



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Regulatory need for non-animal approaches for skin sensitisation hazard assessment

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Skin sensitization

- A skin sensitizer can cause an allergic response following skin contact
 - allergic contact dermatitis (ACD)
 - Type IV delayed type immune response
- Most common form of immunotoxicity
 - prevalence 19% in EU
- Important consumer and occupational health problem
- Low-molecular weight chemicals and some metals
 - nickel, fragrances and preservatives most common causes





Legislation

- Information on skin sensitisation hazard needed for
 - *UN Globally Harmonised System for Classification, labelling and packaging (GHS/CLP)*
 - *REACH*
 - *Cosmetics Directive*
- Info traditionally obtained with animal tests
 - Guinea pig tests, Local Lymph Node Assay (LLNA)
- Information on potency if a substance is a skin sensitiser often required
 - Intrinsic strength to induce sensitisation
 - Potency subcategorisation, e.g. strong/moderate/weak; cat1A vs 1B
 - LLNA provides potency information



Regulatory need for non-animal methods

- Cosmetics Directive – ban on animal testing
- Cosmetics are a common cause of contact allergy



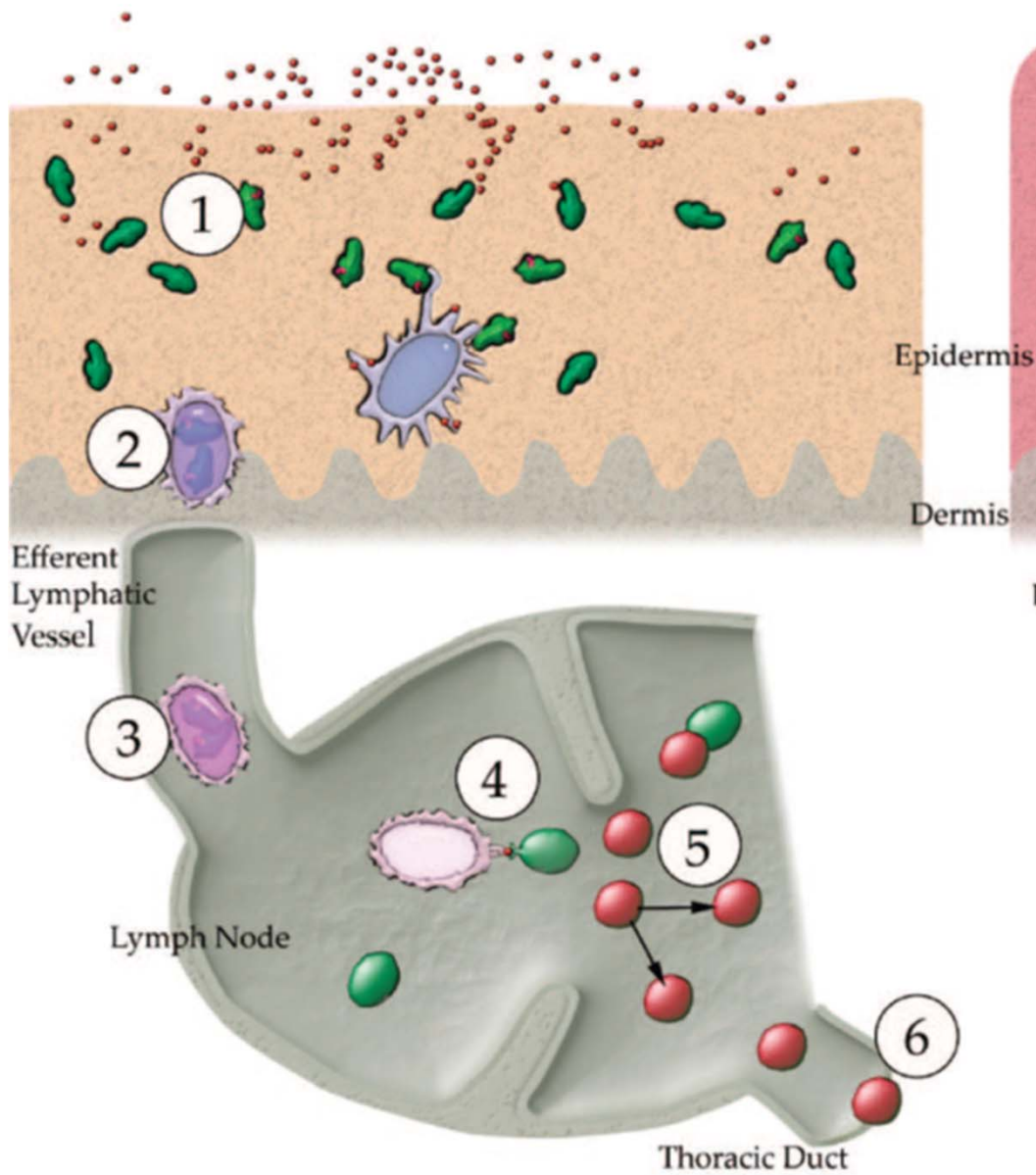
- Recent change REACH Information Requirements
 - Potency must be assessed in case substance is a skin sensitizer
 - Generation new data: start with *in vitro*/*in chemico* methods, unless
 - *in vitro*/*in chemico* test methods are not applicable for the substance (test method specific limitations)
 - *in vitro*/*in chemico* methods are not adequate for classification and risk assessment, including potency assessment (Cat 1A vs 1B)



