REPORT FOR THE INTERNATIONAL COOPERATION ON COSMETICS REGULATION

ALTERNATIVE SAFETY ASSESSMENT TOOLS FOR IDENTIFYING POTENTIAL DERMAL ALLERGENS

Authors:

Julcemara Gresselle De Oliveira (1), Ana Carolina Perdigao Faleiros (1); Silvia Casati (2); Guru Saravanabhavan (3); Hajime Kojima (4), Reiko Teshima (4); Stanislav Vukmanovic# (5); Momeena Omarjee (6); Pedro Amores Da Silva- (7), Ariadne Morais (7); Beta Montemayor (8); Petra Kern (9), Florian Schellauf (9); Masato Hatao (10), Tetsuya Kambe (10) Yutaka Kasai (10); Jay Ansell# (11); Dershana Valla (12).

1. Brazilian Health Regulatory Agency (ANVISA), Brazil
2. European Commission, Joint Research Centre (JRC), Europe
3. Health Canada (HC), Canada
4. National Institute of Health Sciences, Japan
5. US Food and Drug Administration (US FDA), USA
6. South African Department of Health, South Africa
7. Brazilian Association of the Cosmetic, Toiletry and Fragrance Industry (ABIPHEC), Brazil
8. Cosmetics Alliance Canada (CA Canada), Canada
9. Cosmetics Europe, Europe
10. Japan Cosmetic Industry Association (JCIA), Japan
11. US Personal Care Products Council (PCPC), USA
12. South Africa CTFA, South Africa

Co-chairs
- Former Joint Working Group member

TABLE OF CONTENTS

1. SELECTED ABREVIATIONS AND DEFINITIONS 3

2. BACKGROUND ..... Error! Bookmark not defined.

3. PURPOSE.....4

4. INTRODUCTION 5

5. METHODS.....8

5.1 OECD TG 442C - *In chemico skin sensitization: Direct Peptide Reactivity Assay (DPRA)* ……..…9

5.2 OECD TG 442D- *In vitro skin sensitisation: ARE-Nrf2 Luciferase*……*9*

5.3* OECD TG 442E - *In vitro* skin sensitization: *In vitro* skin sensitization assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for skin sensitization *Test Method*……*10

6. DISCUSSION.....11

6.1 Limitations……11

6.2 Use within an Integrated Approaches to Testing and Assessment (IATA) approach……..12

6.3 Last Note……13

7. CONCLUSION.....14

8. REFERENCES 15

1. SELECTED ABREVIATIONS AND DEFINITIONS

ADRA	Amino Acid Derivative Reactivity Assay
AOP	Adverse Outcome Pathway
ARE	Antioxidant Response Element
CONCEA	National Animal Experimentation Control Board
DA	Defined Approaches
DC	Dendritic Cells
DPRA	Direct Peptide Reactivity Assay
DMSO	Dimethyl sulfoxide
h-CLAT	Human Cell Line Activation Test
HRIPT	Human Repeated Insult Patch Test
HPLC	High Performance Liquid Chromatography
IATA	Integrated Approach to Testing and Assessment
ICCR	International Cooperation on Cosmetics Regulation
IL8-Luc	Interleukin-8 Reporter Gene Assay
JWG	Joint Working Group
LLNA	Local Lymph Node Assay
MIE	Molecular Initiating Event
NAM	Non-Animal Methods
OECD	Organization for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive Oxygen Species
WoE	Weight of Evidence

2. BACKGROUND

The International Cooperation on Cosmetics Regulation (ICCR) held its sixth annual meeting (ICCR-6) July 2012. At this meeting, it was agreed that a Joint Working Group (Allergens) would be established and tasked with developing a white paper assessing the current relevant regulatory policies with regard to cosmetic allergenicity in effect in the ICCR member jurisdictions. This white paper was presented at the 2014 ICCR-8 meeting and accepted for posting to the ICCR web site[1].

It was also agreed that additional work on this topic was warranted. Accordingly, a new JWG (Allergens II) was established and tasked with developing a survey of approaches to identify relevant authoritative lists that ICCR jurisdictions may use to monitor risks posed by potential allergens in cosmetics. This report was presented at the 2016 annual meeting (ICCR-10) and accepted for posting to the ICCR web site[2].

Again, both regulators and industry agreed that the topic of allergens in the context of cosmetics remains a topic of high importance and yet another new JWG (Allergens III) was formed to identify emerging testing and risk assessment methodologies that may be used in evaluating and identifying potential skin sensitization risks.

3. PURPOSE

Examine how the combination of non-animal methods below, recently adopted by OECD, may be used within Integrated Approaches to Testing and Assessment (IATA) to adequately substitute for animal tests in the evaluation of skin sensitization potential:

- OECD TG 442C- *In chemico* skin sensitisation: Direct Peptide Reactivity Assay (DPRA);
- OECD TG 442D- *In vitro skin* sensitisation: ARE-Nrf2 Luciferase Test Method;
- OECD TG 442E- *In vitro* skin sensitization: *In vitro* skin sensitization assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitization.

It is recognized that there are a number of governmental and non-governmental organizations actively working in this area. This work includes both the development of new alternative methods as well as guidance for the use of these methods in a defined approach (DA) within the context of an IATA to support regulatory decision-making. For example OECD's working party on Chemicals, Pesticides and Biotechnology published its 2016 guidance document on defined approaches and individual information sources to be used within an IATA for skin sensitization[3]; and the Japanese Ministry of Health, Labor and Welfare issued the guidance on use of combination of *in vitro* skin sensitization assays in the safety assessment of cosmetics and quasi-drugs[4].

