe	Safe	Un-safe

7 Uncertainty

The underlying principle for this skin sensitisation IATA is based in the skin sensitisation AOP (OECD, 2014). The mechanistic understanding of skin sensitisation and description of the AOP has enabled the development and regulatory acceptance of a multitude of NAM and DA, which can be applied for hazard or potency assessment of chemicals as well as being used within a NGRA or IATA.

The uncertainty of this IATA case study has been qualitatively assessed by reviewing and discussing the uncertainty in each element of the IATA (Figure 2). The Table 5 below describes the uncertainty along each step of the NGRA framework. Where uncertainty needed to be specified for the individual DA applied, this is clearly indicated in the table below.

Table 5. Uncertainty in the elements of the NGRA case study for DEA.

NGRA element	Uncertainty (low, medium, high)	Comment
Tier 0		
Consumer exposure	Low	Deterministic worst-case consumer exposure assessment according to the SCCS NoG. Exposure from single product only, aggregate exposure was not considered.
Case study chemical	Low	Well characterized chemical, 100% pure.
NAM data used in the IATA	Medium	Medium level of uncertainty due to inconsistent NAM results. A negative outcome for skin sensitisation potential was obtained from the DPRA and KeratinoSens™. A positive outcome for skin sensitisation was obtained from the U-SENS™ and h-CLAT. Due to the possibility that DEA could be a pro-hapten the use of the DPRA and KeratinoSens™ data requires careful review, as this is a recognized limitation of these test systems (OECD 2018; OECD 2021a).
Analogue data/ Read across	NA	Not considered.
Tier 1		
Hypothesis	Medium	Uncertainty in the hypothesis that DEA is a non-sensitiser, due to inconsistent NAM information.
Tier 2		
DA outputs	Medium	The application of the two versions of the ITS DA resulted in inconsistent outcomes due to different *in silico* tools being applied; ITSv1 (Derek Nexus) predicts DEA to be a skin sensitiser (UN GHS Cat. 1B) and ITSv2 (OECD automated workflow for DASS) predicts DEA as inconclusive. The application of the DPRA and KeratinoSens™ in the ITS, ANN, STS, BN-ITS and SARA DA creates some uncertainty due to the

		possibility that DEA is a pro-hapten.
Extrapolating PoD values from DA outcomes	Low	The uncertainty is considered low as PoD were derived from the most conservative predicted LLNA EC3 values. For the non-sensitiser predictions a LLNA EC3 of 100% was used. For the SARA an ED01 with a 95% confidence interval was used.
MoE of DA-based risk assessment	Low/high	In the case of the risk assessments based upon the BN, ANN and STS the overall uncertainty is considered low as the calculated MoE values for the DA were considered acceptable (greater than the arbitrary value of 100) and confidence in the risk assessment conclusion high (Table 4). In the case of the SARA and ITS, a high level of uncertainty was evident in that SARA prediction of p(low risk) being only 0.5 and in case of ITS, the MoE being <100. In this both cases this resulted in the conclusion of un-safe to ensure that the risk assessment is protective. .
Overall, for IATA 0.8% DEA in a deodorant	Low	Some DA outcomes result in risk assessment conclusion as "safe", while others conclude "un-safe". However, in both cases the uncertainty is low, because the differences in the predictions are due to the inherent differences of the DA objectives (hazard identification vs potency prediction) and the associated level of conservatism in the PoD derivation.

NA: not applicable.

8 Strategy and integrated risk assessment conclusion

For new cosmetic ingredients consumer safety risk assessments are no longer based upon a hazard characterisation by *in vivo* animal tests, but by NAM and DA. Previously, we published a NGRA framework for skin sensitisation to aid the construction of risk assessments based upon NAM and DA (Gilmour and Kern et al. 2020) and an increasing number of case studies are being applied to build knowledge and confidence in application of these new information sources (Assaf Vandecasteele et al. 2021; Gautier et al. 2020; Gilmour et al. 2020; Gilmour et al. 2022; Natsch et al. 2018; Reynolds et al. 2021; OECD, 2021c). The present case study was selected based upon observed inconsistent outcomes in the existing NAM data set (Hoffmann et al. 2022), allowing exploration of how these inconsistencies may impact the DA and the risk assessment outcomes.

Whilst the use of read-across analogues with historical data has previously been shown to reduce uncertainty (Gautier et al. 2020) for these purposes of this case study it was considered as out of scope to allow a focus on how to deal with the inconsistent NAM information. It is intended that read-across will form the topic of a subsequent IATA case study.

Information regarding the chemistry, i.e., the mechanism by which a chemical may interact with protein, is a critical element to understand the applicability domain of the NAM. It also defines the confidence in using the NAM information within the risk assessment. DEA was predicted by the *in silico* tools and expert chemistry review to be a pro-hapten, i.e., it would require metabolic activation to convert to a reactive intermediate which can then react with protein.

The limited ability of the DPRA and KeratinoSens™ to detect pro-haptens, as the test systems lack metabolic capacity, is well documented (OECD 2018; OECD 2021a). The negative results observed in both of these test methods combined with the positive results in U-SENS™ and h-CLAT (the cell-based assays) for DEA introduced some uncertainty in the assessment, resulting in a conclusion that it was not possible to confidently define DEA as a non-sensitiser based upon the weight of evidence and exit the NGRA framework at Tier 1. This uncertainty as well as the possibly relevant consumer exposure from a deodorant product precluded the application of the DA, which defined DEA as a non-sensitiser, also because a definitive conclusion as non-sensitiser would allow that there is no maximum limit to the use concentration. To address this uncertainty a conservative approach was applied in this case study and instead of defining DEA as a non-sensitiser based on the STS and BN-ITS predictions, a PoD was derived using a LLNA EC3 of 100% (25000 µg/cm^2 for subsequent risk assessment.

Many of the DA (all but the SARA) applied within this case study utilise information from *in silico* tools. Uncertainty can be introduced when different versions of *in silico* tools are used as demonstrated by the different outcomes of the two versions of the ITS DA. ITSv1 (with Derek Nexus) predicted DEA to be a skin sensitiser (Cat. 1B) and the ITSv2 (with OECD automated workflow for DASS) was inconclusive. The OECD automated workflow for DASS is an open access software application, whereas the Derek Nexus software requires a commercial licence for use. This does raise the challenge as to when the risk assessor has access to both *in silico* tools and the predictions are in domain, but differs in outcome and subsequently result in different UN GHS categories. It is not only

the use of different tools in a DA, the *in silico* tools are regularly updated with new expert knowledge resulting in updated predictions which may influence the output of the DA which utilise *in silico* predictions. Thus, it is important to document the versions of *in silico* tools used in the risk assessment.

Within the NGRA framework, there is always the possibility to generate additional NAM information with the aim to increase confidence e.g; on potential metabolites (Reynolds G, 2021), this was however not considered in this case study. One way to account for uncertainty in risk assessment is the use of safety assessment factors (SAF). For example, the quantitative risk assessment for skin sensitisation utilises SAF to account for uncertainty in the extrapolation from the PoD to no expected sensitisation induction level (NESIL) which is commonly derived from *in vivo* data, either human repeat insult patch test (HRIPT) or LLNA, to a consumer product use scenario. Uncertainties considered within these SAF include human variability (increased population size), and the way the product is used compared to the HRIPT exposure (frequency, anatomical site). In the NGRA based on the use of NAM, it is still under discussion as to what SAF, if any, should be used to extrapolate from hazard prediction based on NAM information. In the present case study, we have applied a different approach, i.e., we derived a MoE and then evaluated possible areas of uncertainty within the risk assessment process, namely applicability domain of NAM, the impact this could have on DA outcome, and the relative conservatism in deriving a PoD from the DA outcome.

The SARA model translated the MoE into a risk metric [p(low risk)] based upon the model regressing the MoE for the case study ingredient DEA against the MoE for established high/low risk benchmark exposures, this feature means the benchmarks determine whether the MoE is sufficiently high. For the risk assessments using the information from the other five DA an arbitrary value of 100 was first assigned to see whether the MoE was sufficiently high. It should, however, be noted that it remains to be determined as to whether this value is acceptable for skin sensitisation risk assessments based upon NAM. The weight of evidence uncertainty assessment outlined here is a simplistic framework which was applied to explore how such an approach could be further developed to increase transparency in the decision making as to whether an exposure should be considered as safe or un-safe. Work is ongoing to expand upon this type of uncertainty assessment to ensure that our NGRA reaches the desired level of transparency and adequately addresses all of the associated uncertainties.

Whilst the inconsistencies in the NAM information led to differences in the DA outputs, there was less impact on the risk assessment outcomes. For the scenario of 0.8% DEA in a deodorant product 4 of the 7 applied DA resulted in a conclusion of safe (STS, BN-ITS and the two ANN versions) and 3 resulted in a conclusion of un-safe (ITSv1, ITSv2, SARA) (Table 4). The mostly likely explanation for this observation is that this is largely dependent upon the rules that have been applied within this case study. For example, in the process of applying DA which were developed to derive UN GHS categories a conservative approach such as demonstrated here in the case of the ITSv1 has to be applied when a material is classified as a skin sensitiser. A PoD value of >500 µg/cm^2 was applied as a most conservative estimate of a true threshold since it is not possible to determine where the exact threshold would lie. Furthermore, whilst the SARA model has integrated high/low risk benchmarks which provide empirical support for whether a MoE is sufficient and provides a p(lowrisk), The other DA to do not incorporate this functionality, thus an arbitrary value of 100 was set as the ‘acceptable’ MoE so that the NGRA process could be illustrated. If, for example a higher or lower value was applied as the ‘acceptable’ MoE, then the risk assessment outcomes for both exposures may be different. As noted above, this is all ‘work still to be done’. It is envisaged that this will become evident as we evolve a systematic weight of evidence uncertainty assessment approach and it may ultimately transpire that the ‘acceptable MoE’ value is dependent upon the DA applied, the uncertainty associated with the NAM data used within the DA and available additional NAM information, not used in the DA.

Overall, this IATA case study demonstrates that our NGRA framework can be successfully applied to more complex cases, as shown by the predominantly comparable risk assessment outcomes across

the different DA. Most importantly, the framework is transparent enough to explain exactly what has been done and why. This NGRA framework will continue to evolve and thus, be adaptable to different scenarios. Other case studies will follow to further challenge our NGRA framework and to increase the confidence in the risk assessment for skin sensitisation of cosmetic ingredients based on NAM.

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Annex I: Individual information sources used

Direct Peptide Reactivity Assay (DPRA)

Name of the information source	Direct Peptide Reactivity Assay (DPRA) - OECD TG 442C (OECD, 2021a)
Mechanistic basis including AOP coverage	The DPRA measures *in chemico* the binding of test chemicals to model synthetic peptides containing either lysine or cysteine (Gerberick et al. 2004, 2009). Within the skin sensitisation AOP the covalent binding of electrophilic chemicals with nucleophilic sites of amino acids in skin proteins is postulated to be the molecular initiating event (MIE) (i.e. key event 1 – protein binding reactions) leading to skin sensitisation. In skin proteins many amino acids contain electron-rich groups capable of reacting with sensitisers. Lysine and cysteine are those most often quoted but others such as arginine, histidine, methionine and tyrosine can react with electrophilic chemicals.
Description	Solutions of cysteine and lysine containing synthetic heptapeptides are incubated with a 100mM solution of the test chemical at 1:10 and 1:50 ratio respectively for 24-hours at room temperature. At the end of the incubation period unreacted peptide concentration is measured by high- performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Each test chemical is tested at a single concentration in triplicate. The positive control cinnamic aldehyde is tested concurrently, and the positive control results are used as one of the run acceptance criteria. Solvent is used as the negative control. From the determined concentration of unreacted cysteine- and lysine-containing peptides the percent peptide depletion, relative to unreacted peptide control samples is calculated (OECD, TG 442C).
Response(s) measured	Direct peptide reactivity, expressed as: % cysteine depletion, % lysine depletion.
Prediction model	The mean cysteine and lysine peptide percent depletion value of 6.38 is used to discriminate between peptide non-reactive and peptide reactive chemicals (OECD TG 442C). Within structured approaches to data integration the % cysteine and % lysine depletion values or the % of unreacted peptides are often directly used as input parameters instead of the reactivity prediction derived as described above.
Metabolic competence (if applicable)	No metabolic competent system.
Status of development, standardisation, validation	Evaluated in a EURL ECVAM validation study for reliability (Casati et al. 2013) and officially adopted test method (OECD TG 442C).
Technical limitations and limitations with regard to predictivity	Technical limitations: - The method is not suitable for testing highly hydrophobic chemicals. - Test chemicals must be stable under the test conditions (e.g. DPRA uses highly alkaline conditions for lysine reactivity). - Test chemicals having the same retention time as the cysteine and the lysine peptides provide inconclusive results. - The method cannot be used for the testing of complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e UVCB substances) due to the defined molar ratios of test chemicals and peptides. Limitations with regard to predictivity - Test chemicals requiring to be metabolically activated to act as sensitisers (pro-haptens) cannot be detected as being reactive in the DPRA. - Metals are considered outside the applicability of the DPRA since they react with proteins

	with mechanisms different than covalent binding.
Weaknesses and Strengths	Strengths: - Evaluated in a validation study for reliability (Casati et al. 2013) and detailed protocol publicly available at: http://ecvam- dbalm.jrc.ec.europa.eu/ (DB-ALM protocol N°154). - Large dataset (N>150) publicly available (e.g. Natsch et al. 2013). - Implemented and in use by several industry laboratories. - Relatively cheap and easy to perform by personnel experienced with HPLC analysis. Weaknesses: - Since a single concentration of the test chemical is assessed at a single time point, reaction kinetic information cannot be derived. The current version of TG 442C contains a kinetic DPRA. - Evaluation of the reactivity of the electrophile is limited to cysteine and lysine. Test chemicals with selective reactivity towards other nucleophiles may not be detected by the assay. - Test chemicals requiring to be abiotically activated to act as sensitisers (pre-haptens) are reported to be in most cases correctly identified. Strict pro-haptens may be underestimated.
Reliability (within and between laboratories) (if applicable)	The reproducibility in predictions (reactive/non-reactive) that can be expected from the method is in the order of 85% within-laboratories an 80% between-laboratories (OECD TG 442C).
Proprietary aspects	The test method does not have proprietary elements.
Predictive capacity (if applicable)	Results generated in the validation study (Casati et al. 2013) and published studies (Natsch et al. 2013) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS cat. 1) from non- sensitisers is 80% (N='157)' with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. False negative predictions in the DPRA generally concern pro-haptens and chemicals showing a low to moderate sensitisation potency *in vivo*. It has to be noted that the DPRA is not proposed as a stand-alone replacement method and therefore the predictive performance values are reported for indication only.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within a Defined Approach (Guideline 497), (OECD, 2021b). For the purpose of certain regulations, a positive DPRA prediction can be used to classify a chemical into UN GHS category 1. DPRA data can be used within a Defined Approach to support potency prediction.
Potential role within an IATA	The DPRA is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the DPRA is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study DPRA data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment.