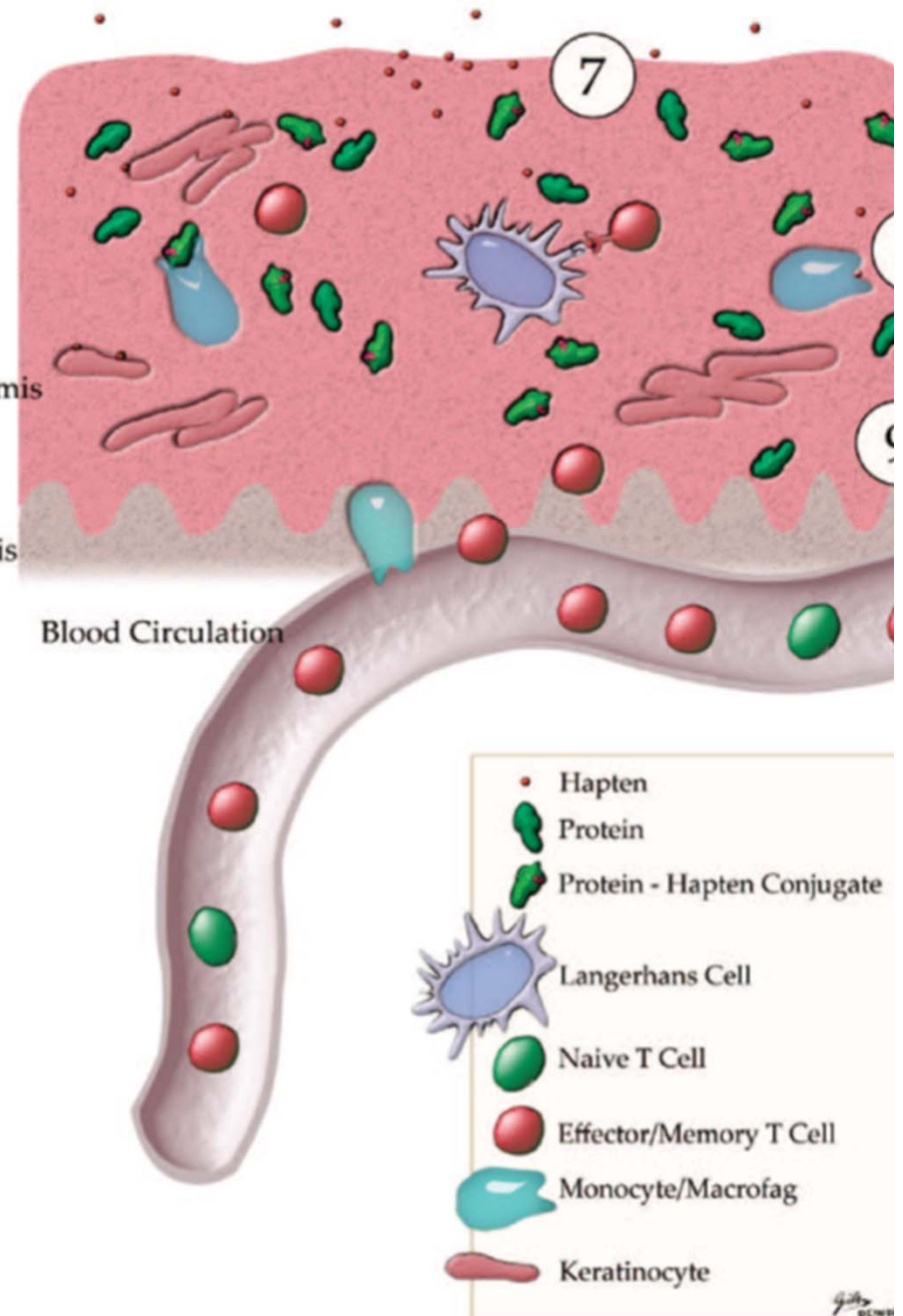
Scientific rationale to move away from animals