In Brazil, the National Animal Experimentation Control Board (CONCEA), established by the Law Nº 11.794,Oct 8, 2008[5], published the Normative Resolution Nº 18, Sep 24, 2014[6] and Normative Resolution Nº 31, Aug 18, 2016[7] recognizing 24 validated alternative methods that replace, refine or reduce tests with live animals[5], including OECD TG 429, OECD TG 442A, OECD TG 442B, OECD TG 442C and OECD TG 442D methods. The Resolution Anvisa RDC nº 35, Aug 7, 2015[8] provides for the acceptance of alternative methods recognized by the CONCEA.

The Ordinance 491, Jul 03, 2012, of Ministry of Science, Technology, Innovation and Communications, established the National Network of Alternative Methods – RENAMA, that promotes the development, validation and certification of new alternative methods to the use of animals in Brazil[9].

Furthermore, a recent review of international regulatory requirements for skin sensitization testing illustrates opportunities for the use of non-animal alternative methods across various regions and chemical sectors[10].

The authors note that this report is not intended to be a comprehensive overview of the entire field of activity but is rather focused on the use of the three specified OECD validated methods for evaluation of sensitization potential.

4. INTRODUCTION

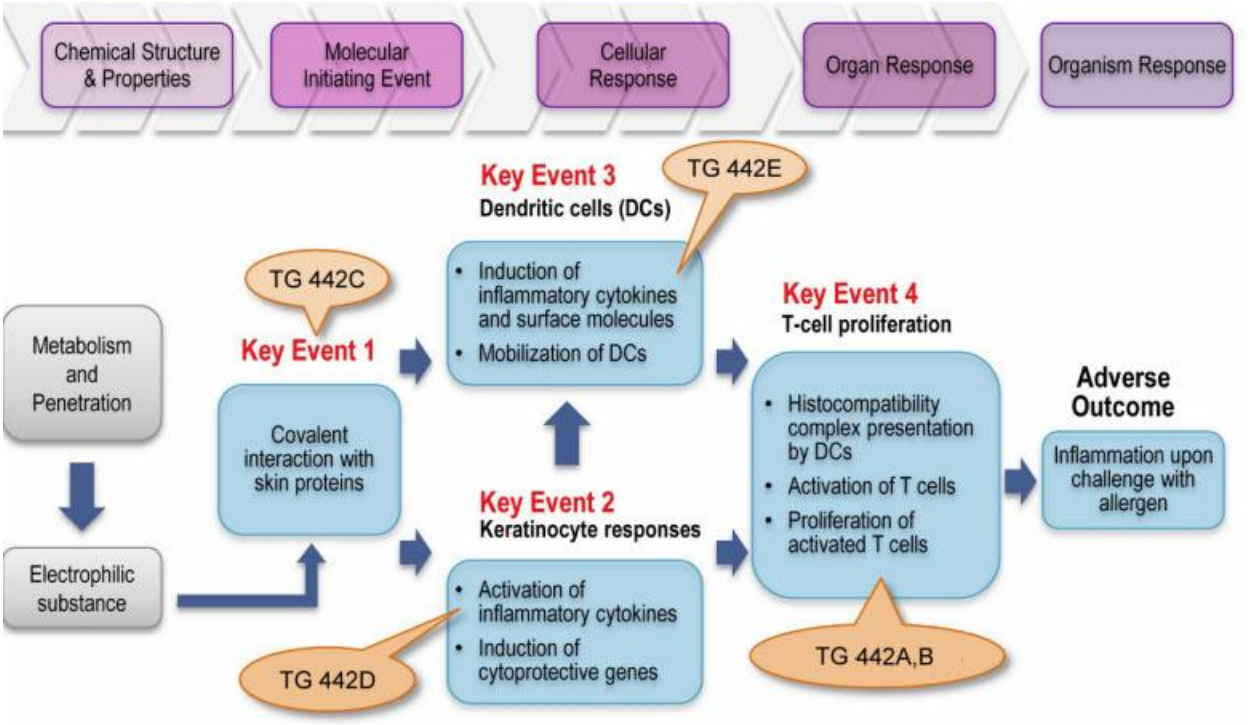
The evaluation of sensitization potential of cosmetic ingredients is integral to the safety assessments of cosmetic products. In the past, data for evaluating sensitization has been obtained using experimental animals and human subjects. The use of human subjects for the identification of a sensitization risk is shadowed by scientific and ethical considerations; hence the use of human subjects in the repeated insult patch test (HRIPT) is subject to increasing criticism[11]. Also, the use of animals for ingredient testing is under significant scrutiny[12, 13], leading to strong commitments by both industry and regulators to the principles for refining, reducing and replacing animal testing, where appropriate (3Rs). Examples of these commitments may be found in the ban on animal testing for cosmetics in the European Union[14], the interagency Memorandum of Understanding between the US National Toxicology Program, the U.S. Environmental Protection Agency and NIH Chemical Genomics Center to develop test methods for toxicity testing that are more scientifically and economically efficient, reducing or replacing animals[15] , the amended Japanese Act on Welfare and Management of Animals requiring alternative methods than the use of animals to reduce the number of animals provided for such use as much as possible[16] and the publication of the federal law n.º 11.794, 2008, that regulates the scientific use of animals in Brazil[5].

Thus, there is a need for alternative (*in vitro*, *in chemico* and *in silico*) assays for the assessment of sensitization potential that would greatly reduce the time and cost of required ingredient testing in addition to the benefit of animal welfare.

Reliable *in vitro* skin sensitization tests have been developed over the past decade based on mechanism(s) of sensitization induction. The Adverse Outcome Pathway (AOP) of skin sensitization[17] describes the following four key events that serve as focus points for the development of alternative tests (as illustrated in Figure 1):

1. covalent modification of epidermal proteins;
2. keratinocyte signaling and activation;
3. dendritic cell activation, migration and antigen processing, and
4. antigen recognition and ensuing T-cell response.