KeratinoSens™

Name of the information source	KeratinoSens™ OECD TG 442D (OECD, 2018)
Mechanistic basis including AOP coverage	The KeratinoSens™ test method addresses one of the biological mechanisms described under key event 2 (events in keratinocytes) of the skin sensitisation AOP by measuring the activation of the Keap1-Nrf2- ARE pathway (Emter et al. 2010). The Keap1-Nrf2-ARE regulatory pathway is reported to be a major regulator of cyto-protective responses to electrophile and oxidative stress by controlling the expression of detoxification, antioxidant and stress response enzymes and proteins. Small electrophilic substances such as skin sensitisers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by e.g., covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor- erythroid 12-related factor 2). The dissociated Nrf2 can then activate ARE- dependent genes such as those coding for phase II detoxifying enzymes. The KeratinoSens™ is performed using an immortalised adherent cell line derived from HaCaT human keratinocytes stably transfected with a selectable plasmid containing the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element. The quantitative measurement by luminescence detection of the luciferase gene induction is used as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to electrophilic test chemicals.
Description	Cells are exposed to 12 concentrations of the test chemical for 48 hours. At the end of the incubation period quantification of luciferase gene induction is performed by luminescence analysis. Each test chemical is tested in three parallel replicate plates and a fourth replicate plate is used for cytotoxicity determination (with the MTT assay). The positive control cinnamic aldehyde is tested concurrently

	and the positive control results are used as one of the run acceptance criteria. Solvent is used as the negative control. Test chemicals are considered positive in the KeratinoSens™ if they induce a statistically significant induction of the luciferase gene above a given threshold (i.e. >1.5 fold) over solvent negative controls, at a concentration which does not significantly affect cell viability and below the concentration of 1000 M.
Response(s) measured	EC1.5 corresponding to the concentration needed for a statistically significant luciferase gene induction above the 1.5-fold threshold. Imax corresponding to the maximal fold induction of the luciferase gene over solvent control. % cytotoxicity.
Prediction model	Test chemicals are identified as potential skin sensitisers if the Imax is statistically significantly higher than 1.5-fold as compared to the basal luciferase activity and the EC 1.5 value is below 1000 M in at least two out of the three repetitions. In addition, at the lowest concentration with a gene induction above 1.5 fold the cellular viability should be above 70% and the dose-response for luciferase induction should be similar between the repetitions (OECD TG 442D).
Metabolic competence (if applicable)	Limited metabolic capacities.
Status of development, standardisation, validation	Evaluated in a validation study for reliability (EURL ECVAM, 2014) and officially adopted test method (OECD TG 442D).
Technical limitations and limitations with regard to predictivity	Technical limitations: The test method is not applicable to the testing of chemicals which are not soluble or do not form a stable dispersion either in water or DMSO. Highly cytotoxic chemicals cannot always be reliably assessed. Test chemicals that strongly interfere with the luciferase enzyme cannot be reliably tested. Limitations with regard to predictivity: Test chemicals with cLogP above 7 fall outside the known applicability of the method.
Weaknesses and Strengths	Strengths: Validated method for reliability (EURL ECVAM, 2014) and detailed protocol publicly available at: http://ecvam-dbalm.jrc.ec.europa.eu/ (DB-ALM protocol N°155). Large dataset (N> 300) publicly available (e.g. Natsch et al. 2013; EURL ECVAM, 2014). Provides dose-response information. Easy to perform. Implemented and in use by several industry laboratories. Weaknesses: Because of the limited metabolic capacity of the cell line and the experimental conditions, test chemicals requiring enzymatic activation (pro-haptens) or requiring autoxidation to act as sensitisers (pre- haptens) may provide negative predictions. Substances with an exclusive reactivity towards lysine-residues are likely to give negative results in the KeratinoSens™. Test chemical stressors other than electrophilic chemicals may activate the Keap1-Nrf2-ARE pathway leading to false positive predictions in the KeratinoSens™.
Reliability (within and between laboratories) (if applicable)	The reproducibility in predictions (positive/negative that can be expected from the method is in the order of 85% within- and between-laboratories (OECD TG 442D).
Predictive capacity (if applicable)	The accuracy of the KeratinoSens™ (EURL ECVAM, 2014) in discriminating sensitisers (i.e. UN GHS cat. 1) from non-sensitisers is 77% (N='201)' with a sensitivity of 78% (71/91) and a specificity of 76% (84/110) when compared to LLNA results. False negative predictions in the KeratinoSens™ generally concern pro-haptens or chemicals showing low to moderate skin sensitisation potency *in vivo*. It has to be noted that the KeratinoSens™ is not proposed as a stand-alone replacement method and therefore the predictive performance values are reported for indication only.
Proprietary aspects	The KeratinoSens™ is a proprietary method for which a license agreement is needed. It is now widely offered by CRO's. The plasmid encoding for the luciferase gene is proprietary to Promega, but a license for use in sensitisation assessment is included in the MTA of KeratinoSens™.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within a Defined Approach (Guideline 497), (OECD, 2021b). For the purpose of certain regulations. KeratinoSens™ prediction can be used to classify a chemical into UN GHS category 1. KeratinoSens™ data can be used within a Defined Approach to support potency prediction.
Potential role within an IATA	The KeratinoSens™ is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the KeratinoSens™ is part of the integrated decision strategy

	for skin sensitisation hazard or potency identification. In this case study KeratinoSens™ data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment.

Human Cell Line Activation Test (h-CLAT)

Name of the information source	Human Cell Line Activation Test (h-CLAT) - OECD TG 442E (OECD, 2022)
Mechanistic basis including AOP coverage	The h-CLAT quantifies in vitro changes in the expression of the CD86 and CD54 membrane phenotypic markers in a human monocytic leukemia cell line (THP-1 cells) (Ashikaga et al. 2010). THP-1 cells are monocyte-derived cells that have shown to produce DC- like responses following exposure to skin sensitising chemicals, including upregulation of surface markers (e.g. CD86 and CD54) and cytokine production (e.g. TNF-a). The CD86 (a co-stimulatory molecule) and the CD54 (an adhesion molecule) are upregulated in activated Dendritic Cells (DC) and play a critical role in DC presentation of antigens to T cells (T-cell priming). By studying the potential of test chemicals to up-regulate markers of DC activation, the h-CLAT generates information addressing key event 3 (dendritic cell activation) of the skin sensitisation AOP.
Description	Qualified THP-1 cells are exposed for 24 hours to eight serial concentrations of test chemicals selected on the basis of a predetermined CV75 (concentration of test chemical yielding 75% cells survival). At the end of the incubation period, cells are stained with FITC-labelled anti- CD86, anti-CD54 and mouse IgG1 antibodies (for measurement of non- specific background signal). Changes of CD86 and CD54 surface markers expression are measured by flow cytometry analysis. Each chemical is tested in singlicate in at least two independent runs to derive a positive or negative prediction. The positive control 2,4-dinitrochlorobenzene (DNCB) is tested concurrently at a single concentration yielding approximately 70- 90% of cell viability and positive control's results are used as one of the run acceptance criteria. Solvent is used as the negative control. Cytotoxicity is measured in parallel (with propidium iodide staining). The calculated relative fluorescence intensity (RFI) is used as indicator of CD86 and CD54 expression.
Response(s) measured	CD86 relative fluorescence intensity. CD54 relative fluorescence intensity. % cell viability.
Prediction model	An h-CLAT prediction is considered positive if: the RFI of CD86 is equal to or greater than 150% at any tested dose (with cell viability = 50%) in at least two independent runs or if the RFI of CD54 is equal to or greater than 200% at any tested dose (with cell viability = 50%) in at least two independent runs or the RFIs of both markers exceed the respective thresholds at any tested dose (with cell viability = 50%) in at least two independent runs. For test chemicals predicted as positives, two Effective Concentrations (EC) values, the EC150 for CD86 and EC200 for CD54, i.e. the concentration at which the test chemicals induced a RFI of 150 or 200, can be calculated.
Metabolic competence (if applicable)	Limited metabolic capacities (Fabian et al. 2013).
Status of development, standardisation, validation	Evaluated in a EURL ECVAM validation study for reliability (Casati et al. 2015) and officially adopted test method (OECD TG 442E).
Technical limitations and limitations with regard to predictivity	Technical limitations: The method is not applicable to the testing of chemicals which are not soluble or do not form a stable dispersion in a solvent compatible with the experimental system. Highly cytotoxic chemicals cannot be tested. Strong fluorescent test chemicals emitting at the same wavelength as FITC may interfere with the flow-cytometry light-signal acquisition. Limitations with regard to predictivity: Test chemicals with a Log P of greater than 3.5 tend to produce false negative results. Negative results with these test chemicals should be considered as inconclusive.
Weaknesses and Strengths	Strengths: Validated method for reliability (Casati et al. 2015) and detailed protocol publicly available at: http://ecvam-dbalm.jrc.ec.europa.eu/ (DB-ALM protocol N°158). Large dataset (N>140) publicly available (e.g. Takenouchi et al. 2013). Provides dose-response information. Implemented and in use by several industry laboratories. Weaknesses:

	Because of the limited metabolic capacity of the cell line and the experimental conditions, test chemicals requiring enzymatic bioactivation (pro-haptens) or autoxidation (pre-haptens) to induce sensitisation may produce false negative results. Need of expensive instruments.
Reliability (within and between laboratories) (if applicable)	The reproducibility in predictions (positive/negative) that can be expected from the method is in the order of 80% within- and between-laboratories (Casati et al. 2015).
Predictive capacity (if applicable)	Results generated in the validation study (Casati et al. 2015) and published studies (Takenouchi et al. 2013) overall indicate that the accuracy of the h-CLAT in discriminating sensitisers (i.e. UN GHS cat. 1) from non-sensitisers is 85% (N='142)' with a sensitivity of 93% (94/101) and a specificity of 66% (27/41) when compared to LLNA results. Published data indicate and accuracy of 83% (N='66)' in predicting responses in humans (Nukada et al. 2011). The relatively low rate of false negative predictions in the h-CLAT generally concern pro-haptens or chemicals showing low to moderate skin sensitisation potency *in vivo*.
Proprietary aspects	The test method has intellectual property rights protected by Patent N. 4270702 only in Japan.
	It has to be noted that the h-CLAT is not proposed as a stand-alone replacement method and therefore the predictive performance values are reported for indication only.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within a Defined Approach (Guideline 497), (OECD, 2021b). For the purpose of certain regulations a positive h-CLAT prediction can be used to classify a chemical into UN GHS category 1. h-CLAT data can be used within a Defined Approach to support potency prediction.
Potential role within an IATA	The h-CLAT is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the h-CLAT is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study h-CLAT data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment.

U-SENS™

Name of the information source	U-SENS™ , OECD TG 442E (OECD, 2022)
Mechanistic basis including AOP coverage	Skin sensitisers have been reported to induce the expression of cell membrane markers associated with activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers. Dendritic cell activation upon exposure to sensitisers leads to functional changes. For example, there are clear changes in cytokine secretion (e.g. TNF-a IL-1Tand in the expression of some chemokine receptors such as CCR7 and CXCR4. Additionally, during dendritic cell maturation, co-stimulatory and intercellular adhesion molecules such as HLA-DR, HLA-ABC, CD40, CD80, CD83, CD86 and ICAM-1/CD54 can be up-regulated. Most of the in vitro test methods measure the activation of the cell surface marker CD86, which has been established as mechanistically relevant and predictive (Ade et al. 2006; Piroird et al. 2015). The U-SENS™ assay quantifies the induction of the CD86 protein marker expression, associated with DC maturation *in vivo*. The assay is performed on the human myeloid U937 cell line, closely related to monocytes and dendritic cells. The assay therefore addresses one of the biological mechanisms covered by key event 3 of the skin sensitisation AOP (OECD, 2014; OECD 2016).
Description	The U-SENS™ method is an in vitro assay that quantifies changes of CD86 cell surface marker expression on a human histiocytic lymphoma cell line, U937 cells, following 45±3 hours exposure to at least four concentrations of test chemical selected amongst usable concentrations pre-defined in the DB-ALM protocol N°183 (2017). The CD86 surface marker is one typical marker of U937 activation. CD86 is known to be a co-stimulatory molecule that may mimic monocytic activation, which plays a critical role in T-cell priming. The changes of CD86 cell surface marker expression are measured by flow cytometry following cell staining typically with fluorescein isothiocyanate (FITC)-labelled antibodies (CD86-IgG1 percent of positive cells measurement). Cytotoxicity measurement is also conducted (e.g. by using propidium iodide) concurrently to assess whether upregulation of CD86 cell surface marker expression occurs at sub-ytoctoxic concentrations (cell viability = 70%). Each test chemical is tested in at least four concentrations and in at least two independent runs (performed on a different day) to derive a single prediction (NEGATIVE or POSITIVE). The stimulation index (S.I.) of CD86 cell surface marker compared to solvent/vehicle control is calculated and used in the prediction model, to support the discrimination between sensitisers and non-sensitisers.