- Animal tests have advantages and limitations
 - LLNA has an accuracy of $\sim 80\%$ for hazard identification
 - Relative low specificity/high number of false-positives
 - Potency estimation is less accurate and prone to variability
- In toxicology shift towards mechanism-based human relevant non-animal toxicity methods
- Immunological mechanism of skin sensitisation relatively well known
- Replacement of LLNA:
 - combination of non-animal methods that cover biology needed
 - information for hazard identification and potency estimation

Sensitization



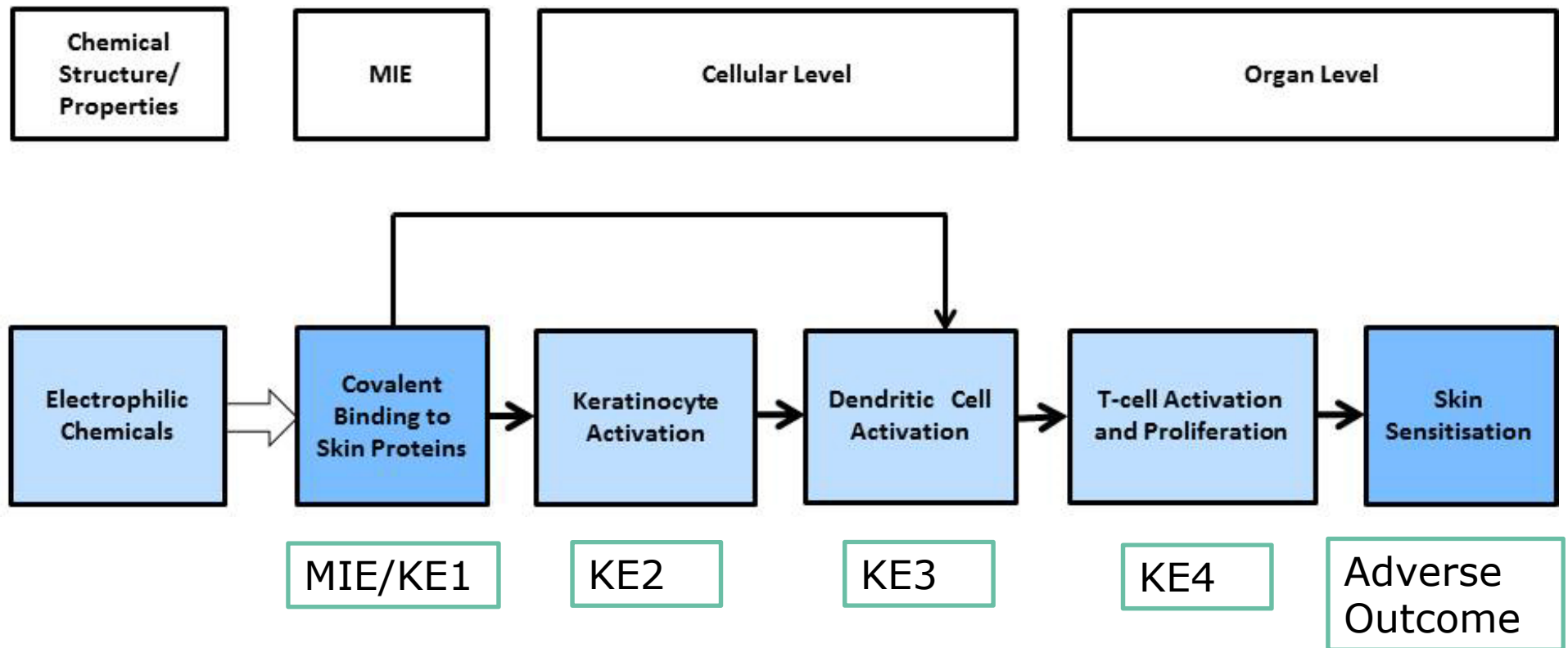
Elicitation



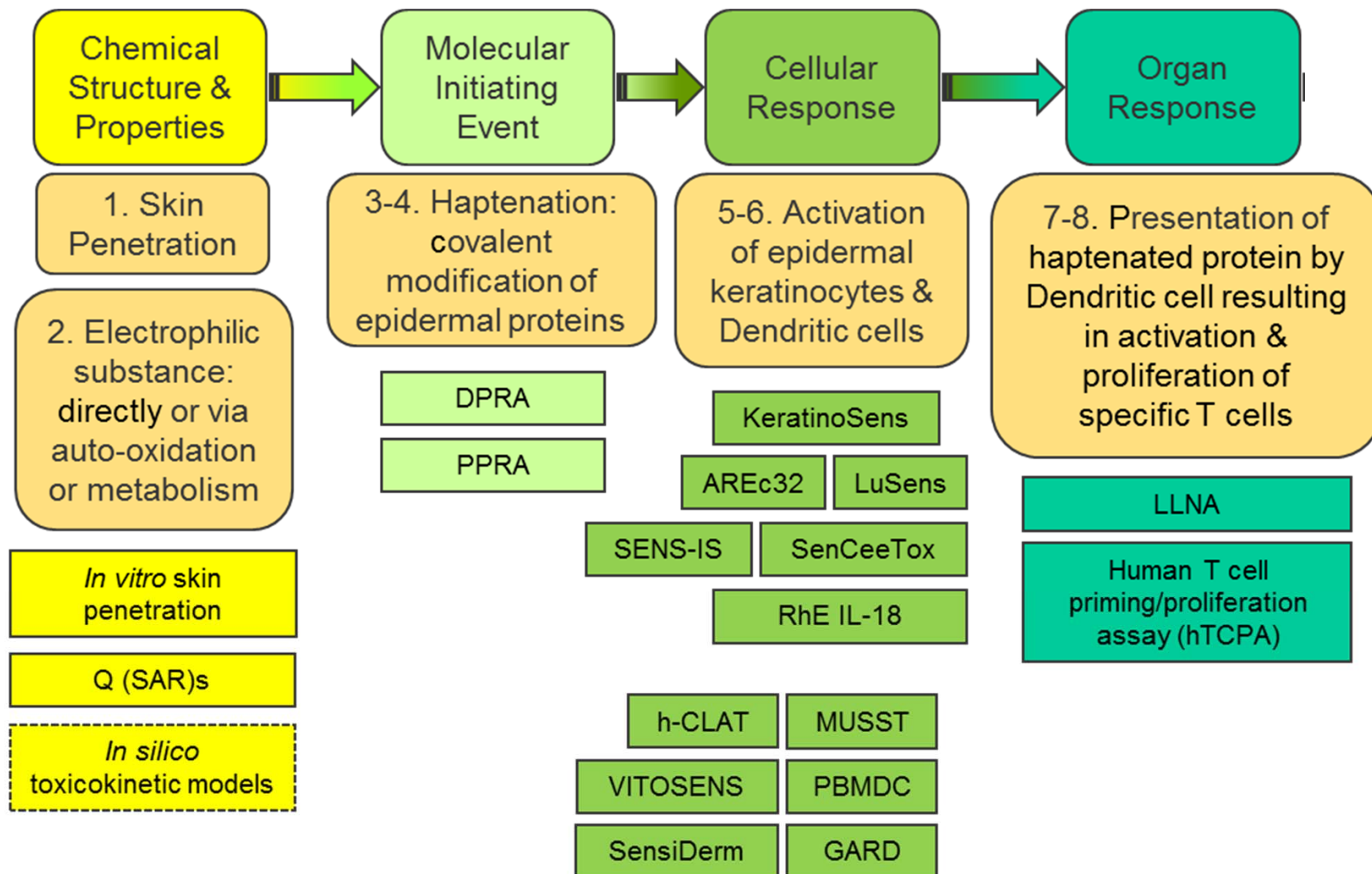
- Hapten
- Protein
- Protein - Hapten Conjugate
- Langerhans Cell
- Naive T Cell
- Effector/Memory T Cell
- Monocyte/Macrophag
- Keratinocyte