FIGURE 1: THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITIZATION INITIATED BY COVALENT BINDING TO PROTEINS [21]



This mechanistic understanding of the AOP has enabled development of alternative assays that test the impact of chemical compounds on defined key mechanisms of the AOP and at least serve as first-tier assays designed to significantly reduce the skin sensitization testing performed in animals. Table 1 lists 16 assays analyzed during the first phase of the Cosmetics Europe method evaluation program [18], as well as 21 additional assays discovered through literature survey. Five of these assays (DPRA, KeratinoSens™, LuSens, h-CLAT and U-SENS™) have been validated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and/or peer reviewed by the EURL ECVAM Scientific

Advisory Committee (ESAC)[19]. The IL-8 Luc assay has also been validated and reviewed by the Japanese Center for Alternatives to Test Methods [20].

TABLE 1: LIST OF AVAILABLE IN VITRO ASSAYS AIMED AT TESTING DISTINCT ASPECTS OF THE AOP

(OECD validated assays are bolded for easier identification)

Groups of assays	Principle	Key Event	List of tests
In *chemico* assays for haptenation	Depletion of reactant peptide containing cysteine or lysine in the presence of test chemical; fluorescence-based quantification of test chemical-bound reactant peptide	1	**DPRA** [35, 36], PPRA [37], HTKP [38], kinetics assay [39], SenCeeTox [40] (Part 1), APIA [41], ADRA & ADRA-DM [42, 43], adduct detection assays [44, 45, 46, 47, 48], fluorescence based electrophile detection [49]
ARE (Antioxidant Response Element)-based assays	Activation of the Keap1/Nrf2/ARE pathway. Covalent binding to Keap 1 cysteine allows nuclear translocation of Nrf2 and subsequent binding to ARE enhancers, inducing expression of ARE-sensitive genes	1 and 2	AREc32 [50], **KeratinoSens™** [51, 52], **Lu-Sens** [28], HaCaSens [53], Sens-IS [54]
Keratinocyte activation assays	Induction of proinflammatory (cytokines, chemokines costimulatory and other) molecules	2	SenCeeTox [40] (Part 2), NCTC2544 [55, 56], Sens-IS [54], EE Potency [57, 58], EpiSensA [59]
Dendritic cell activation assays	Induction of costimulatory or other activation markers; cytokine production; induction of ROS; chemokine induced migration patterns	3	GARD [60], **h-CLAT** [61], mMUSST [28], , **U-SENS** [62], MUTZ-3 [63], PBMDC [64], SensiDerm™ [18], VITO-SENS [65], ROS assay [66], K-DC coculture assay [67], TLR synergy [68], Low density cDNA array [69], MUTZ-LC migration assay [70], **IL-8 Luc** assay [71, 72]
T cell activation assays	Proliferation (detected by tritiated thymidine incorporation or CFSE dilution); cytokine production; T-cell mediated damage to co-cultured skin explant	4	Proliferation with or without pre-expansion or regulatory cell removal [73, 74], hTCPA [75], LCSA-ly [76], co-culture of T cells and skin explants [77]

It should be stressed that the individual methods adopted so far by OECD should not be used in isolation either for excluding the skin sensitization potential or for potency predictions. For these purposes, they should be used in the context of Defined Approaches as potential elements within IATA.

5. METHODS

The AOP of skin sensitization describes that covalent binding of sensitizers to nucleophilic centers in skin proteins is the molecular initiating event (MIE) of the sensitization process. Good correlation between protein reactivity and sensitization potential has been observed for a wide range of sensitizers. In the Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C) the protein reactivity of test chemicals is evaluated by determining their reactivity towards synthetic model peptides containing either lysine or cysteine using a fixed ratio between chemical and peptide and fixed reaction time.

Inflammatory responses and gene expressions associated with specific signaling pathways in keratinocytes are regarded as the second key event in the AOP of sensitization. Current mechanistic evidences suggest that skin sensitizers could alter sensory protein Keap1 (Kelch-like ECH-associated protein 1) by binding to its highly reactive cysteine residues leading to the dissociation of Keap1 from the transcriptional regulator Nrf2. Accumulation of Nrf2 in the nucleus results in the upregulation of antioxidant/electrophile response element (ARE)-dependent genes which codes for phase II detoxifying enzymes. The ARE-Nrf2 Luciferase Test methods (KeratinoSens™ and LuSens; both described in OECD TG 442D), assess the ARE-dependent gene expressions in immortalized adherent cell lines derived from - human keratinocytes stably transfected with selectable plasmids. The cell lines contain the luciferase gene under the transcriptional control of ARE element. Thus, activation of Nrf2 dependent genes by sensitizers induces the luciferase gene which can be quantitatively measured using luminescence techniques after reaction with the luciferase substrate.

Activation of dendritic cells (DC) is regarded as the third key event in the sensitization process. Activation of DC induces the expression of cell membrane markers such as CD40, CD54, CD80, CD83 and CD86, and the induction of proinflammatory cytokines such as IL-1β, TNF-α, and chemokines such as IL-8 and CCL3. There are three validated assays described in OECD TG 442E, namely the human Cell Line Activation Test (h-CLAT) assay (measures CD86 and CD54 markers in the human monocytic leukemia cell line THP-1), U-SENS™ assay (measures CD86 in human histiocytic lymphoma cell line U937) and IL8-Luc assay (measures IL-8 expression in the THP-1-derived IL-8 reporter cell line), to quantify changes in the expression of these markers in human cell lines following exposure to sensitizers.

A brief description of these *in vitro* assays is presented below. Additionally, each of the methods have been characterized to the extent possible for their applicability domain as currently identified in the OECD guidelines. For more information please see the respective OECD guidance cited.