Response(s) measured	- Cell viability using Propidium Iodide to calculate the concentration at which a chemical reaches the cytotoxicity threshold of 70% (CV70). - CD86 stimulation index: CD86 relative value of intensity in chemical-treated cells compared to solvent/vehicle treated cells to calculate the concentration at which a chemical reaches the CD86 positive threshold of 150%(EC150). The EC150 and CV70 values are calculated - for each run: the individual EC150 and CV70 values are used as tools to investigate the concentration response effect of CD86 increase, - based on the average viabilities, the overall CV70 is determined, - based on the average S.I. of CD86 values, the overall EC150 is determined for the test chemical predicted as POSITIVE with the U-SENS™.
Prediction model	For CD86 expression measurement, each test chemical is tested in at least four concentrations and in at least two independent runs to derive a single prediction (NEGATIVE or POSITIVE). - The individual conclusion of an U-SENS™ run is considered Negative (hereinafter referred to as N) if the S.I. of CD86 is less than 150% at all non-cytotoxic concentrations (cell viability = 70%) and if no interference is observed (cytotoxicity, solubility or colour regardless of the non-cytotoxic concentrations at which the interference is detected). In all other cases: S.I. of CD86 higher or equal to 150% and/or interferences observed, the individual conclusion of an U-SENS™ run is considered Positive (hereinafter referred to as P). - An U-SENS™ prediction is considered NEGATIVE if at least two independent runs are negative (N). If the first two runs are both negative (N), the U-SENS™ prediction is considered NEGATIVE and a third run does not need to be conducted. - An U-SENS™ prediction is considered POSITIVE if at least two independent runs are positive (P). If the first two runs are both positive (P), the U-SENS™ prediction is considered POSITIVE. - If, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only, the run is then considered to be concluded NOT CONCLUSIVE (NC), and additional concentrations should be tested in additional runs. In case a run is identified as NC, at least 2 additional runs should be conducted, and a fourth run in case runs 2 and 3 are not concordant (N and/or P independently).
Metabolic competence (if applicable)	Characterized (Fabian et al. 2013): although the activities of some xenobiotic metabolizing enzymes are not detected, U937 have functional NAT-1 and esterases.
Status of development, standardisation, validation	Evaluated in a EURL ECVAM validation study for reliability (Casati et al. 2017) and officially OECD adopted test method (OECD TG 442E).
Technical limitations and limitations with regard to predictivity	Technical limitations: The method is not applicable to the testing of chemicals which are not soluble or do not form a stable dispersion in a solvent compatible with the experimental system. Highly cytotoxic chemicals cannot always be reliably assessed. Strong fluorescent test chemicals emitting at the same wavelength as FITC may interfere with the flow-cytometry light-signal acquisition. Limitations with regard to predictivity: Membrane disrupting substances (like surfactants), Theoretically, substances that may interfere with CD86 induction pathways due to their own biological activity.
Weaknesses and Strengths	Strengths: Transferability, intra- and inter-reproducibility demonstrated Available to CROs Possible automation demonstrated for this method Large dataset tested (175 chemicals) publicly available Provide dose-response information Pre or pro-haptens correctly predicte False negatives in majority amongst weak or rare sensitisers. Weaknesses: Because of the limited metabolic capacity of the cell line and the experimental conditions, test chemicals requiring enzymatic bioactivation (pro-haptens) or autoxidation (pre-haptens) to induce sensitisation may produce false negative results. Need of expensive instruments (flow cytometer).
Reliability (within and between laboratories) (if applicable)	A validation study (combining two multicentric studies conducted in 2013 and 2014) including four laboratories and testing up to 38 chemicals designed to assess reliability was carried out according to internationally agreed OECD principles. The level of reproducibility in predictions that can be expected from the test method is in the order of 90% and 84% within and between laboratories, respectively (Alépée et al. 2015).
Predictive capacity (if applicable)	Results generated in the validation study (Alépée et al. 2015) and other published studies (Piroird et al. 2015) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 86% (N='166)' with a sensitivity of 91%

	(118/129) and a specificity of 65% (24/37). Compared with human results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 77% (N='101)' with a sensitivity of 100% (58/58) and a specificity of 47% (20/43). False negative predictions compared to LLNA with the U-SENS™ are more likely to concern chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (Alépée et al. 2017). It is important to note that the predictive values given here for the U-SENS™ as a stand-alone test method are only indicative, since the test method should be considered in combination with other sources of information in the context of an IATA.	
Proprietary aspects	The test method does not have proprietary elements.	
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within an IATA. For the purpose of certain regulations, a positive U-SENS™ prediction can be used to classify a chemical into UN GHS category 1. U-SENS™ data can be used within IATA to support potency prediction.	
Potential role within an IATA	The U-SENS™ is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the U-SENS™ is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study U-SENS™ data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment.	

TIMES-SS

Name of the information source	TIMES-SS (V2.30.1.11)
Mechanistic basis including AOP coverage	Chemical reactivity of xenobiotics (and their metabolites) with proteins can be predicted from their chemical structure as is the molecular initiating event of skin sensitisation and Key event 1 of the AOP.
Description	TIMES-SS is a software package to predict skin sensitisation.
Response(s) measured	i. Amount of protein-hapten adduct formation ii. Total Structural domain
Prediction model	Automatic prediction of the amount of protein-hapten adduct formation per mole of hapten.
Metabolic competence (if applicable)	In silico predicted metabolism and abiotic oxidation.
Status of information source development, standardisation, validation	Commercially available software, compliant with the OECD principles for QSAR validation (OECD, 2004a).
Technical limitations and limitations with regard to applicability	A defined chemical structure is needed. Unreliable predictions for chemicals falling outside the applicability domain of the model. This is indicated by the output of the software in each prediction. However, our results show that the defined approach is not affected by the applicability domain of TIMES-SS.
Weaknesses and Strengths	Strengths: - Includes prediction of metabolism, indicates whether molecule is within applicability domain. High predictive capacity. - 100% reproducibility - Fast - No high expertise needed - Can be used on any computer Weakness: - Cannot calculate mixtures, metals, polymers, and natural products.
Reliability	Not applicable
Predictive capacity (if applicable)	According to Patlewicz et al. 2007, the skin sensitisation prediction of the model performs as shown below. However, the skin sensitisation prediction readout was not used in the defined approach, but the amount of protein-hapten. Accuracy (75%, 30/40) Sensitivity (56%, 9/16) Specificity (87.5%, 21/24) In our dataset, if we assigned a positive prediction to the chemicals predicted by TIMES to be reactive to proteins and viceversa, the predictive power of the "amount of protein-hapten" was the following: All comp. (269) Accuracy= 87% Sensitivity= 92% Specificity= 78% comp. not in training set of TIMES (92). 80% 86% 70%.

Proprietary aspects	Need for a License
Proposed regulatory use	- To support the discrimination between sensitising and non- sensitising chemicals within the defined approach. - The structural alerts also included in the readouts of the software package can contribute to classification of chemicals into mechanistic domains to support read-across.
Potential role within an IATA	TIMES-SS is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the TIMES-SS is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study TIMES-SS data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment.

Toxtree

Name of the information source	Toxtree (from Ideaconsult Ltd)
Mechanistic basis including AOP coverage	The classifications that are attributed by the Toxtree Skin Sensitisation Alerts decision tree are giving an indication of the reactivity potential/behavior of the tested chemical derived from its structure. Reactivity determines the capacity of the substance to modify/haptenize skin proteins, which is the molecular initiating event defined in the AOP. (Aptula and Roberts, 2006)
Description	In silico prediction software containing Skin Sensitisation Alerts based on the Reaction Mechanistic Domains classification.
Response(s)	Five mechanistic alerts for reactivity. With “SNAr”, “SN2”, “Acyl transfer agent”, “Michel acceptor” and “Shiff base formation” reactivity alerts the chemical is classified as sensitiser, with “no skin sensitisation alert” it is classified as non-sensitiser.
Prediction model	Toxtree’s Skin Sensitisation Alerts decision tree which relies on a Reaction Mechanistic Domains classification, will output alerts for a parent chemical structure. (Aptula and Roberts, 2006)
Metabolic competence (if applicable)	No
Status of information source development, standardisation, validation	Open source software, no official validation. Toxtree’s Skin Sensitisation Alerts follow OECD in silico models’ validation principles. The approach is published in peer-reviewed journals.
Technical limitations and limitations with regard to applicability	The method can only be applied to chemicals with a defined structure (no mixtures, no polymers). Its domain mostly covers small organics, rarely inorganics. Currently there is no definition of model domain integrated.
Weaknesses and Strengths	Strengths: Mechanism based classification; freely available software; transparency of the algorithms used to generate data; the approach is published in peer-reviewed journals. Limitations: Currently there is no definition of model domain integrated.
Reliability	Not applicable
Predictive capacity (if applicable)	Ex. literature study (Safford et al. 2011) : Based on the Reaction Mechanistic Domains classification (Aptula and Roberts, 2006) and the LLNA skin sensitisation classification (Sensitiser/Non Sensitiser) for 363 chemicals, the reaction mechanistic domain classification was said to have a sensitivity of 86% and a specificity of 64% as a predictive tool for skin sensitisation (S/NS).
Proprietary aspects	Open source software from Ideaconsult Ltd.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within an IATA. The alerts can contribute to classification of chemicals into mechanistic domains to support read-across.
Potential role within an IATA	Toxtree is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the Toxtree is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study Toxtree data was integrated with other information in Tier 1 of the sequential testing strategy.

Derek Nexus

Name of the information source	Derek Nexus (version 2.0 from Lhasa Limited)
Mechanistic basis including AOP coverage	The skin sensitisation alerts that are given by Derek Nexus are mainly giving an indication of the reactivity potential/behavior of the tested chemical derived from its structure. Reactivity determines the capacity of the substance to modify/haptenize skin proteins, which is the molecular initiating event defined in the AOP (Langton et al. 2006)
Description	In silico knowledge-based toxicity alerting software comprising alerts on skin sensitisation.
Response(s)	Mechanistic alerts for Skin Sensitisation. Binary conclusions: Positive alert (='Probable,' Plausible, Equivocal alerts) or Inconclusive (absence of alert).
Prediction model	Derek Nexus is a knowledge based expert system designed to alert on the toxicity of a chemical from its structure. An alert is given if a structural feature or toxicophore associated with the occurrence of skin sensitisation has been recognized. To each alert there is a certainty level is associated. Chemicals with a skin sensitisation alert with a “certain”, “probable”, “plausible”, or “equivocal” certainty level are conservatively regarded as potential sensitisers.
Metabolic competence (if applicable)	Not applicable.
Status of information source development, standardisation, validation	Commercially available software, no official validation. Derek Nexus skin sensitisation alerts follow OECD in silico models’ validation principles (OECD, 2004a). The approach is published in peer-reviewed journals.
Technical limitations and limitations with regard to applicability	The method can only be applied to chemicals with a defined structure (no mixtures, no polymers). Its domain mostly covers small organics, rarely inorganics. To each alert there is a certainty level is associated. Chemicals with a skin sensitisation alert with a “certain”, “probable”, “plausible”, or “equivocal” certainty level are conservatively regarded as potential sensitisers.
Weaknesses and Strengths	Strengths: Mechanism based alerts; the results are extensively documented; the approach is published in peer-reviewed journals; transparency of the algorithms used to generate data; only the chemical structure is needed as input. Weaknesses: Commercial software; no calculations on structurally unidentified substances and mixtures possible.
Reliability	Not applicable
Predictive capacity (if applicable)	Alerting system, not prediction model.
Proprietary aspects	A license agreement is needed for Derek Nexus, commercially available software from Lhasa Limited.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within a Defined Approach. The alerts can contribute to classification of chemicals into mechanistic domains to support read-across.
Potential role within an IATA	Derek Nexus is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the Derek Nexus is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study Derek Nexus data was integrated with other information in the ITSv1 DA to predict skin sensitisation potency and derive a PoD for risk assessment.

OECD Toolbox v4.4.

Name of the information source	OECD QSAR Toolbox v4.4. (Skin sensitisation predictions for DA, Automated workflow for DASS)
Mechanistic basis including AOP coverage	The skin sensitisation alerts given by the QSAR Toolbox mainly give an indication of the reactivity potential/behavior of the tested chemical derived from its structure.
Description	In silico knowledge-based toxicity alerting software comprising alerts on skin sensitisation.
Response(s)	Skin sensitiser or non-sensitiser.
Prediction model	The QSAR toolbox is a knowledge based in silico tool designed to alert on the toxicity of a chemical from its structure.
Metabolic competence (if	If the target compound has no protein binding alerts, QSAR Toolbox can be used to predict

applicable)	auto-oxidation products and skin metabolites.
Status of information source development, standardisation, validation	The Toolbox is not a validated NAM, but is recommended as a method to fill toxicity data gaps in the assessment of chemical hazards. It is evaluated on a case-by-case basis for regulatory applications. It can be used as a stand-alone prediction, as part of a weight-of- evidence approach or in an IATA.
Technical limitations and limitations with regard to applicability	Toolbox is not applicable to substances that have no associated chemical structure such as substances of unknown composition.
Weaknesses and Strengths	Weaknesses: • No predictions can be made for structurally unidentified substances and mixtures possible. Strengths: • Uses publicly available software that is supported by OECD. • Mechanism based alerts; the results are extensively documented; the approach is published in peer-reviewed journals; transparency of the algorithms used to generate data.
Reliability	NA
Predictive capacity (if applicable)	Alerting system, not prediction model.
Proprietary aspects	NA
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within a Defined Approach. The alerts can contribute to classification of chemicals into mechanistic domains to support read-across.
Potential role within an IATA	The Toolbox is foreseen to be combined with complementary information and evaluated in the context of IATA. In this case study QSAR toolbox data (Automated workflow for DASS) was integrated with other information in the ITSv2 DA to predict skin sensitisation potency and derive a PoD for risk assessment (as in OECD GD 497).