Skin sensitisation AOP (OECD, 2012)



AOP (OECD)



From Casati EURL-ECVAM

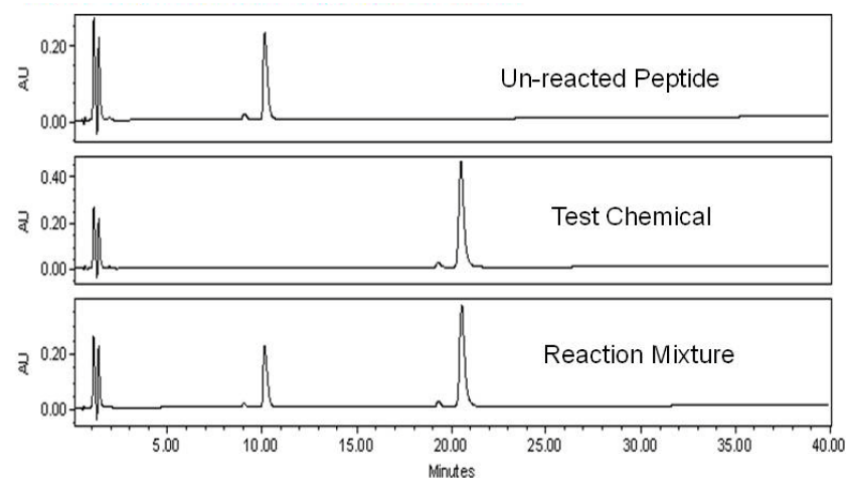
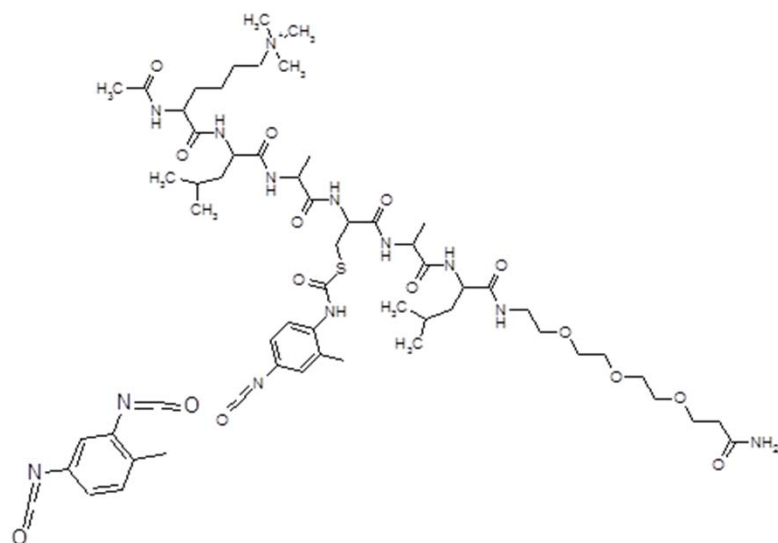
OECD test guidelines + work programme