5.1 OECD TG 442C - *In chemico skin sensitization: Direct Peptide Reactivity Assay (DPRA)* [22]

This Test Guideline is under revision to include the Amino Acid Derivative Reactivity Assay (ADRA) test method, as well as the Direct Peptide Reactivity Assay (DPRA). Only the latter is currently described. The DPRA involves incubation of fixed concentrations of test chemicals and synthetic model peptides (containing cysteine and lysine residues) solutions. After incubation, the solution is analyzed using High Performance Liquid Chromatography (HPLC) and UV detector to quantify the depletions of both peptides containing intact cysteine or lysine residues. One or more positive controls (e.g. cinnamic aldehyde), reference controls (peptides alone), and co-elution controls (test chemical alone) are included in each HPLC run sequence. Linear calibration curves developed using varying concentrations of lysine or cysteine peptides are used to measure the concentration of intact lysine or cysteine peptides in the test samples. Based on these data, the percent cysteine and lysine depletion are estimated. Acceptance criteria were developed based on the linearity of the standards calibration curve, the mean percent peptide depletion value and the standard deviation for reference controls.

Predictive models based on a reference dataset of chemicals with known reactivity properties, which are correlated with *in vivo* testing data from Local Lymph Node Assay (LLNA), have been developed to support the discrimination between sensitizing and non-sensitizing chemicals. In addition, the calculated lysine and/or cysteine depletion values of the measured unreacted peptides, in association with other information, can be used to inform potency predictions. Based on this predictive model, test chemicals with mean peptide depletion values of 0 to ≤ 6.38%, 6.38 to ≤ 22.62%, 22.62 to ≤ 42.47%, and 42.47 to ≤ 100% are classified as minimal, low, moderate and high reactivity respectively. DPRA was able to discriminate sensitizers from non-sensitizers with an accuracy of 80% (sensitivity: 80%; specificity: 77%) when compared to LLNA data during validation and inter-laboratory comparison exercises.

5.2 OECD TG 442D- *In vitro skin sensitisation: ARE-Nrf2 Luciferase Test Method* [23]

The KeratinoSens™ method involves incubating HaCaT human keratinocytes cells (80-90% confluent) with varying concentrations of test chemicals dissolved in dimethyl sulfoxide (DMSO), water or culture medium. Positive control (cinnamic aldehyde) and negative control (DMSO) are tested simultaneously with the test chemicals in each experiment. After incubation, the luciferase substrate is added to the cell lysates and luminescence is measured using a luminometer. Test chemicals are considered positive if they

induce a statistically significant induction of luciferase activity (> 1.5-fold or 50% increase above the solvent control) below 1000 µM and at a concentration at which the cellular viability is above 70%. In addition, an apparent dose-response relationship should be observed for all chemicals considered positive.

KeratinoSens™ assay was able to discriminate sensitizers from non-sensitizers with an accuracy of 77% (sensitivity: 78%; specificity: 76%) when compared to LLNA data during validation and inter-laboratory comparison exercises. Each of the methods has been characterized to the extent possible for their applicability domain as current identified in the OECD guidelines.

The LuSens is a similar method to the KeratinoSens™ that has recently been added to OECD TG 442D and can be used interchangeably with the KeratinoSens™ in the context of defined approaches or IATA.

5.3 OECD TG 442E - *In vitro* skin sensitization: *In vitro* skin sensitization assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for skin sensitization [24]

OECD TG 442E covers assays addressing activation of dendritic cells: the human Cell Line Activation Test (h-CLAT); Interleukin-8 Reporter Gene Assay (IL8-Luc); and Myeloid U937 Skin Sensitization Test (U-SENS™). In **h-CLAT assay**, varying concentrations of test chemicals in DMSO or culture medium are added to human monocytic leukemia cell line, THP-1. After exposure, the cells are isolated by centrifugation and treated with a blocking solution. Then the cells are stained with FITC-labelled anti-CD86, anti-CD54 or mouse IgG1 (isotype) antibodies. After washing with staining buffer, the cells are treated with propidium iodide staining solution. The expression levels of CD86 and CD54, and cell viability are analyzed using flow cytometry. The test chemical is considered positive when one or both of the following acceptance criteria is fulfilled: (a) The relative fluorescence intensity (RFI) of CD86 is equal to or greater than 150% at any tested concentration with cell viability ≥ 50%; (b) The RFI of CD84 is equal to or greater than 200% at any tested concentration with cell viability ≥ 50%. At least two independent runs are carried out consisting of test samples, positive (2, 4-dinitrochlorobenzene) and negative (solvent) controls. Results from the validation studies indicate that the accuracy of this assay for distinguishing sensitizers from non-sensitizer is 85% with a sensitivity of 93% and specificity of 66%.

The **U-SENS™ assay** utilizes human histiocytic lymphoma cell (U937 cells) line and measures specific cell surface marker CD86. In this test method, the U937 cell suspension is mixed with test chemicals dissolved in culture medium or DMSO. After incubation, the cells are isolated by centrifugation, washed with staining buffer and mixed with FITC-labelled anti-CD86 or mouse IgG1 (isotype) antibodies at 4°C. After washing with the staining buffer, the cells are treated with propidium iodide solution (for cytotoxicity

assessment). The expressions of CD86 and cell viability are measured using flow cytometry. A stimulation index is estimated based on the percentage of FL-1 positive cells in the treated and control samples in the population of viable cells. The test chemical is declared positive when the stimulation index is equal to or greater than 150% at all non-cytotoxic concentrations. At least two independent runs are carried out each consisting of test samples in triplicate, positive (picrylsulfonic acid) and negative (solvent) controls. Results from the validation studies indicate that the accuracy this assay for distinguishing sensitizers from non-sensitizer is 86% with a sensitivity of 91% and specificity of 65%.