Volatility through MPBPVP model in EPI Suite™ software

Name of the information source	Volatility through MPBPVP model in EPI Suite™ software (from US EPA).
Mechanistic basis including AOP coverage	"Volatility" expressed through the vapor pressure calculated by the MPBPVP model (based on the structure of a given chemical) was identified by the different statistical models we applied for its informative value to predict skin sensitisation hazard in combination to the *in silico*, *in chemico* and *in vitro* information sources used in the Integrated Testing Strategy. Although there is no evident link between this "volatility" parameter and a chemical/biological mechanism related to skin sensitisation, our hypothesis is that it might have an impact on the stability/bioavailability of the substances in defined test conditions (differences between *in vivo* and in *chemico / in vitro* test conditions).
Description	EPI Suite™ is a computer platform that contains over 13 different predictive calculation modules and databases for physicochemical properties (amongst which vapor pressure) and environmental fate.
Response(s)	Measured vapor pressure data at 25°C in mmHg, if available. Calculated vapor pressure at 25°C in mmHg. Next, we transform these values into volatility classes according to Spicer (Spicer et al. 2002): VP<10-7 mmHg = non- volatile; VP between 10-7 and 10-1 mmHg = semi volatile; VP between 10-1 and 380 mmHg = volatile; VP>380 mmHg = very volatile (These last two groups are for the stacking meta-model purpose grouped together into a "very volatile" class).
Prediction model	Based on the structure of a given chemical, the EPI Suite™ MPBPVP model estimates vapor pressure from various physicochemical equations (US EPA, 2021). In turn these equations all use as input data, measured or calculated boiling points derived from group contribution QSAR methods. A final "suggested" vapor pressure estimation is chosen depending on the fact whether the chemical is a solid, liquid or gas.
Metabolic competence (if applicable)	No

Status of information source development, standardisation, validation	Open source software, no official validation. The calculated vapor pressure model MPBPVP from EPI Suite™ follows OECD in silico models' validation principles. The approach is published in peer-reviewed journals. In addition, the EPI Suite™ platform has undergone detailed review by a panel of EPA's independent Science Advisory Board.
Technical limitations and limitations with regard to applicability	The method can only be applied to chemicals with a defined structure (no mixtures, no polymers). Its domain mostly covers small organics, rarely inorganics. Currently there is no definition of model domain integrated.
Weaknesses and Strengths	Strengths: the calculated vapor pressure model is freely available; the approach is published in peer-reviewed journals. Limitations: currently there is no definition of model domain integrated; estimation error can be introduced by poor boiling point estimates or values.
Reliability	Not applicable
Predictive capacity (if applicable)	Ex. literature study (Dearden et al. 2003 and 2007) : Results from a 100-compound test set showed that for the MPBPVP vapor pressure model the average absolute prediction error on log VP (Pa) was 0.285; 18 compounds had errors between 0.4 and 0.6, 4 compounds had errors between 0.6 and 1.0, and 2 compounds had errors > 1.0 (the worst was cy-anogen with an error of 3.339). It is noted that estimation error can be introduced by using poor boiling point estimates or values as input data.
Proprietary aspects	Open source software from US EPA.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within an IATA. More in particular integrate stability and/or bioavailability characteristics that could potentially impact *in vitro/in vivo* correlation due to different testing conditions.
Potential role within an IATA	The calculated vapor pressure is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the calculated vapor pressure is part of the integrated strategy for skin sensitisation hazard identification (Tier 1) based on *in silico*, *in chemico*, and *in vitro* data in the sequential testing strategy DA.

Measured pH

Name of the information source	Measured pH
Mechanistic basis including AOP coverage	The measured pH was identified by the different statistical models we applied for its informative value to predict skin sensitisation hazard in combination to the *in silico*, *in chemico* and *in vitro* information sources used in the Integrated Testing Strategy. Although there is no evident link between this parameter and a chemical/biological mechanism related to skin sensitisation, our hypothesis is that it might have an impact on the stability/bioavailability of the substances in defined test conditions (differences between *in vivo* and in *chemico / in vitro* test conditions).
Description	Measured quantitative pH value in water obtained with a method adapted from OECD Guideline for the Testing of Chemicals No. 122 (OECD, 2013). The pH-measurement is done at 21±2°C with a specific combined glass electrode developed for Hamilton pH-module instrument. It is calibrated before each measurement with 3 standard buffers (pH4, pH7 and pH9,2). The pH measurement of a sample is repeated 8 times with 8 electrodes and a mean and standard deviation is calculated.
Response(s) measured	Measured pH (quantitative variable): value between 1 and 14
Prediction model	Not applicable
Metabolic competence (if applicable)	No
Status of information source development, standardisation, validation	The pH measuring method is adapted from the OECD Guideline for the Testing of Chemicals No. 122.
Technical limitations and limitations with regard to applicability	Its domain mostly covers small organics, rarely inorganics.
Weaknesses and Strengths	Strengths: applicable to a wide range of chemicals; simple method. Weakness: contrary to the OECD Guideline, with our adapted method, if the pH is lower than 4 or higher than 10, the pH values are not reconfirmed by a titration method with a standardized strong base/acid.
Reliability (within and between laboratories) (if	A variation on the absolute value of ±0,3 units could be observed between 2 laboratories and 2 measurement instruments calibrated with the same buffers.

applicable)	
Predictive capacity (if applicable)	Not applicable
Proprietary aspects	L'Oréal internal method adapted from OECD Guideline for the Testing of Chemicals No. 122.
Proposed regulatory use	To support the discrimination between sensitising and non-sensitising chemicals within an IATA. More in particular integrate stability and/or bioavailability characteristics that could potentially *impact in vitro/in vivo* correlation due to different testing conditions.
Potential role within an IATA	The pH is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the pH is part of the integrated strategy for skin sensitisation hazard identification (Tier 1) based on *in silico, in chemico*, and *in vitro* data in the sequential testing strategy DA.

Molecular weight

Name of the information source	Molecular weight
Mechanistic basis including AOP coverage	The calculated molecular weight (MW) is applied for its informative value to discriminate UN GHS cat 1A from UN GHS cat. 1B in combination to the *in silico, in chemico* and *in vitro* information sources used in the TIER 2 of the Sequential Testing Strategy. Although there is no evident link between this parameter and a chemical/biological mechanism related to skin sensitisation, our hypothesis is that it might have an impact on the bioavailability of the substances and thus potentially impact their sensitising potency.
Description	The MW is calculated using Biovia software.
Response(s)	Molecular weight of the cleaned* structure of a given chemical (quantitative variable). * structure without any counter ion and with neutralized acid/basic functions
Prediction model	Using the cleaned* structure of a given chemical, the MW model estimates its molecular weight based on the constituting atom's measured weight contributions. * structure without any counter ion and with neutralized acid/basic functions
Metabolic competence (if applicable)	Not applicable
Status of information source development, standardisation, validation	The MW calculation is commercially available through software from Biovia.
Technical limitations and limitations with regard to applicability	The method can only be applied to chemicals with a defined structure (no mixtures, no polymers).
Weaknesses and Strengths	Weaknesses: no calculations on structurally unidentified substances and mixtures possible. Strengths: high predictive capacity; 100% reproducibility; fast; no high expertise needed; can be used on any computer; applicable to all structurally defined substances.
Reliability	Not applicable
Predictive capacity (if applicable)	The precision of the molecular weight is determined by the precision of the least precise atomic mass value.
Proprietary aspects	For molecular weight calculations commercially available software from Biovia can be used, but multiple freeware molecular weight calculators are also available.
Proposed regulatory use	To support the discrimination between UN GHS cat. 1A from UN GHS cat. 1B sensitisers within Tier 2. More in particular integrate bioavailability characteristics in it that could potentially impact sensitising potency.
Potential role within an IATA	The MW is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the MW is part of the integrated strategy for skin sensitisation potency prediction (Tier 2) based on *in silico, in chemico,* and *in vitro* data in the sequential testing strategy DA.

ClogP

Name of the information source	ClogP
Mechanistic basis including AOP coverage	The octanol-water partition coefficient calculated by the ClogP model is applied for its informative value to discriminate UN GHS cat 1A from UN GHS cat. 1B in combination to the *in silico, in chemico* and *in vitro* information sources used in the Tier 2 of the Sequential testing strategy. Although there is no evident link between this parameter and a chemical/biological mechanism related to skin sensitisation, our hypothesis is that it might have an impact on the bioavailability of the substances and thus potentially impact sensitising potency.
Description	The ClogP software calculates the octanol-water partition coefficient (Pow).
Response(s)	Logarithm of calculated octanol-water partition coefficient (quantitative variable).
Prediction model	Using the structure of a given chemical, the ClogP model estimates its octanol-water partition coefficient based on the theoretical fragmentation of the structure into suitable substructures for which reliable log Pow increments are known. The calculated log Pow is obtained by summing the fragment values and the correction terms for intramolecular interactions. These fragment values are originally derived from measured octanol-water partition coefficient data.
Metabolic competence (if applicable)	Not applicable
Status of information source development, standardisation, validation	The ClogP software is commercially available from the BioByte Corp. The used approach is described and referenced in the OECD Guideline for the Testing of Chemicals No. 117 (OECD, 2004b).
Technical limitations and limitations with regard to applicability	The method can only be applied to chemicals with a defined structure (no mixtures, no polymers). Its domain mostly covers small organics, rarely inorganics. In general, the applicability of the calculation method decreases as the complexity of the compound under study increases. There is an error number with description given as an output to help evaluate the applicability of the model to the given structure.
Weaknesses and Strengths	Weaknesses: no calculations on structurally unidentified substances and mixtures possible. Strengths: high predictive capacity; 100% reproducibility; fast; no high expertise needed; can be used on any computer; error number with description given to evaluate the applicability to a given structure.
Reliability	Not applicable
Predictive capacity (if applicable)	A comparison by Dearden et al. (2003) found that for ClogP, using a 138-chemical test set, the percentage of chemicals with a calculated log Pow predicted within ± 0.5 log units of the measured log Pow value was 88,4 % with a standard deviation of 0,29.
Proprietary aspects	A license agreement is needed for ClogP, commercially available software from BioByte Corp.
Proposed regulatory use	To support the discrimination between UN GHS cat. 1A from UN GHS cat. 1B within the Tier 2 of the sequential testing strategy DA. More in particular integrate bioavailability characteristics in it that could potentially impact sensitising potency.
Potential role within an IATA	The calculated octanol-water partition coefficient is foreseen to be combined with complementary information and evaluated in the context of IATA. In such context, the calculated octanol-water partition coefficient is part of the integrated decision strategy for skin sensitisation hazard or potency identification. In this case study calculated octanol-water partition coefficient data is integrated with other information in skin sensitisation defined approaches for potency prediction to derive a PoD for risk assessment .

Annex II: Defined Approaches

1: Sensitiser potency categorization based on test methods addressing KE 1+3 and *in silico* prediction (ITSv1 and ITSv2 DA)

Summary

The DA is constructed as ITS version 1 (ITSv1) and ITS version 2 (ITSv2) for the prediction of the skin sensitisation potential and potency of a substance (Takenouchi et al. 2015). Both DA were recently adopted as an OECD guideline No. 497 (OECD 2021b). The DA include an *in silico* prediction (Derek Nexus in ITSv1 and the OECD automated workflow for DASS in ITSv2) and uses test methods that address two key events (KE) 1 and 3 as defined in OECD Adverse Outcome Pathway (AOP) of skin sensitisation: KE1 of protein binding is evaluated using the Direct Peptide Reactivity Assay (DPRA; OECD TG 442C); KE3 of dendritic cell activation is evaluated using the human cell line activation test (h-CLAT; OECD TG 442E).

Derek Nexus in ITSv1 predicts the probability that a substance will be a Sensitisers / Non-Sensitisers (S/NS) by an alert. The OECD automated workflow for DASS in ITSv2 is an open access software application, which predicts protein binding alerts and skin sensitisation hazard (https://qsartoolbox.org). The peptide depletion in the DPRA and the quantitative dose-response outcome in the h-CLAT do correlate to sensitising potency based on the EC3 values in the LLNA. The alert in Derek Nexus or OECD automated workflow and the quantitative outcomes in the DPRA and h-CLAT are converted to a score of 0 to 3. The summed score of three information sources can be used to predict the skin sensitising potential (hazard identification; S/NS) and potency of a substance. The potency prediction is given as sub-categorisation according to the UN GHS: UN GHS Cat. 1A (EC3<=2% in LLNA), UN GHS Cat. 1B (EC3>2%), Not Classified (Non-Sensitisers (NS)).

Rationale underlying the construction of the defined approach

Based on the adverse outcome pathway of skin sensitisation defined by OECD, the molecular initiating event (KE1) and the cellular response of dendritic cells (KE3) are taken into account in the ITSv1 and ITSv2 DA. KE1 leading to skin sensitisation is postulated to be covalent binding of electrophilic chemical species to selected nucleophilic molecular sites of action in skin proteins. The covalent binding to skin proteins is evaluated using the Derek Nexus, OECD automated workflow and the DPRA. The activation of dendritic cells (DC) is typically assessed by expression of specific cell surface markers, chemokines and cytokines. The h-CLAT is proposed to address the KE3 (dendritic cell activation) of the skin sensitisation AOP and is OECD TG 442E. An assay related to KE2 is not included, but DPRA cysteine depletion (KE1) and KeratinoSens™ covering KE2 are mechanistically relevant (Jaworska et al. 2013). The key molecular pathway (Nrf2-ARE pathway) induced in KeratinoSens™ corresponds to cysteine reactivity with the Keap1 sensor protein. In addition, the Nrf2 activation is induced by sensitisers and not by non-sensitisers in THP-1 cells and could function as one of the danger signals to lead to the phenotypic alterations on THP-1 cells (Migdal et al. 2013; Ade

et al. 2009). Thus, there is a mechanistic rationale that DPRA and h-CLAT could be linked to KeratinoSens™ (KE2).

In the ITSv1 and ITSv2 DA, the outcomes or quantitative parameters in each of the individual test methods are assigned to scores, by modifying the weight of evidence approach proposed by Jowsey et al. (2006) and Natsch et al. (2009) in order to define a sensitising potential (hazard identification; sensitisers vs non-sensitisers) and potency (three rank classes: EC3<=2% in LLNA (Strong), EC3>2% (Weak to moderate), Non-Sensitisers (NS)) of a substance. The underlying rationale of this is that either a medium score (2) in the individual test (i.e., DPRA or h-CLAT) or a low score (1) in two test methods out of three is considered enough evidence for judging a substance as a sensitiser.

Description of the individual information sources used

Derek Nexus (in ITSv1): *in silico* knowledge-based toxicity alerting software comprising alerts on skin sensitisation (version 6.1.0 from Lhasa Limited). Derek Nexus is mainly addressing structural features and whether a hapten has a potential for electrophilic binding to skin proteins either directly or following metabolism (Langton et al. 2006). To each alert, a likelihood level is associated. Substances with causative structural alert(s) (i.e., certain, probable, plausible, or equivocal) are conservatively considered to be positive.

OECD QSAR Toolbox v 4.4 (in ITSv2): *in silico* software application, which predicts protein binding alerts and skin sensitisation hazard based in a read across approach (https://qsartoolbox.org).