AOP KE	OECD TG	Test methods
MIE Peptide binding	442C	Direct peptide reactivity assay (DPRA)
KE2 Keratinocyte activation	442D	ARE-Nrf2 Luciferase Test methods (Keratinosens, LuSens) <i>SENS-IS (validated, under peer review)</i>
KE3 Dendritic cell activation	442E	Human Cell Line Activation Test (h-CLAT) <i>Myeloid U937 Skin Sensitization Test (U-SENS)</i> <i>IL-8 Luc assay</i>
KE4 T cell activation and proliferation	429	Local Lymph Node Assay (LLNA)
AO Skin sensitisation	406	Guinea Pig test methods (GPMT, Buehler test)



MIE - DPRA

- *In chemico* method to assess protein reactivity of a test substance with synthetic peptides containing either lysine or cysteine
- % peptide depletion is measured with a HPLC
- No metabolic capacity





KE2 – keratinocyte-based assays

❖ Measurement of molecular pathways regulated by skin sensitisers

- **Keratinosens and LuSens**

- Reporter cell lines in human keratinocytes
- activation of Keap-1-Nrf2 pathway using luciferase signalling

- **SENS-IS**

- Episkin® RhE model - reconstructed epidermis (3D)
- Gene expression measurements (RT-PCR)
- REDOX panel: 17 genes Keap1-Nrf2- genes
- SENS-IS panel: 21 genes (inflammation, cell migration)
- Validated, currently under peer review
- Provides info on potency





KE3 – dendritic cell-based assays 2

- ❖ Flow cytometric assessment of maturation markers on dendritic cells
- ❖ Measurement of cytokine gene regulation in dendritic cells

- **h-CLAT**
 - THP-1 human monocytic cell line
 - Expression levels of CD54 and C86 cell surface markers

- **U-SENS**
 - Human myeloid U937 cell line
 - Expression levels of C86 cell surface marker

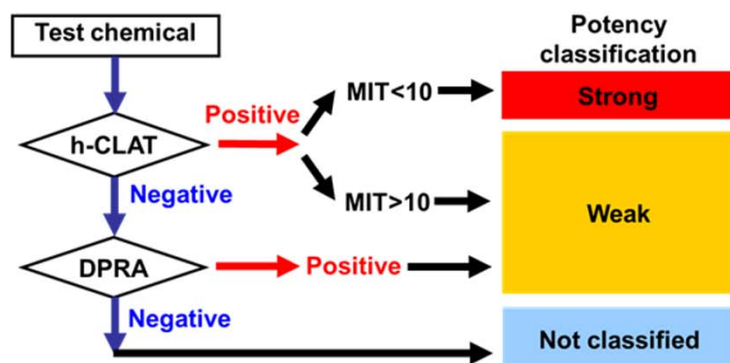
- **IL-8 Luc assay**
 - Reporter cell line in THP-1 cell line
 - Luciferase gene under the control of the IL-8 promoters



Individual test methods and testing strategies

- No non-animal methods for KE4 - T cell activation
- *In vitro* assays identify prehaptens and prohaptens tested so far
- Non-animal methods do provide quantitative data
 - Stand-alone: info not sufficient to predict skin sensitising potency
 - In combination with other methods → potency estimation possible
- AOP is used as a backbone to develop testing strategies:
 - Combining methods that cover MIE, KE2, KE3
 - Additional info (QSARs, phys-chem properties)