Unlike h-CLAT and U-SENS assays, **IL8-Luc** assay quantifies changes in the expression of IL-8 cytokines associated with the activation of dendritic cells using the THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocytic leukemia cell line (THP-1) that harbors the Stable Luciferase Orange (SLO) and Stable Luciferase Red (SLR) luciferase genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters respectively. In this method, the test chemicals are dissolved in X-VIVO™ 15, a commercially available serum-free medium with Triplu c® Luciferase assay reagent added to the cell suspension and the plate placed in the luminometer and bioluminescence is measured to quantify the luciferase activity. The measured values are used to calculate the normalized IL8LA (SLO luciferase activity reflecting IL-8 promoter activity). Induction of IL8LA (Ind-IL8LA) is calculated as the ratio of the normalized IL8LA measurements of THP-G8 cells treated with chemicals to the normalized IL8LA measurements of untreated cells. The test chemical is considered positive when the Ind-IL8LA is equal to or greater than 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA is ≥ 1.0. Data generated during validation studies suggest that the IL-8 Luc assay has an accuracy of 86% with a sensitivity of 96% and specificity of 41% when compared to LLNA. Negative results should be interpreted with caution as several test chemicals such as anhydrides do not dissolve in X-VIVO™. Moreover, results from previous studies indicate that surfactants (e.g. cationic, anionic or non-ionic) may produce false positive results in this assay.

6. DISCUSSION

6.1 Limitations

Overall, all the *in vitro* test methods discussed above show relatively good inter- and intra-laboratory reproducibility and accuracy in discriminating sensitizers from non-sensitizers when compared to LLNA data. Although these assays are very good at identifying strong sensitizers, sensitizers with moderate and low potencies may not be detected. Moreover, since each of these test methods focuses on a single key event of the sensitization process, none of them should be considered as stand-alone alternatives to skin

sensitization potential. The predictive models, as is the case in all tests based on animal data, need to be carefully evaluated for relevance to humans.

In addition, recent analyses have shown that the adopted methods are able to correctly identify the majority of pro-haptens (chemicals that require enzymatic bioactivation to become sensitizers) and pre-haptens (chemicals that require abiotic activation to become sensitizers) [25] [26] tested so far. In certain cases, e.g. with slow oxidizing agents and chemicals requiring enzymatic activation for example via P450 enzymes, detection of the sensitizing potential may prove challenging as these assays have limited metabolic capacity.

Although simple mixtures with known chemical compositions could be tested, these *in vitro* methods are not always suitable for testing either complex mixtures or unknown or variable composition, complex reaction products or biological materials. Thus, negative results from these assays should be corroborated with complimentary information on test chemicals from other testing or non-testing methods. False positive predictions are also possible – for example, in DPRA chemicals that alter proteins via oxidation and dimerization mechanisms show higher percent protein depletion leading to assignments in higher reactivity class. Moreover, chemicals such as phytoestrogens could potentially inhibit or enhance luciferase activity in luciferase-based reporter gene assays thereby complicating interpretation of the results.

6.2 Use within an Integrated Approaches to Testing and Assessment (IATA) approach [27]

To assess the predictive value of these assays, the *in vitro* data has been compared to the existing *in vivo* data to determine the degree of their association [26, 28, 29]. The overall consensus based on the results of *in vitro* tests performed so far is that no single assay is sufficient to characterize the sensitization potential of any chemical and that the use of an IATA approach is required [30, 31, 32].

One proposed approach using only data from these three assays is the weight of evidence (WoE) approach, where the results of two concordant out of three performed tests are considered [28, 29, 33]. As summarized in Table 2, depending on the study (list of chemicals tested) and the *in vivo* data used, the overall concordance is reported in the range from 74 to 92%. The rate of concordance is slightly better if HRIPT data are used instead of the LLNA, but for many chemicals this may not be possible due to lack of data on humans. A number of other integrated approaches have been proposed with various success rates. Due to the number of these approaches, rather than discussing it here, the reader is referred to a recent review article devoted solely to this topic[34].

TABLE 2: SUMMARY OF CONCORDANCE OF WOE WITH LLNA OR HRIPT DATA

Study	Study´s Methods	Sensitizers based on LLNA and/or HRIPT data	WoE scores for chemicals with available LLNA data		WoE scores for chemicals with available HRIPT data	
			Tested positive / expected positive#	Tested negative/ expected negative[&]	Tested positive/ expected positive#	Tested negative/ expected negative[&]
Bauch et al. [28]	DPRA, KeratinoSens™, Lu-Sens, hCLAT, mMUSST.	Yes	29/35	6/0	25/27	2/0
		No	2/0	17/19	2/0	21/23
		Overall concordance[@]	46/54 (85%)		46/50 (92%)	
Natsch et al. [29]	Lu-Sens, DPRA , KeratinoSens™.	Yes	95/103	18/0		
		Negative	10/0	33/43		
		Overall concordance[@]	128/146(88%)			
Urbisch et al. [26]	DPRA, KeratinoSens™, Lu-Sens, h-CLAT, (m)MUSST	Yes	116/143	37/0	65/71	6/0
		No	14/0	43/57	3/0	5/8
		Overall concordance[@]	159/200 (79%)		70/79 (89%)	
Asturiol et al. [78]	DPRA, KeratinoSens™ h-CLAT)	Yes	94/113	19/0	50/66	16/0
		No	11/0	34/45	1/0	11/12
		Overall concordance[@]	128/158 (81%)		61/78 (78%)	
Patlewicz et al. [79]	DPRA, KeratinoSens™ h-CLAT.	Yes	75/90	15/0	43/49	6/0
		No	10/0	27/37	5/0	17/22
		Overall concordance[@]	102/127 (80%)		60/71 (84%)	

#- All sensitizers (extreme, strong, moderate, and weak) are expected to test positive in WoE setting.
[&]- All chemicals classified as having no sensitizing potency are expected to score negative in WoE setting.
[@]- Overall concordance is summation of tested/expected positive for sensitizers and tested/expected negative for non-sensitizers.

6.3 Last Note

Development and validation of new alternative methods and corresponding integrated approaches is a rapidly evolving field. While this is true for many scientific fields, this is especially the case here with new assays continuously being developed and proposed for validation and subsequent inclusion in various integrated strategies. Consequently, the list of assays presented in Table 1 should not be taken as a final list of available alternative methods. Further, validated *in vitro* assays other than those presented in this paper are likely to become available in the near future. Among these, the SENS-IS and the Genomic Allergen Rapid Detection (GARD) assay, both based on gene expression analyses, are being considered by the OECD and included in its work program for the development of the respective test guidelines.