DPRA is addressing the peptide binding. Haptens applied to the skin are covalently binding to nucleophilic residues (i.e. cysteine, lysine) in dermal proteins. Binding of chemicals to the skin protein is an essential step for sensitiser to obtain allergenicity (OECD, 2021a). Substances that induced mean peptide depletion of cysteine- and lysine-containing peptide above 6.38% (or in the case of co-elution, cysteine-only depletion above 13.89%) are considered to have peptide reactivity of sensitiser.

h-CLAT is addressing DC activation. When a hapten is applied to the skin, surface molecules (i.e. CD54, CD86) on skin DCs were up-regulated through the maturation process. Since CD54 is involved in DC migration to draining lymph nodes and CD86 stimulates T cell activation during antigen-presentation by DC, both molecules are essential in the induction of skin sensitisation. Substances inducing a fold induction greater than 2-fold for CD54 and/or 1.5-fold increase for CD86 at cell viabilities above 50% are predicted to have a DC activating potential of sensitiser (Ashikaga et al. 2010). From the dose-dependency curves of experiments, the median concentration(s) inducing 1.5- and/or 2-fold induction of CD86 and/or CD54 are calculated, and the resulting lower value is defined as minimal induction threshold (MIT).

Data interpretation procedure applied

The quantitative parameters or outcomes of the individual test methods are assigned to scores, by modifying the weight of evidence approach proposed by Jowsey et al. (2006) and Natsch et al. (2009) in order to define a sensitising potential and potency of a substance. The quantitative parameters of h-CLAT and DPRA are converted into a score from 0 to 3 as shown in Table ANNEX II.1. The thresholds for the scores from 0 to 3 were set in order to span the whole dynamic range on the individual assays and were also derived from the values needed for significant results. For h-CLAT, the minimum induction thresholds (MITs) are converted to a score from 0 to 3 based on the cut-offs of 10 and 150 µg/ml. For DPRA, the mean percent depletion for the cysteine and lysine peptides is converted to a score from 0 to 3, based on OECD guideline 497. In cases where co-elution occurs

only with the lysine peptide, the depletion for only cysteine peptides is converted to a score from 0 to 3. If co-elution occurs with cysteine or both peptides, the result is inconclusive. For Derek Nexus (ITSv1) and OECD automated workflow (ITSv2), an alert is assigned a score of 1; absence of an alert was assigned a score of 0. Having only an alert outcome is regarded as not sufficient evidence to predict a test substance as a sensitiser. When the sum of these scores have been assessed, a total battery score from 0 to 7, calculated by summing the individual scores, is used to predict the sensitising potential (hazard identification; sensitisers vs non-sensitisers) and potency (three rank classes: UN GHS Cat. 1A, Cat. 1B, Non-Sensitisers (NS)). The positive criteria are set as a total battery score of 2 or greater. Furthermore, a total battery score is classified into three ranks: score of 6 or 7 is defined as a strong (UN GHS Cat. 1A) sensitiser; score of 5, 4, 3, or 2 as a weak to moderate (UN GHS Cat. 1B) sensitiser; score of 1 or 0 as not-classified (non-sensitiser). The summed score yields a qualitative result (positive/negative and three rank classes).

Table ANNEX II.1. Conversion of the outcome in h-CLAT, DPRA, and DEREK for the ITSv1 and v2 DA.

Score	h-CLAT MIT (µg/mL)	DPRA Depletion (%) (Cysteine-only)	Derek Nexus	OECD QSAR Toolbox
3	=10	=42.47 (=98.24)		
2	>10, =150	=22.62, <42.47 (=23.09, <98.24)		
1	>150, =5000	=6.38, <22.62 (=13.89, <23.09)	Positive	Positive
0	Not calculated	<6.38 (<13.89)	Negative	Negative
	Potency	**Total Battery Score**		
	UN GHS Cat. 1A	6-7		
	UN GHS Cat. 1B	2-5		
	Not classified	0-1		

Limitations in the application of the defined approach

The limitations on the individual *in chemico* and *in vitro* test methods are described in the individual test guidelines (TG 442C, TG 442E). Chemicals that fall outside the applicability domains of the DPRA and h-CLAT cannot be applicable to the ITSv1 or ITSv2 DA.

For *in silico* information source predictions from Derek Nexus, negative predictions that contain misclassified and/or unclassified features are considered to be outside applicability domain.

The level of confidence of the ITSv1 and ITSv2 DA is dependent on the total battery score and applicability domain of the individual information sources. The DA predictions with low confidence are considered inconclusive for prediction of the hazard potential and/or potency sub-categorisation.

Pre- and pro-haptens might not be reliably predicted due to lack of metabolic capacities in both the DPRA and h-CLAT. When information from the different individual data sources is integrated in the ITSv1 and ITSv2 DA, the individual limitation can be minimised, and the DA can lead to correct classification of pre- /pro-haptens.

Consideration of uncertainties associated with the application of the defined approach

DIP structure

- KE4 is not included due to lack of available tests.
- The ITSv1 and ITSv2 DA cover KE1 and KE3 of AOP and is originally based on a dataset of 139 chemicals.
- In some cases, the confidence is lower for chemicals with log P > 3.5. Negative results for chemicals with LogP >3.5 should not be considered, since they are outside the applicability domain of the h-CLAT (OECD, 2021b).
- The confidence might be lower for pre-haptens and pro-haptens due to limited metabolic capacities of test methods.

The information sources used within the defined approach

- The DPRA and h-CLAT has been validated under the ECVAM. Reproducibility of peptide reactivity and CD86/CD54 measurements are high.

Benchmark data

- The ITSv1 and ITSv2 DA for hazard identification is based on the data from LLNA. The variability of EC3 values of LLNA has been reported depending on vehicle used and laboratories. Therefore, the uncertainty in misprediction of EC3 values is taken into account.

Impact of uncertainty on the DIP's prediction

Some uncertainty might cause under- or over-estimation of hazard identification and potency classification for the ITSv1 and ITSv2 DA. For a new test chemical, similar chemicals with *in vitro* or *in vivo* data should be checked.

Prediction for DEA

DEA was predicted to be a UN GHS Cat. 1B (score of 2) by the ITSv1 DA and Not Classified/Non-Sensitiser (score of 1) by the ITSv2 DA. This difference is due to the inconsistent *in silico* predictions in the in Derek Nexus (sensitiser) and OECD toolbox (non-sensitiser).

2: Sensitiser potency prediction based on KE 1+2+3: The artificial neural network model for predicting LLNA EC3 (ANN)

Summary

The artificial neural network (ANNN) DA is an integrated testing strategy for prediction of the skin sensitisation potential and potency (predicted LLNA value) of a substance (Hirota et al. 2018). The combination of test methods used covers the first three KE of the AOP leading to skin sensitisation as formally described by the OECD: KE 1: protein binding (e.g. via the DPRA; OECD TG 442 C); KE 2: keratinocyte activation (e.g. via the KeratinoSens™; OECD TG 442D); and KE3: dendritic cell activation (e.g. via the h-CLAT; OECD TG 442E). As well an *in silico* prediction from TIMES-SS or

Toxtree is used as an input parameter in the model. The ANN is based on a nonlinear statistical data-modelling tool which can be used to subcategorise skin sensitisers in cat 1A and 1B for GHS/CLP and potency estimations (extreme and strong, moderate, weak, and non-sensitiser) useful to derive a point of departure for risk assessment.

Rationale underlying the construction of the defined approach

Skin sensitisation is the result of a complex multifactorial sequence of events and has long been the focus of research. The molecular initiating event is defined as the covalent binding of the hapten to skin proteins. This step is evaluated using the Direct Peptide Reactivity Assay (DPRA; OECD TG 442C). Inflammatory and protective responses by the first cells coming into contact with the substance, the keratinocytes, are essential for downstream events to take place. Keratinocyte activation is evaluated via the KeratinoSens™ (OECD TG 442D). Dendritic cells (DCs) transport the hapten to the regional lymph nodes, present the hapten on the cell surface and, when activated (mature DCs), are able to present the antigen in the proper context (upregulated cell surface markers, e.g. CD86 and CD54) to activate naïve T-cells thereby triggering their proliferation. The potential of a substance to cause DC activation is assessed using the h-CLAT (OECD TG 442E). TIMES-SS prediction is used as *in silico* parameter. Each *in vitro* test is corresponding protein binding, Keratinocyte activation and Dendritic cell activation. These indicator and tests are covering the AOP key events. Our ANN model can contribute to building a new quantitative risk assessment (QRA) evaluation system by predicting EC3 without animal testing. Because the mechanisms of skin sensitisation are too complex, based on immune system, it is widely recognized that a single *in vitro* test is insufficient to replace and that integration of results from various *in vitro* tests, as well as *in silico* methods, is needed for prediction of skin sensitisation potency. Therefore, the ANN approach would be to play important role in this field, where the commonly used approaches hardly work. It is widely accepted that ANN approach is effective for estimation of complex reaction consisting of multi steps such a toxicological process (Valkova et al. 2004).

Description of the individual information sources used

A) Either TIMES-SS or Toxtree: *in silico* parameter.

B) AOP key event 1: i.e. DPRA is addressing a protein binding potential.

C) AOP Key event 2: i.e. KeratinoSens™ is addressing an activation of the keratinocyte (Natsch and Emter, 2008).

D) AOP Key event 3: i.e. h-CLAT: human Cell Line Activation Test (*in vitro*) h-CLAT is addressing a dendritic cell activation.

E) LLNA (*in vivo*) EC3 values are used as *in vivo* indicators. In case of non-sensitiser, maximum applied doses were used as well. In general, descriptors that are correlated with each other are not used in ANN analysis, because combinations of independent descriptors are expected to improve the predictive performance of ANN models, as compared with combinations of correlated descriptors (e.g., cytotoxicity of each *in vitro* test).

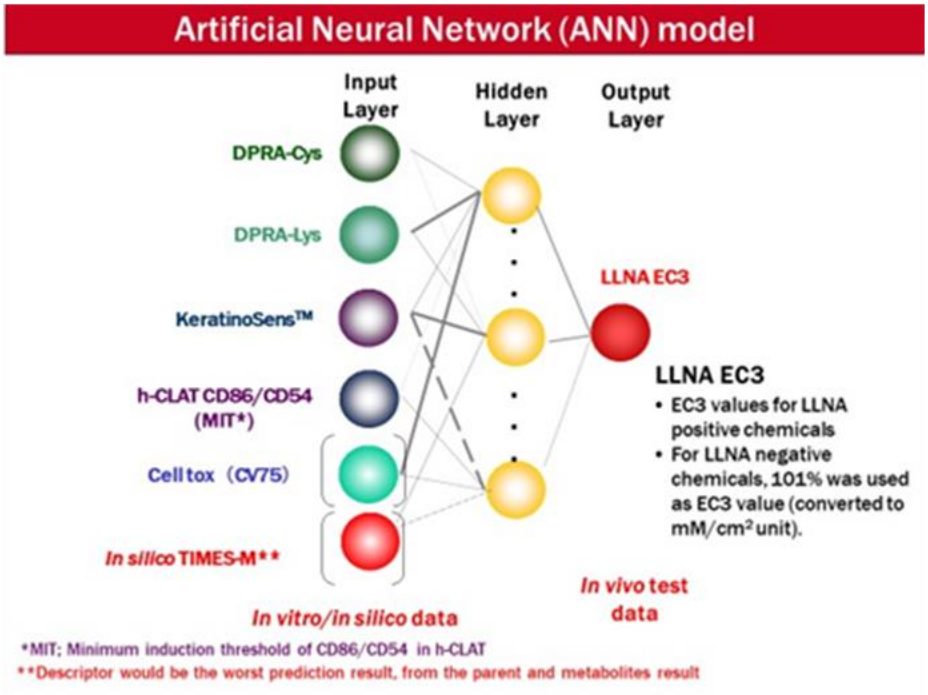
Data interpretation procedure applied

These individual tests give us several parameters. Then these parameters are integrated by using artificial neural network (Figure ANNEX II.1). Therefore, it is possible to say that our model is a kind

of integrated approach in order to derive an interim conclusion. Please note that we cannot see how much each information source contributed to the decision. The artificial neural network consists of input layer, hidden layer and output layer. Parameters resulting from the three *in vitro* tests and *in silico* data (TIMES-SS) are input. All calculations were performed using QwikNet Ver. 2.23. In this mode, there are two hidden layers. Published LLNA thresholds are used as the output layer. Our DIP model is quantitative.

Figure ANNEX II.1. Conceptual diagram of the artificial neural network consisting of input layer, hidden layer and output layer.

The LLNA Threshold is EC3 value (%).



Limitations in the application of the defined approach

Chemicals that fall outside the applicability domains of each *in vitro* test adopted in this model cannot be applicable.

- The strengths and technical limitations on the individual test inputs are detailed in the respective individual data sources.
- Physical state may preclude testing e.g., gases, highly lipophilic substances (cell culture). Substances with a high logP (e.g., exceeding 3.5 in the h-CLAT and 5.0 in the KeratinoSens™ assay) may pose problems due to the aqueous nature of the cell culture medium and solubility issues.
- Substances must be stable under test conditions e.g., the DPRA uses high alkaline conditions for lysine reactivity.

Substance related limitations:

- Pre- and pro-haptens might not be reliably predicted due to lack of metabolic capacities in both the DPRA and h-CLAT.
- Substances that only react with lysine and not with cysteine can lead to false negative predictions as both the DPRA and KeratinoSens™ use cysteine reactivity as a read-out.

In silico limitations:

- It is impossible to see how much each information source contributed to the decision. The number of the dataset is limited (134 chemicals). Increasing the size of the dataset might be effective to improve the predictive capacity.

Consideration of uncertainties associated with the application of the defined approach

DIP structure

- Key event 4 is not included due to lack of available tests.
- The confidence is lower for chemicals which are out of the technical limitations (e.g., log P > 3.5).
- The confidence is lower for pre-haptens and pro-haptens due to limited metabolic capacities of test methods.
- The predictive capacity can vary depending on the dataset used.
- In this approach, there is a hidden layer between input layer and output layer. Therefore, it is impossible to see from outside how the different data were weighted and combined.
- There is not an agreed approach to the validation of *in silico* methods such as neural network analysis.

The information sources used within the defined approach

- The DPRA, KeratinoSens™ and h-CLAT are approved within OECD test guidelines and have been validated by ECVAM. Reproducibility of peptide reactivity and CD86/CD54 measurements are high.

Benchmark data

- The Integrated Testing Strategy (ITS) for hazard identification is based on LLNA data. The variability of EC3 values of LLNA has been reported depending on vehicle used and laboratories. Therefore, the uncertainty in misprediction of EC3 values is taken into account.