7. CONCLUSION

1. Allergens remain a topic of great interest to both regulators and industry.

2. Both the use of human subjects and animal models to assess the allergenic potential of ingredients are under scientific and ethical scrutiny driving the development of new alternative strategies.

3. Modern methods of risk assessment argue that reliable alternative tests should be based on mechanism(s) of sensitization induction.

4. This report focuses on three OECD validated methods:

- OECD TG 442C- *In chemico* skin sensitization: Direct Peptide Reactivity Assay (DPRA)
- OECD TG 442D- *In vitro* skin sensitization assays: ARE-Nrf2 Luciferase Test Method
- OECD TG 442E-*In vitro* skin sensitization assays: *In vitro* skin sensitization assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitization

5. While these assays are very good at identifying strong sensitizers, sensitizers with moderate and low potencies may not be detected.

6. Since each of these test methods focuses on a single key event of the sensitization process, they should not be used as a stand-alone alternative to identifying potential sensitizers.

7. The Scientific community will continue to investigate how best to structure a testing framework utilizing the three studies to increase the predictive power.

8. Overall these models should provide a valuable tool in an Integrated Approaches to Testing and Assessment (IATA).

8. REFERENCES

1. Milstein, S.R. *et al.* International Cooperation on Cosmetics Regulation: "Allergens in Cosmetics and Personal Care Products: Comparison of Jurisdictional Regulatory Approaches". 2014 [cited]Available from: http://www.iccr-cosmetics.org/files/8414/1407/7467/2014-07_Allergens_-_Comparison_of_Regulatory_Approaches.pdf

2. Ansell, J. *et al.* International Cooperation on Cosmetics Regulation: "Survey of Approaches Undertaken to Develop Authoritative Lists of Potential Allergens in Cosmetics and Personal Care Products – Allergens II: Part 1". 2017 [cited]Available from: http://www.iccr-cosmetics.org/files/5214/8717/1045/Allergens_II__Part_1_Final_Jan_2017.pdf

3. OECD. Working Party On Chemicals, Pesticides And Biotechnology, "Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation", Series on Testing & Assessment, No. 256, ENV/JM/MONO(2016)29. 2016 [cited]Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)29&doclanguage=en

4. Pharmaceuticals and Medical Devices Agency. [cited]Available from: http://www.pmda.go.jp/files/000222425.pdf

5. Law Nº 11.794, Oct 08, 2008.2008 [cited]Available from: http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm

6. Ministry of Science, Technology, Innovation and Communications.. Normative Resolution Nº 18, Sep 24, 2014. 2014 [cited]Available from: http://www.agrarias.ufpr.br/portal/wp-content/uploads/2015/02/RN18-2014-Reconhece-m%C3%A9todos-alternativos-para-uso-de-animais-em-pesquisa.pdf

7. Ministry of Science, Technology, Innovation and Communications. Normative Resolution Nº 31, Aug 18, 2016. 2016 [cited]Available from: http://www.lex.com.br/legis_27179133_RESOLUCAO_NORMATIVA_N_31_DE_18_DE_AGOSTO_DE_2016.aspx

8. Brazilian Health Regulatory Agency (ANVISA). Resolution Nº 35, Aug 7, 2015. 2015 [cited]Available from: https://ww2.icb.usp.br/icb/wp-content/uploads/bioterio_etica/Resolucao_RDC_35.pdf

9. Ministry of Science, Technology, Innovation and Communications.Ordinance 491, Jul 03, 2012. 2012[cited]Available from: https://www.mctic.gov.br/mctic/opencms/legislacao/portarias/migracao/Portaria_MCTI_n_491_de_03072012Portaria_MCTI_n_491_de_03072012.html

10. Daniel, A.B. *et al.* International regulatory requirements for skin sensitization testing. *Regul Toxicol Pharmacol* **95**, 52-65 (2018).

11. Basketter, D.A. The human repeated insult patch test in the 21st century: a commentary. *Cutan Ocul Toxicol* **28**, 49-53 (2009).

12. Hoffmann, S. LLNA variability: An essential ingredient for a comprehensive assessment of non-animal skin sensitization test methods and strategies. *ALTEX* **32**, 379-383 (2015).

13. Dumont, C., Barroso, J., Matys, I., Worth, A. & Casati, S. Analysis of the Local Lymph Node Assay (LLNA) variability for assessing the prediction of skin sensitisation potential and potency of chemicals with non-animal approaches. *Toxicol In Vitro* **34**, 220-228 (2016).

14. European Commission. Ban on Animal Testing. 2013 [cited 2015 1 December]Available from: http://ec.europa.eu/growth/sectors/cosmetics/animal-testing/index_en.htm

15. National Institute of Environmental Health Sciences. Memorandum of Understanding on High Throughput Screening, Toxicity Pathway Profiling, and Biological Interpretation of Findings. 2008 [cited 2015 1 December]Available from: http://www.niehs.nih.gov/about/highlights/assets/docs/memorandum_of_understanding_508.pdf

16. Ministry of the Environement. Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973) Last revision: Act No. 46 of May 30, 2014:. 2014 [cited]Available from: https://www.env.go.jp/nature/dobutsu/aigo/1_law/files/aigo_kanri_1973_105_en.pdf

17. Organisation for Economic Co-operation and Development. The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. 2012 [cited 2015 1 December]Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)10/part1&doclanguage=en

18. Reisinger, K. *et al.* Systematic evaluation of non-animal test methods for skin sensitisation safety assessment. *Toxicol In Vitro* **29**, 259-270 (2015).

19. European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). Recommendations. Skin Sensitization. 2015 [cited]Available from: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

20. JaCVAM. The Japanese Center for Validation of Alternative Methods. 2017 [cited 2018]Available from: http://www.jacvam.jp/en/index.html

21. EPA's Office of Chemical Safety and Pollution Prevention: Office of Pesticide Programs Office of Pollution Prevention and Toxics. Interim Science Policy: Use of Alternative Approaches for Skin Sensitization as a Replacement for Laboratory Animal Testing. 2018 [cited 2018]Available from: https://www.regulations.gov/document?D=EPA-HQ-OPP-2016-0093-0090

22. OECD. Test No. 442C: In chemico Skin Sensitisation. 2015 [cited]Available from: /content/book/9789264229709-en http://dx.doi.org/10.1787/9789264229709-en

23. OECD. Test No. 442D: *In vitro* Skin Sensitisation. 2018 [cited]Available from: /content/book/9789264229822-en: http://dx.doi.org/10.1787/9789264229822-en

24. OECD. Test No. 442E: *In vitro* skin sensitization assays: *In Vitro* Skin Sensitisation. 2018 [cited]Available from: /content/book/9789264264359-en; http://dx.doi.org/10.1787/9789264264359-en

25. Casati, S. *et al.* Ability of non-animal methods for skin sensitisation to detect pre- and pro-haptens: Report and recommendations of an EURL ECVAM expert meeting; EUR 27752 EN. 2016 [cited 2018]Available from: https://publications.europa.eu/en/publication-detail/-/publication/c849106d-deb0-11e5-8fea-01aa75ed71a1/language-en

26. Urbisch, D. *et al.* Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul Toxicol Pharmacol* **71**, 337-351 (2015).

27. OECD. Guidance Document on the Reporting of Defined Approaches and Individual Information Sources to be Used within Integrated Approaches to Testing and Assessment (IATA) for Skin Sensitisation. 2017 [cited]Available from: /content/book/9789264279285-en

http://dx.doi.org/10.1787/9789264279285-en

28. Bauch, C. *et al.* Putting the parts together: combining *in vitro* methods to test for skin sensitizing potentials. *Regul Toxicol Pharmacol* **63**, 489-504 (2012).

29. Natsch, A. *et al.* A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *J Appl Toxicol* **33**, 1337-1352 (2013).

30. Basketter, D. *et al.* Skin sensitisation--moving forward with non-animal testing strategies for regulatory purposes in the EU. *Regul Toxicol Pharmacol* **67**, 531-535 (2013).

31. Mehling, A. *et al.* Non-animal test methods for predicting skin sensitization potentials. *Arch Toxicol* **86**, 1273-1295 (2012).

32. Rovida, C. *et al.* Integrated Testing Strategies (ITS) for safety assessment. *ALTEX* **32**, 25-40 (2015).

33. van der Veen, J.W. *et al.* Evaluating the performance of integrated approaches for hazard identification of skin sensitizing chemicals. *Regul Toxicol Pharmacol* **69**, 371-379 (2014).

34. Kleinstreuer, N.C. *et al.* Non-animal methods to predict skin sensitization (II): an assessment of defined approaches (*). *Crit Rev Toxicol* **48**, 359-374 (2018).

35. Gerberick, G.F. *et al.* Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci* **81**, 332-343 (2004).

36. Gerberick, G.F. *et al.* Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. *Toxicol Sci* **97**, 417-427 (2007).

37. Gerberick, G.F. *et al.* Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. *Toxicol Sci* **112**, 164-174 (2009).

38. Roberts, D.W. & Natsch, A. High throughput kinetic profiling approach for covalent binding to peptides: application to skin sensitization potency of Michael acceptor electrophiles. *Chem Res Toxicol* **22**, 592-603 (2009).

39. Chipinda, I. *et al.* Rapid and simple kinetics screening assay for electrophilic dermal sensitizers using nitrobenzenethiol. *Chem Res Toxicol* **23**, 918-925 (2010).

40. McKim, J.M., Jr., Keller, D.J., 3rd & Gorski, J.R. An *in vitro* method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. *Cutan Ocul Toxicol* **31**, 292-305 (2012).

41. Dietz, L. *et al.* Proteomic allergen-peptide/protein interaction assay for the identification of human skin sensitizers. *Toxicol In vitro* **27**, 1157-1162 (2013).

42. Fujita, M. *et al.* Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives. *J Pharmacol Toxicol Methods* **70**, 94-105 (2014).

43. Yamamoto, Y. *et al.* A novel in chemico method to detect skin sensitizers in highly diluted reaction conditions. *J Appl Toxicol* **35**, 1348-1360 (2015).

44. Ahlfors, S.R., Sterner, O. & Hansson, C. Reactivity of contact allergenic haptens to amino acid residues in a model carrier peptide, and characterization of formed peptide-hapten adducts. *Skin Pharmacol Appl Skin Physiol* **16**, 59-68 (2003).

45. Alvarez-Sanchez, R. *et al.* Effect of glutathione on the covalent binding of the 13C-labeled skin sensitizer 5-chloro-2-methylisothiazol-3-one to human serum albumin: identification of adducts by nuclear magnetic resonance, matrix-assisted laser desorption/ionization mass spectrometry, and nanoelectrospray tandem mass spectrometry. *Chem Res Toxicol* **17**, 1280-1288 (2004).

46. Chittiboyina, A.G., Avonto, C., Rua, D. & Khan, I.A. Alternative testing methods for skin sensitization: NMR spectroscopy for probing the reactivity and classification of potential skin sensitizers. *Chem Res Toxicol* **28**, 1704-1714 (2015).

47. Nilsson, A.M., Bergstrom, M.A., Luthman, K., Nilsson, J.L. & Karlberg, A.T. A conjugated diene identified as a prohapten: contact allergenic activity and chemical reactivity of proposed epoxide metabolites. *Chem Res Toxicol* **18**, 308-316 (2005).