Impact of uncertainty on the DIP's prediction

The different sources of uncertainty could cause to under- or over-estimate skin sensitisation potential.

Prediction for DEA

DEA was predicted to be a weak sensitiser by the ANN DA with an EC3 value of 59.1% using Toxtree as *in silico* input parameter and with an EC3 value of 81.5% using TIMES-SS as *in silico* input parameter.

3: Sequential testing strategy for hazard identification (Tier 1) and potency (UN GHS cat. 1A / 1B) categorization (Tier 2) of skin sensitisation

Summary

In this sequential testing strategy DA, hazard identification and potency (UN GHS cat. 1A/1B/no Cat.) categorization is based on the combination of multiple *in vitro* and *in silico* parameters covering the Adverse Outcome Pathway (AOP's) key events 1 to 3 leading to skin sensitisation. The DA is constructed as a tiered approach with a decision point at the end of each tier, allowing stepwise and efficient information gathering (Figure ANNEX II.2). The first tier, which combines protein reactivity and skin sensitisation predictions (TIMES-SS, Toxtree), Direct Peptide Reactivity Assay (DPRA), U-SENS™ and KeratinoSens™ data as well as physicochemical parameters (pH, volatility), was built on 219 chemicals having a LLNA-based No Cat./Cat.1 classification (Del Bufalo et al. 2018; Tourneix et al. 2019). The second tier, which combines physicochemical parameters (Molecular Weight, volatility and clogP) as well as DPRA, U-SENS™, KeratinoSens™ and in Tier 2 optionally SENS-IS data, was built on 100 chemicals having a LLNA-based UN GHS Cat. 1A/1B classification. For each of those tiers, the combination of the different input parameters was achieved using a meta-model stacking five different statistical methods (Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring), providing a probability to belong to the group of interest ("to be a sensitiser" Tier 1, "to be a cat. 1A" Tier 2).

Rationale underlying the construction of the defined approach

The sequential testing strategy was constructed to allow a stepwise gathering of information using a tiered approach on skin sensitisation hazard (Tier 1) and potency (Tier 2) (Gautier et al. 2020; Assaf Vandecasteele et al. 2021). Both tiers integrate information covering the skin sensitisation AOP KE1 (MIE), KE2 and KE3. As such, the sequential testing strategy is based on both intrinsic physicochemical properties of the chemical and descriptors of early innate immune cell responses key events, as described below.

Intrinsic physicochemical properties of the chemical

- Descriptors allowing to integrate stability and/or bioavailability characteristics. As such, the measured pH and the calculated volatility, cLogP and MW were considered as relevant variables to combine with *in silico*, *in chemico* and *in vitro* NAM, as defined in a splitting statistical analysis (Gomes et al. 2012). See individual information sources for rationale description ("Mechanistic basis including AOP coverage" section).
- Chemical reactivity (which is directly linked to the initial key event: haptenation of skin proteins): the Toxtree protein binding alerts. The Times-SS predictions also mainly take into account electrophilic binding to skin proteins either directly or following metabolism. Finally, the *in chemico*

DPRA (OECD Test Guidelines 442C), related to AOP key event 1, is a method giving a measurement of MIE as cysteine and lysine peptides modifications by the chemical.

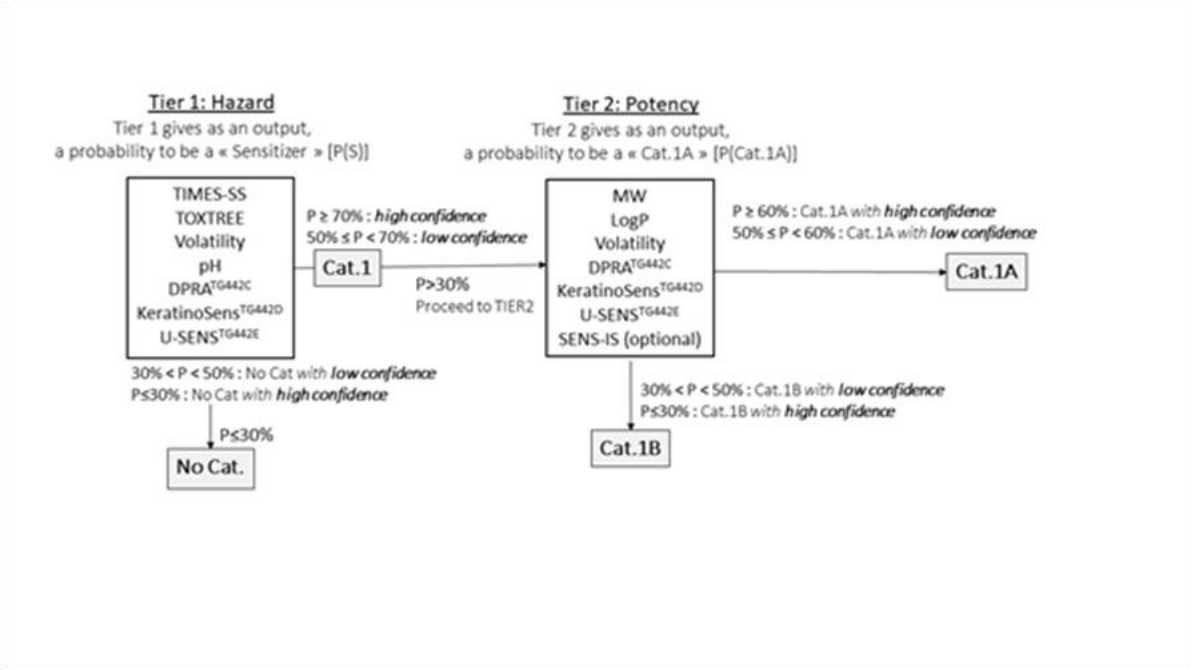
Descriptors of early innate immune cell responses

- AOP key event 2: i.e. keratinocytes activation, with the KeratinoSens™ assay assessing the induction of the Nrf-2 pathway (OECD Test Guidelines 442D), and optionally the SENS-IS™ assay assessing the transcriptomic activation of keratinocytes within a reconstructed human epidermis (Cottrez et al. 2015; 2016).
- AOP key event 3: i.e. dendritic cells activation, with the existing DC-surrogates based CD86 activation U-SENS™ assay (Piroird et al. 2015; Alépée et al. 2015; Alépée et al. 2017).

In both tiers, the combination of the different input parameters was achieved using a stacking meta-model. From the large number of supervised classification models proposed in the literature, five different methods: Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring were selected (Gomes et al. 2014; Nocairi et al. 2016). These methods have strong differences, but they all produce posterior probability of belonging to the group of interest. Therefore, two stacking models (Tier 1 “to be a sensitiser” and Tier 2 “to be a UN GHS cat. 1A”) were built independently on a proper training set (based on LLNA data). Instead of trying to choose a specific method, these methods were combined by the stacking methodology of Wolpert (1992) and Breiman (1996), in order to obtain a specific stacking meta-model for each tier.

Figure ANNEX II.2. Schematic representation of sequential testing strategy for hazard identification (Tier 1) and potency (UN GHS cat. 1A / 1B) categorization (Tier 2) of skin sensitisation.

There is the option to also use of SENS-IS in Tier 2.



Description of the individual information sources used

The individual data sources have been described in Annex 1. The respective input parameters from the NAM that were combined for the construction of the two tiers are given in Table ANNEX II.2 below.

Table ANNEX II.2. Input parameters of each tier of the sequential testing strategy.

Individual sources	Tier 1 (Hazard)	Tier 2 (Potency)
Physicochemical properties	-Volatility class (Very-volatile, Semi-volatile, Non-volatile, INC (Qualitative) - pH (Quantitative)	- Volatility class: Very-volatile, Semi-volatile, Non-volatile, INC (Qualitative) - cLogP (Quantitative) - MW (Quantitative)
TIMES-SS	- P/N/INC outcome (Qualitative)	
ToxTree	- R/NR outcome (Qualitative)	
DPRA	- R/NR/INC (Qualitative)	- Reactivity classes: HR, MR, LR (Qualitative)
KeratinoSens™	- P/N/INC outcome (Qualitative)	- $EC_{1.5}$ in µM (Quantitative)
U-SENS™	- P/N outcome (Qualitative)	- EC 150 in µg/mL (Quantitative)
SENS-IS™ (optional*)		- Potency class: weak, moderate, strong (Qualitative)

INC: Inconclusive; R: reactive; NR: No or minimal Reactivity; LR: Low Reactivity; MR: Moderate Reactivity; HR: High Reactivity; P: Positive; N: Negative. *SENS-IS used in case of borderline Tier 2 predictions.

Data interpretation procedure applied

The respective input parameters of each tier (Table ANNEX II.2) are entered into the model where they are run in five different supervised classification models (Boosting, Naïve Bayes, SVM, Sparse PLS-DA and Expert Scoring), each of it providing a probability of being a skin sensitiser (Tier 1) or a probability of being a UN GHS cat. 1A skin sensitiser (Tier 2). These intermediate probabilities that are evidently highly positively correlated (Gomes et al. 2012; Nocairi et al. 2016) are then used in a stacking meta-model that provides a final probability to be a skin sensitiser (Tier 1) or to be a UN GHS Cat. 1A skin sensitiser (tier 2) (primary outcomes of the meta-models). Optimal predictive capacities based on LLNA reference data were obtained by setting the following probability thresholds:

Tier 1 (Hazard identification):

- Chemicals with probability to be sensitiser ≥ 70% are predicted “Sensitiser” with high confidence;
- Chemicals with probability to be sensitiser comprised between ≥50% and <70% are predicted “Sensitiser” with low confidence;
- Chemicals with probability to be sensitiser comprised between (>30 and < 50%) are predicted “Non-Sensitiser” with low confidence;
- Chemicals with probability to be sensitiser ≤ 30% are predicted “Non-Sensitiser” with high confidence.

Tier 2 (Potency prediction):

- Chemicals with probability to be sensitiser ≥ 60% are predicted “UN GHS cat.1A” with high confidence;

- Chemicals with probability to be sensitiser comprised between ≥50% and <60% are predicted “UN GHS cat.1A” with low confidence;
- Chemicals with probability to be sensitiser comprised between (>30 and < 50%) are predicted “UN GHS cat. 1B” with low confidence;
- Chemicals with probability to be sensitiser ≤ 30% are predicted “UN GHS cat. 1B” with high confidence.

Based on these predictions, the decision rules for a sequential testing strategy are the following:

Tier 1 (Hazard identification):

- Chemicals with a probability to be sensitiser ≤ 30%, are classified “Non-Sensitiser” with high confidence and no further testing is needed.
- Chemicals with a probability > 30%, proceed to Tier 2.

Tier 2 (Potency prediction):

- Chemicals with a probability to be UN GHS cat. 1A ≥ 60 % are classified “UN GHS cat. 1A” with high confidence.
- Chemicals with probability to be sensitiser comprised between ≥50% and <60% are predicted “UN GHS cat.1A” with low confidence;
- Chemicals with probability to be sensitiser comprised between (>30 and < 50%) are predicted “UN GHS cat. 1B” with low confidence;
- Chemicals with a probability ≤ 30% are classified “UN GHS cat. 1B” with high confidence.

Limitations in the application of the defined approach

The strengths and limitations on the individual test inputs are detailed in the respective individual data sources. Potentially interferences for volatiles, color, highly cytotoxicity, low solubility, pre- or pro-haptens, membrane disrupting chemicals might occur depending on the individual data sources. By integrating the different individual data sources, the stacking meta model minimises individual limitations and allows a correct classification of pre- pro-haptens, dyes and low soluble chemicals. DA can be used for chemicals with defined structures, for which physicochemical properties can be calculated and *in silico* predictions (TIMES-SS, TOXTREE) can be made. Complex mixtures without a defined structure and a defined MW could not be processed in the sequential testing strategy. Overall applicability domain of the defined approach comprises several classes of cosmetic chemicals (fragrances, dyes, preservatives, actives, surfactants and Ultra-Violet filters), and non-cosmetic organic chemicals. Results should be interpreted with care for agrochemicals, metals, nanomaterials, since the representation of these categories in the training set is low or even absent. The DA is not applicable for gases.

Consideration of uncertainties associated with the application of the defined approach

DIP structure

The workflow aims to infer the sensitising outcome of the LLNA. The variability of the LLNA will of course affect the accuracy and confidence in a sensitisation prediction for humans. The correlation of the LLNA with human NOELs and LOELs is far from perfect.

Phase I metabolic pathways are not fully represented.

The information sources used within the defined approach

In the DA, all *in chemico* / *in vitro* methods used have been shown to be reliable (intra- and inter-laboratories) and relevant (S/NS) through multicentre studies evaluations. DPRA, KeratinoSens™ and U-SENS™ assays have been regulatory accepted by OECD (TG 442C, 442D, 442E respectively).

The uncertainties for the defined approach that are related to the DIP information sources include the following:

- Inconsistent results in the source data for a given chemical would reduce the confidence in the hazard predictions.
- Volatility was predicted rather than measured.
- Results from TIMES for predicted auto-oxidation products or skin metabolites may rely on those that are not biologically important.

Benchmark data

The benchmark data used to develop the test methods (219 chemicals for Tier1 and 100 chemicals for Tier2) was primarily based on data obtained from the murine LLNA. The variability of the reference *in vivo* data inevitably affects the accuracy of prediction. This variability originates from the intrinsic variability of the biological model and from the testing variability (between- and within-laboratory variability). The LLNA between-laboratory concordance for sensitiser/non-sensitiser classifications is around 80% (ICCVAM, 1999). In the original validation study, the LLNA (and guinea pig tests) was reported to have an accuracy of 72% when compared to human data (Dean et al. 2001). Variability in the EC3 values of the LLNA has been reported (Dumont et al. 2016). Around those uncertainties, the defined approach was developed using the most prevalent reference result for LLNA hazard (Tier1) and UN GHS Cat. 1A/1B potency classification when multiple data were available.

Impact of uncertainty on the DIP's prediction

The meta stacking model approach is designed to account for uncertainties in the underlying data and is directly incorporated into the final probability prediction (with low and high probability confidence). The user will have higher confidence in predictions by those chemicals:

- That lie within the applicability domain of *in silico* tools, *in vitro* tools.
- That are performed without any technical limitations in the running of *in chemico* or *in vitro* methods.
- That are direct acting in nature and do not require metabolic or chemical activation.