48. Natsch, A. & Gfeller, H. LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicol Sci* **106**, 464-478 (2008).

49. Avonto, C., Chittiboyina, A.G., Rua, D. & Khan, I.A. A fluorescence high throughput screening method for the detection of reactive electrophiles as potential skin sensitizers. *Toxicol Appl Pharmacol* **289**, 177-184 (2015).

50. Wang, X.J., Hayes, J.D. & Wolf, C.R. Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of nrf2 by cancer chemotherapeutic agents. *Cancer Res* **66**, 10983-10994 (2006).

51. Emter, R., Ellis, G. & Natsch, A. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. *Toxicol Appl Pharmacol* **245**, 281-290 (2010).

52. Natsch, A. *et al.* The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers i*n vitro*: Results of a ring-study in five laboratories. *Toxicology in Vitro* **25**, 733-744 (2011).

53. Chung, H. *et al.* Intra- and inter-laboratory reproducibility and predictivity of the HaCaSens assay: A skin sensitization test using human keratinocytes, HaCaT. *Toxicol In Vitro* **46**, 304-312 (2018).

54. Cottrez, F., Boitel, E., Auriault, C., Aeby, P. & Groux, H. Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay. *Toxicol In Vitro* **29**, 787-802 (2015).

55. Corsini, E. *et al.* Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol In Vitro* **23**, 789-796 (2009).

56. Galbiati, V. *et al.* Further development of the NCTC 2544 IL-18 assay to identify in vitro contact allergens. *Toxicol In Vitro* **25**, 724-732 (2011).

57. dos Santos, G.G. *et al.* A potential in vitro epidermal equivalent assay to determine sensitizer potency. *Toxicol In Vitro* **25**, 347-357 (2011).

58. Gibbs, S. *et al.* An epidermal equivalent assay for identification and ranking potency of contact sensitizers. *Toxicol Appl Pharmacol* **272**, 529-541 (2013).

59. Saito, K. *et al.* Development of a new in vitro skin sensitization assay (Epidermal Sensitization Assay; EpiSensA) using reconstructed human epidermis. *Toxicol In Vitro* **27**, 2213-2224 (2013).

60. Johansson, H., Albrekt, A.S., Borrebaeck, C.A. & Lindstedt, M. The GARD assay for assessment of chemical skin sensitizers. *Toxicol In Vitro* **27**, 1163-1169 (2013).

61. Ashikaga, T. *et al.* A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern Lab Anim* **38**, 275-284 (2010).

62. Piroird, C. *et al.* The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. *Toxicol In Vitro* **29**, 901-916 (2015).

63. Python, F., Goebel, C. & Aeby, P. Comparative DNA microarray analysis of human monocyte derived dendritic cells and MUTZ-3 cells exposed to the moderate skin sensitizer cinnamaldehyde. *Toxicol Appl Pharmacol* **239**, 273-283 (2009).

64. Reuter, H. *et al.* In vitro detection of contact allergens: development of an optimized protocol using human peripheral blood monocyte-derived dendritic cells. *Toxicol In Vitro* **25**, 315-323 (2011).

65. Hooyberghs, J. *et al.* A cell-based in vitro alternative to identify skin sensitizers by gene expression. *Toxicol Appl Pharmacol* **231**, 103-111 (2008).

66. Saito, K., Miyazawa, M., Nukada, Y., Sakaguchi, H. & Nishiyama, N. Development of an in vitro skin sensitization test based on ROS production in THP-1 cells. *Toxicol In Vitro* **27**, 857-863 (2013).

67. Hennen, J. *et al.* Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization. *Toxicol Sci* **123**, 501-510 (2011).

68. Dearman, R.J. *et al.* Synergistic effects of chemical insult and toll-like receptor ligands on dendritic cell activation. *Toxicol In Vitro* **22**, 1927-1934 (2008).

69. Cluzel-Tailhardat, M. *et al.* Chemicals with weak skin sensitizing properties can be identified using low-density microarrays on immature dendritic cells. *Toxicol Lett* **174**, 98-109 (2007).

70. Gibbs, S., Spiekstra, S., Corsini, E., McLeod, J. & Reinders, J. Dendritic cell migration assay: a potential prediction model for identification of contact allergens. *Toxicol In Vitro* **27**, 1170-1179 (2013).

71. Takahashi, T. *et al.* An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol Sci* **124**, 359-369 (2011).

72. Kimura, Y. *et al.* Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. *Toxicol In Vitro* **29**, 1816-1830 (2015).

73. Dietz, L. *et al.* Tracking human contact allergens: from mass spectrometric identification of peptide-bound reactive small chemicals to chemical-specific naive human T-cell priming. *Toxicol Sci* **117**, 336-347 (2010).

74. Vocanson, M. *et al.* Human T cell priming assay: depletion of peripheral blood lymphocytes in CD25(+) cells improves the in vitro detection of weak allergen-specific T cells. *EXS* **104**, 89-100 (2014).

75. Richter, A. *et al.* Human T cell priming assay (hTCPA) for the identification of contact allergens based on naive T cells and DC--IFN-gamma and TNF-alpha readout. *Toxicol In Vitro* **27**, 1180-1185 (2013).

76. Frombach, J. *et al.* Lymphocyte surface markers and cytokines are suitable for detection and potency assessment of skin-sensitizing chemicals in an in vitro model of allergic contact dermatitis: the LCSA-ly. *Arch Toxicol* **92**, 1495-1505 (2018).

77. Ahmed, S.S. *et al.* An in vitro human skin test for assessing sensitization potential. *J Appl Toxicol* **36**, 669-684 (2016).

78. Asturiol, D., Casati, S. & Worth, A. Consensus of classification trees for skin sensitisation hazard prediction. *Toxicol In Vitro* **36**, 197-209 (2016).

79. Patlewicz, G. *et al.* Can currently available non-animal methods detect pre and pro-haptens relevant for skin sensitization? *Regul Toxicol Pharmacol* **82**, 147-155 (2016).