On the other hand, predictions with lower confidence are those where the substances fall outside of the TIMES-SS applicability domain, multifunctional chemicals for which assignment of a reaction domain is challenging and assigned as a special case, acylating agents for which assays such as the KeratinoSens™ tend to give rise to false negative results and substances which are associated with data showing them to be corrosive or highly irritating.

If the probability to be a GHS category 1A sensitiser in Tier 2 is very close to the cut offs, the option to integrate a SENS-IS potency class may be used (Table ANNEX II.2). The use of SENS-IS data in Tier 2 is not systematically done, but only in case of borderline predictions, where the inclusion of additional evidence may support classification.

Prediction for DEA

The Tier 1 of the STS DA predicted DEA as a No Cat. / Non-sensitiser, with a probability to be a sensitiser of 13%. This equals to a 87% probability DEA is a non-sensitiser.

4: Sensitiser potency prediction based on Key event 1+2+3: Bayesian Network ITS/DS for hazard and potency identification of skin sensitisers (BN-ITS)

Summary

This DA is based on Bayesian Network (BN) methodology. The Skin Sensitisation AOP structure (i.e., sequence of events, MIE) as well as data related to KE 1 (DPRA), 2 (Keratinosens™), 3 (h-CLAT) are encoded in the BN-ITS. Cysteine and Lysine reactivity are treated as two separate, independent molecular initiating events (MIE). BN ITS uses information on metabolic transformation and auto-oxidation from TIMES-SS in the prediction process. Bioavailability considerations are applied via physicochemical properties, to represent an estimate of the potential to penetrate the stratum corneum and the free concentration respectively. Since the BN-ITS can reason based on partial information, only relevant or available data are used for predictions. This allows explicit consideration of the applicability domains of individual assays. Data outside of domains can be excluded in the integrated prediction or treated with caution according to the prediction process. The prediction is given as potency probability distribution, the pEC3, in 4 potency classes: non-sensitisers (NS), weak (W), moderate (M), strong/extreme (S). Expressing prediction as a probability distribution naturally quantifies prediction uncertainty. In turn, it allows conversion of the prediction into a decision based on the strength of the evidence which is done using Bayes factors. Since the process of adding *in vitro* assay data to the BN ITS can be cumulative, it can also be used to guide and optimize testing strategies before testing is commenced.

Rationale underlying the construction of the defined approach

The BN-ITS DA uses the following data streams as input data:

-Bioavailability using physicochemical properties (logDpH=7, water solubility WspH=7, fraction ionised (calculated using LogP and LogDpH7), Protein Binding PB– ACDlabs)
-*In silico* metabolism, potential for oxidation, potency prediction (TIMES prediction)
-KE 1: Peptide reactivity [OECD 442 C: Direct peptide reactivity test (DPRA)]
-KE 2: Keratinocyte activation [OECD 442D: ARE-Nrf2 luciferase test method (KeratinoSens™)]
-KE 3: Dendritic cell activation [human cell-line activation test (h-CLAT)].

A Bayesian network is a probabilistic graphical model (a type of statistical model) that graphically represents a set of variables and their conditional dependencies (based on Jaworska et al. 2010, 2013). The structure of the BN ITS DA model was developed manually from mechanistic knowledge of the endpoint following the approach outlined in Lucas et al. (2004). The AOP structure (i.e.

sequence of events, MIE) as well as data related to KE 1, 2, and 3 are encoded in the BN ITS which allows chemical specific result interpretation in the biological context. The hypothesis generated by the BN ITS model can be explained based on known mechanisms.

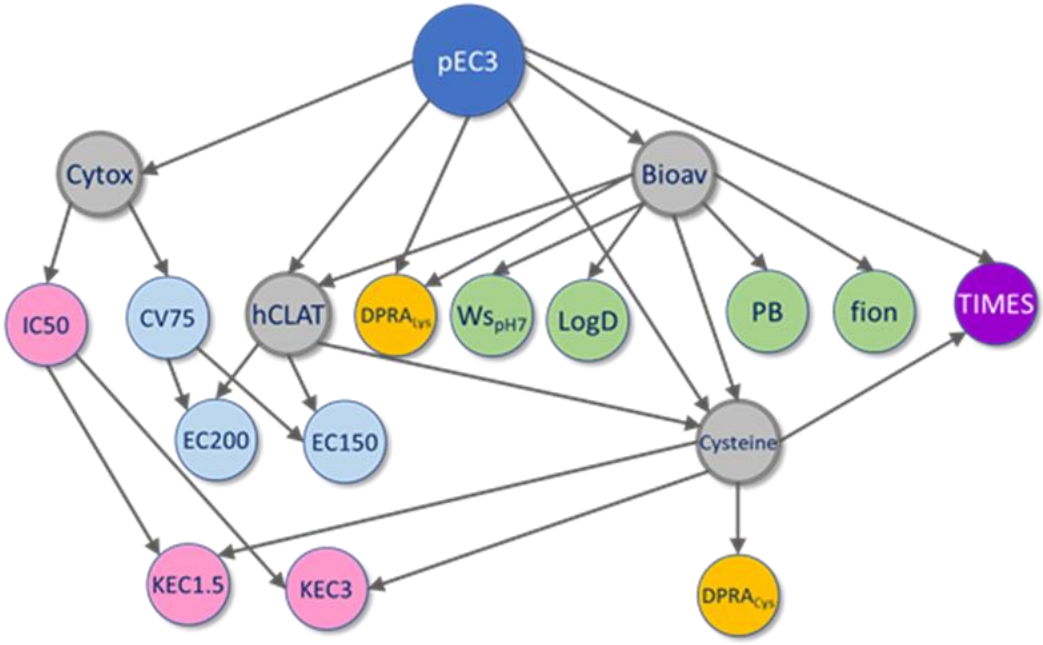
Both the construction method and the resulting structure (Figure ANNEX II.3) of the current BN ITS-4 (Kern et al. 2022 in preparation) are identical to the previous version BN ITS-3 (Jaworska et al. 2015, OECD , 2017). For the BN ITS the mechanistic scheme of the skin sensitisation induction process based on the AOP was translated into a Naïve Bayes network structure assuming that these events are independent. In the network the bioavailability latent node relates to stratum corneum penetration potential as well as free concentration *in vitro*. The Cys latent node and Lys nodes relate to KE 1, peptide binding, and 2, keratinocyte activation (for Cys only). The h-CLAT latent node relates to KE3, DC activation, and combines information from all h-CLAT readouts. Second, the tests used to observe the above process were mapped onto the initial network as manifest variables. There are tests that clearly measure different key events and there are also tests that measure the same KE or part of the process but in different ways.

There are two possible MIE: reaction with cysteine (Cys) and reaction with lysine (Lys), which are represented by two independent nodes. This allows identification of chemicals that act via both MIE as well as only through one MIE. The Cys latent variable represents the event of cysteine haptenation that can be observed via the DPRA-Cys measurement and/or the KeratinoSens™ assay. Reactivity towards cysteine is also measured indirectly in TIMES as electrophilicity molecular descriptors. Further, it has been postulated that the molecular basis of DC stimulation by electrophilic chemicals is a reflection of their ability to bind to sensor proteins (such as Keap1 or others). Therefore, it was even argued that DC-based assays might be a complicated measure of cysteine reactivity (Kimber et al. 2011). To reflect this, arcs connecting Cys latent with h-CLAT, as well as Cys latent and TIMES were introduced.

BN ITS also relates to bioavailability and cytotoxicity. To this end, we decided to generalise the bioavailability latent variable to consider skin penetration in the BN ITS framework structure. The bioavailability latent variable is constructed from the following physicochemical properties: water solubility at pH=7, distribution coefficient, log D at pH=7, fraction ionised at pH=7, and % plasma protein binding (PB). These variables are relevant determinants of skin penetration, cell membrane penetration, and free concentration. The bioavailability latent variable is connected by arcs to LLNA pEC3, Cys, Lys, and h-CLAT nodes.

To account for the presence of the danger signal in the network, we connect the cytotoxicity and pEC3 nodes. The cytotoxicity latent variable is constructed from cytotoxicity measured in the h-CLAT (CV75) and the KeratinoSens™ assay (IC50). The arcs connecting IC50 with the KeratinoSens™ data inputs KEC1.5, KEC3, as well as CV75 with h-CLAT data inputs EC150 and EC200, inform about cell viability in relation to the sensitisation-specific response.

Figure ANNEX II.3. Structure of the BN ITS.



The structure of the BN ITS model represents abstracted AOP and is developed based on mechanistic knowledge with the aim to follow sequence of the mechanistic events in the existing AOP.

Description of the individual information sources used

The respective input parameters are described in Table ANNEX II.3 below.

Table ANNEX II.3. Input parameters for the BN-ITS DA.

Input type	Endpoint	Unit
Bioavailability/ physicochemical properties (ACD lab)	1.Ws-Water solubility at pH='7' 2. Log D- Distribution coefficient at pH='7' 3. Plasma protein binding fraction 4. Fraction ionized	M/l [-] [-] [-]
TIMES-SS	1. Prediction of 3 classes (Non-sensitiser (1), weak (2) or moderate/strong (3) based on the most potent among parent and predicted metabolites.	Classes (NS, W, S)
KE 1: DPRACys, DPRALys	% cysteine- (Cys), and lysine- (Lys) peptide remaining in the DPRA	% remaining peptide
KE 2: KEC1.5, KEC3, IC50	1.5-fold (KEC1.5); 3-fold (KEC3) Induction of Nrf2-dependent luciferase activity in the KeratinoSens™ assay; 50% reduction in cell viability in the KeratinoSens™ assay	µM/l
KE 3: EC150, EC200, CV75	150% induction of the cell surface activation marker CD86 in the h-CLAT; 200% induction of the cell surface activation marker CD54 in the h-CLAT; 25% reduction in cell viability in the h-CLAT.	µM/l

Data interpretation procedure applied

The process of deriving a hazard or potency prediction for a new chemical consists of two steps: gathering evidence and developing a quantitative hypothesis:

1. Gathering evidence
   a) Calculation of physicochemical properties of chemicals.
   b) Prediction of sensitisation potency category using TIMES SS:
      i) Potency is based on the highest potency between the parent molecule and its predicted metabolites
      ii) Review potential of metabolic activation (pro-hapten) and auto-oxidation (pre-hapten) to facilitate interpretation of DPRA, KeratinoSens™ and h-CLAT assay results (not used as data input to the BN-ITS)
   c) Collection of data on KE1, 2, 3 and evaluation of the completeness (in particular for MIE if Lys depletion is missing).
   d) Assessment of applicability domains:
      i) TIMES-SS: predictions are used if parent is within total domain. If parent is out of total domain, metabolite predictions are only used in case of high transformation map reliability.
      ii) If the chemical is deemed a potential pre- or pro-hapten, then DPRA, KeratinoSens™, and h-CLAT data are examined with caution, against potential conflict with other data. A hypothesis without these data can be considered.
      iii) *in vitro* data are used as input if results are reliable within the limits/ applicability domains of the OECD TG restrictions.

2. Integration of all relevant evidence via BN ITS and prediction of the pEC3 probability distribution.
   a) Analysis of the cumulative evidence from all relevant input data: review probability distributions over 4 categories. (Highest probability most likely to determine potency category prediction).
   b) Conversion of probability distribution to Bayes factors (BF) for final interpretation and acceptance of prediction. Highest BF across the 4 groups defines the final potency category. Magnitude of BF determines confidence in prediction. A flat probability curve distribution might now allow derivation of one potency class (similar BF across some categories). In that case probabilities could be pooled for the weak/ moderate and strong categories and compared with the probability for the NS category to obtain a hazard result as sensitiser/ non sensitiser.

Bayes Factors:

The predicted probability distributions are converted to Bayes factors (BF) to: 1) remove prediction bias introduced by the training set, and to 2) express prediction uncertainty. This allows transparent and consistent criteria for accepting the prediction. Details of the BF conversion are given in Jaworska et al 2015. The conversion is done using the following formula:

$$B = \frac{P(H = x|e)/P(H = not\_x|e)}{P(H = x)/P(H = not\_x)} = \frac{posterior\ odds}{prior\ odds}$$

Where:
Prior distribution
P(H=x) - probability of a chemical to be in class x (x=NS, W, M, S) in the training set
P(H=not x) probability of a chemical to not to be in class x
Posterior distribution
P(H=x|e)- probability of a chemical to be in class x (x=NS, W, M, S) given the evidence provided to ITS
P(H=not x) probability of a chemical to not to be in class x given the evidence provided to BN ITS

Table ANNEX II.4. Interpretation of Bayes factors in terms of strength of evidence (Jeffreys, 1961).

Bayes Factor	Strength of evidence
<1	Negative (supports alternative)
1-3	Barely worth mentioning (weak)
3-10	Substantial
>30	Strong

Limitations in the application of the defined approach

BN ITS system requires biological (*in vitro*) data input of reliable consistent quality. The data need to come from within the applicability domains of the individual assays (DPRA, KeratinoSens™, h-CLAT) as described in the respective OECD TG. Technical limitations when conducting the assays need to be considered. TIMES predictions for chemicals outside applicability domain (as described above) should not be used as input or taken with caution. DA can be used for chemicals with defined structures, for which physicochemical properties can be calculated and *in silico* predictions (TIMES-SS) can be made. Derivation of physicochemical properties of e.g., charged molecules should be carefully reviewed. Physicochemical data should be generated using ACD lab software. Physicochemical data generated via other sources can be used, but the BN ITS prediction should be carefully evaluated for potential impact on the predicted potency classes.

Consideration of uncertainties associated with the application of the defined approach

DIP structure

A Bayesian network approach allows converting integrated predictions to transparent, consistent decisions. It has the following features: a) it is adaptive, as it can run with only partial evidence; it allows to add more evidence if needed to make a decision; b) quantifies uncertainty for individual prediction based on the evidence entered. As such it quantifies confidence in decisions; and allows them to be fit for purpose; c) it assesses consistency in evidence and identifies conflict between input data; d) guides potential additional testing by quantifying the additional test information value before testing is commenced.

Table ANNEX II.5. Uncertainty assumptions made for the BN-ITS DA.

Assumption	Direction & Magnitude
Bayesian network structure correctly represents the biological mechanism of the induction of skin sensitisation.	The structure of the DIP model represents abstracted AOP and is developed purely based on mechanistic knowledge with the aim to follow sequence of the mechanistic events in the existing AOP.
The dataset robustly characterizes parameters of the network, the conditional probability tables.	The cross-validation done shows a very stable network.
The physicochemical properties sufficiently characterize bioavailability *in vitro* and *in vivo*.	These parameters are key inputs to skin penetration model as well key parameters for *in vitro* kinetics.
Metabolic activation and autooxidation are sufficiently characterized by TIMES.	False positive will yield overestimation, false negative will yield underestimation.

The information sources used within the defined approach

In the DA, all *in chemico*/*in vitro* methods used have been shown to be reliable and relevant through multicentre studies evaluations. DPRA, KeratinoSens™, h-Clat assays have been regulatory accepted by OECD (TG 442C, 442D, 442E respectively).

The uncertainties for the defined approach that are related to the DIP information sources include the following:

- Inconsistent results in the source data for a given chemical would reduce the confidence in the hazard predictions.
- Results from TIMES for predicted auto-oxidation products or skin metabolites may rely on those that are not biologically important.
- Physicochemical properties calculated from non ACD lab would reduce confidence in predictions.

Benchmark data

The benchmark data used to develop the DA are based on data obtained from the murine LLNA. The variability of the reference *in vivo* data inevitably affects the accuracy of prediction. This variability originates from the intrinsic variability of the biological model and from the testing variability (between- and within-laboratory variability). Around those uncertainties, the defined approach was developed using a representative reference result for LLNA potency classification when multiple data were available.

Impact of uncertainty on the DIP's prediction

Deterministic models have very limited scope for correctly handling intrinsic data uncertainty while probabilistic models have a naturally built-in capability to handle it. The ITS prediction for a new chemical, being probabilistic, inherently includes assessment of uncertainty associated with this prediction. Further, conversion to Bayes factors allows for a consistent acceptance of uncertainty in predictions based on fit for purpose criteria. This uncertainty reflects the combined uncertainty associated with ITS structure and, in part, uncertainty due to the variability of input information sources as well as the target, i.e., LLNA pEC3.

Prediction for DEA

DEA was predicted as a non-sensitiser in the BN-ITS with a high probability (> 99%) and a high Bayes Factor (>30), which provides strong evidence for the prediction.

5: DIP for Skin Allergy Risk Assessment (SARA)

Summary

The SARA model utilises a Bayesian statistical approach to infer a human-relevant metric of sensitiser potency and a measure of consumer risk for any given consumer exposure to a chemical of interest (Reynolds, 2022). It can utilise any combination of data from (historical) HRIPT (Politano and Api 2008), (historical) LLNA (OECD, 2010), DPRA, KeratinoSens™, h-CLAT or U-SENS™ to derive sensitiser potency, which is expressed as the ED01 (the dose in µg/cm2 which is predicted to sensitise

1% of a HRIPT population), with explicit quantification of the uncertainty in the prediction. In addition to potency assessment the SARA model also provides the probability of whether a given exposure is low risk (SARA risk metric). The margin of exposure (MoE) is calculated for the given exposure within the SARA model, by dividing the ED01 by the consumer exposure (in µg/cm2). This MoE is then regressed against the MoE derived for benchmark consumer exposures. The benchmark exposures are historical/current exposures to consumer products which been designated as high or low risk for induction of skin sensitisation based upon the publicly available clinical/patch test data.

Rationale underlying the construction of the defined approach

The SARA model is a DA which uses Bayesian statistics to infer a human-relevant metric of sensitiser potency and allows explicit quantification of the uncertainty in potency estimates (Reynolds et al. 2019). The available inputs into the model were any combination of HRIPT (Politano and Api, 2008), LLNA (OECD, 2010), DPRA (OECD, 2021a), KeratinoSens™ (OECD, 2018), h-CLAT or U-SENS™ (OECD, 2018b) data. Briefly, features of this model include:

- a dataset of 30 chemicals
- sensitiser potency metric defined as the maximum HRIPT dose at which there is no chance of inducing sensitisation
- modelling assumptions pertaining to:
    - population variability of thresholds for sensitisation
    - sampling variability in the HRIPT
    - relationships between the potency metric and data generated in the LLNA and *in vitro* assays for skin sensitisation
    - explicit modelling of variability of LLNA data, but not *in vitro* data.

Since publication, the model has undergone substantial revision. The updated SARA model not only provides a human-relevant metric of sensitiser potency but also provides a probability that a consumer exposure scenario can be classified as low risk, hereon termed the SARA risk metric (Reynolds and Gilmour et al. 2022). Briefly, major updates include:

- Redefining the metric of sensitiser potency from a HRIPT dose with a 0% chance of sensitisation, to a dose with a 1% chance of sensitisation (termed the ED_{01} with units µg/cm^2). The latter metric can be inferred more reliably from the data.
- Expansion of the dataset underpinning the model, from 30 chemicals to 81 chemicals. The dataset is now restricted to chemicals which are currently, or have historically been, used in consumer (household or personal care) goods.
- Expansion of the model to explicitly account for variability in the DPRA, KeratinoSens™, h-CLAT and U-Sens™. This change was necessary because, alongside the revision of the model, the *in vitro* component of the database underpinning the model was expanded to include multiple *in vitro* study results for many chemicals in the database. Variability in the historical in vivo data was a feature of the original published model.
- Expansion of the model to consider correlations between residuals within the regression components of the model. Two tests for skin sensitisation potential or potency may give a misleading result for the same reasons, for example, lack of metabolism for activation of a pre-hapten. This refinement of the model protects against overconfident estimates of potency by allowing for tests to be correlated in their predictive error.

Expansion of the model to incorporate benchmark exposure information. This component of the model is used to calculate a risk metric for an exposure of interest.

The SARA DA draws upon available information spanning all KE (i.e. KE1: DPRA; KE2: KeratinoSens™; KE3: h-CLAT; U-Sens™; KE4: historical (LLNA) and the adverse outcome (AO) (i.e. historical HRIPT and clinical benchmarks) to infer the dose with a 1% chance of sensitisation (termed the ED01 with units µg cm^2). The underlying rationale and assumption in the proposed DA is that, given the coverage of the AOP KE and the well-characterised assays, these data sources contain mechanistically-relevant information for predicting human skin sensitisation potency.

Description of the individual information sources used

Table ANNEX II.6. Input parameters for the SARA model.

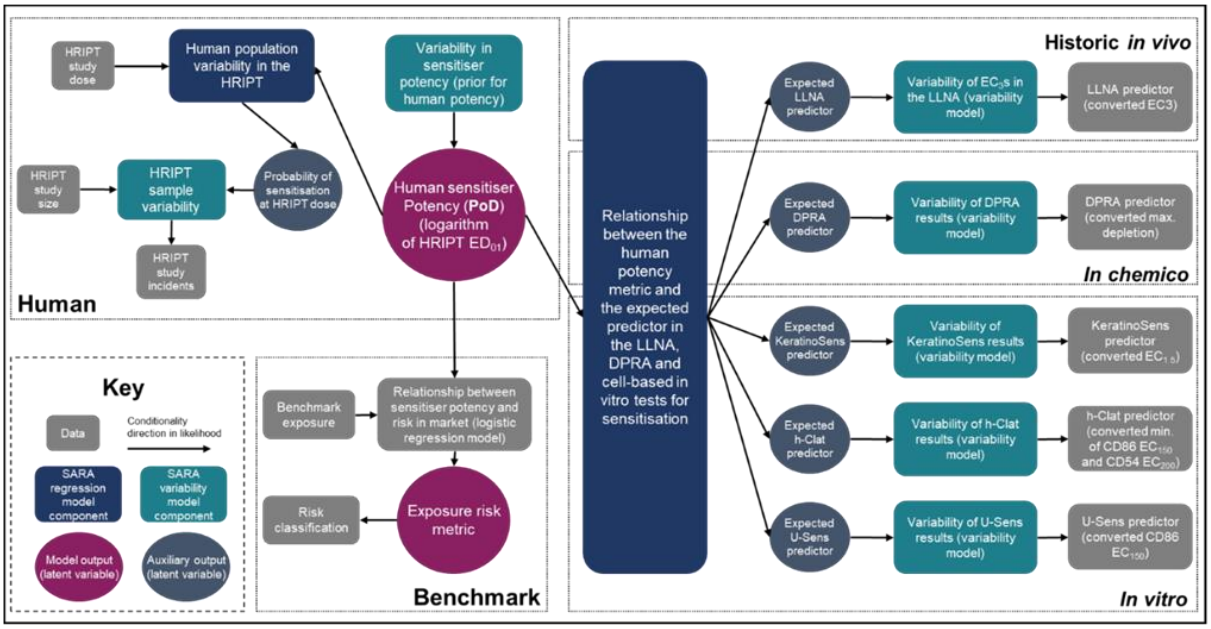
Method	Input
(Historic) Human Repeat Insult Patch Test (HRIPT)	Only studies in which cohort size, number sensitised and applied dose are reported are admissible
(Historic) Local Lymph Node Assay (OECD TG 429)	EC3 values obtained from individual studies are admissible as evidence. Representative or averaged values are not admissible
DPRA (OECD TG 442c)	Maximum % depletion with either cysteine and/or lysine
KeratinoSens™ (OECD TG 442D)	EC1.5 value (M)
h-CLAT (OECD TG 442E)	CD54 EC200, CD86 EC150
U-Sens™ (OECD TG 442E)	Reported CD86 EC150 (g ml-1)
Exposure	Dermal dose applied (µg/cm^2)
Clinical benchmark	High / low risk

Data interpretation procedure applied

The SARA DA utilises a probabilistic (technically a Bayesian multilevel (i.e., hierarchical) model to characterise the relationships between data from sources identified above in Table ANNEX II.6

The approach makes a quantitative prediction of the dose with a 1% chance of sensitisation in the HRIPT (termed the ED_{01} with units µg/cm^2). The Bayesian model allows to explicitly, and quantitatively, describe the uncertainty in potency estimates, conditional on the model and available data. The ED_{01} (µg cm^2), is inferred as a probability distribution. The variance of the distribution reflects the precision in the estimate, conditional on the chemical-specific data available. The ED_{01} is the measure of hazard potency for a chemical and may be used to derive a point-of-departure for risk assessment. A margin of exposure (MOE), (i.e. the measure of how far away the consumer exposure is from the PoD) is calculated and by comparison of this to the MOE for the clinical benchmarks, which are classified as either high or low risk based upon clinical experience a probability that an exposure is low risk is calculated (SARA risk metric). The SARA model structure is shown in Figure ANNEX II.4.

Figure ANNEX II.4. Schematic of the relationships between variables in the SARA DA (see supplementary material for full details of the mathematical model of (Reynolds and Gilmour et al. 2022)).



Limitations in the application of the defined approach

The various assays have technical applicability domains that limits the number of chemicals which can be tested in them. These same limits clearly apply to the DA as well. Underlying the approach is the assumption that for a chemical of interest, the relationship between the ED_{01} and the various assays is represented by the relationships learnt from the wider dataset. The approach is data driven and therefore estimates of the ED_{01} will be biased if the output of an individual assay is itself biased, i.e., a DPRA assay with 0% depletion because a sensitising chemical requires metabolic activation will bias towards underestimating potency. It should be noted however that when data from several assays are used, the influence of a single unrepresentative assay result will be mitigated provided the other assay results are representative. Furthermore, the reliability of each assay is learnt from the dataset if there are many chemicals whose assay outcome is unrepresentative of human potency, the influence of that assay will be automatically decreased in the prediction model.

Consideration of uncertainties associated with the application of the defined approach

DIP structure

The SARA DA utilises a Bayesian multilevel modelling approach. The fundamental assumption behind this modelling approach is that there is a quantifiable relationship between the individual data sources. The rationale for this being the case is underpinned by the fact that each of these data sources corresponds to key events of the AOP or to the adverse outcome itself. However, the strength of the relationship and how informative data from one data source (e.g., *in vitro* data sources) is for predicting another (e.g., HRIPT) is determined by updating the model parameters using the underlying dataset and then assessing model fit.

The information sources within the defined approach

The DA utilises a Bayesian multilevel modelling approach that attempts to account for a) the variability in underlying observed data and b) the uncertainty in the strength of relationship that exists between the data sources. Specifically, variability of *in vitro* and historical *in vivo* data is explicitly accounted for in the model.

Benchmark data

The present dataset is limited to 81 chemicals, restricted to chemicals which are currently, or have historically been, used in consumer (household or personal care) goods. There are ongoing efforts to collate additional data. A fundamental uncertainty associated with this model (and other approaches seeking to learn from past data) is whether the data under consideration is representative of future data. For this approach, two key questions are a) is the distribution of sensitiser potency across the set of chemicals historically tested in the HRIPT representative of the distribution of sensitiser potency of future chemicals and b) will the strength of the association between sensitiser potency and the outcome of the various *in vitro* tests be conserved for future compounds. Confidence in the predictions of the SARA DA is conditional on having a reasonable level of belief that these assumptions hold. However, this is no different to any other scientific approach where the knowledge is built around a limited dataset and then extrapolation made to new scenarios.

The SARA risk metric is established based upon a set of 83 clinical benchmark exposures. The benchmark exposures have been selected based upon common established allergens which have been reviewed extensively in the literature, where it is possible to draw some conclusion about whether an exposure is high or low risk based upon epidemiological information and the profile of allergic reactions observed. The current small number of benchmarks is a limitation and increasing the number of materials within the SARA model with both high and low risk exposures will improve confidence in the reliability of the risk metric identification of additional risk benchmarks is ongoing.

Impact of uncertainty on the DIP's prediction

The modelling approach is designed to account for uncertainties in the underlying data and the relationships between the data sources. Thus, the effect of this uncertainty is directly incorporated into the final prediction of the human sensitiser potency (ED01) which is expressed as a probability distribution and SARA risk metric (probability that a given exposure is low risk) The remaining, unquantified uncertainties, relate to those discussed under benchmark data and to whether the dataset used to build the model is representative in general. This would have to be considered on a case-by-case basis and would likely take into account the representativeness of the underlying dataset with respect to the chemistry of the chemical for which a prediction is required.

Prediction for DEA

The SARA model predicted an ED_{01} of 13000 µg/cm^2 for DEA (95th % confidence interval 530 – 370000 µg/cm^2). This outcome is consistent with a prediction of a weak/moderate skin sensitiser potency.