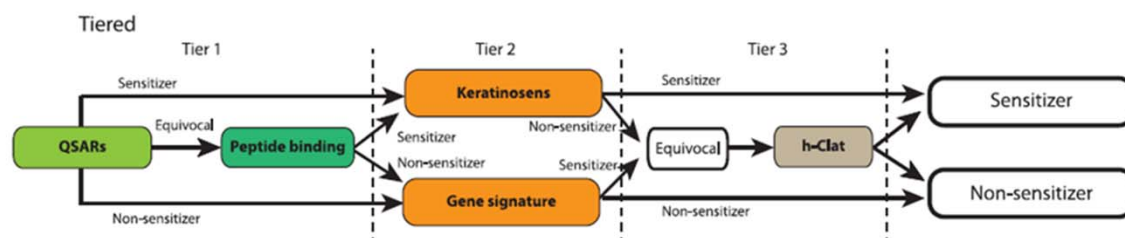
Skin sensitisation: many possibilities of combining information



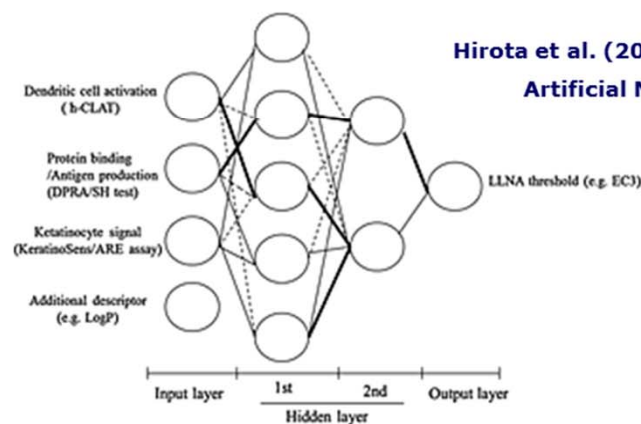
Score	h-CLAT MIT	DPRA depletion	DEREK
3	≤10 µg/mL	≥42.47%	-
2	>10, ≤150 µg/mL	≥22.62, <42.47%	-
1	>150, ≤5000 µg/mL	≥6.376, <22.62%	Alert
0	not calculated	<6.376%	No alert

Potency: Total battery score	Strong :	7
	Weak :	2-6
	Not classified :	0-1

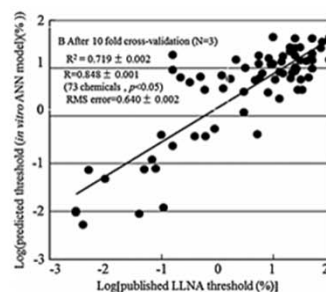
Takenouchi et al. (2015) J. Appl. Toxicol.: STS & ITS



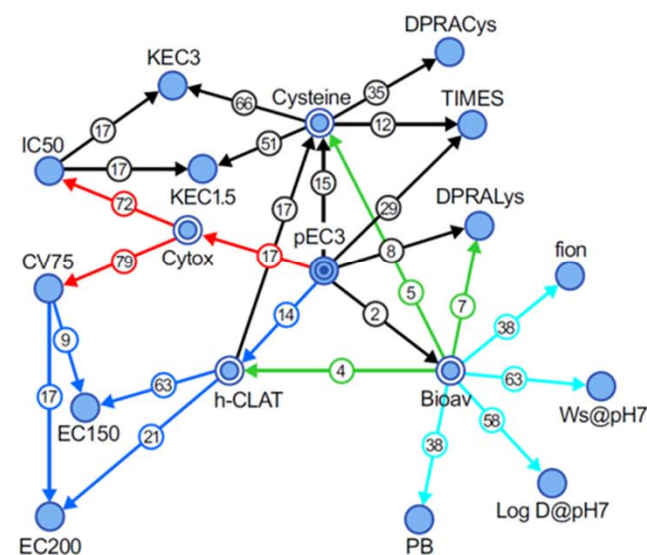
Van der Veen (2014) RTP; RIVM STS



Hirota et al. (2015) J. Appl. Toxicol.:
Artificial Neural Network



Jaworska et al. (2015) Arch. Toxicol.:
Bayesian Network





Intelligent Testing Strategy

IATA

Defined approaches to testing and assessment

Battery approach



Integrated Testing Strategy

Tiered approach



OECD guidance documents on IATA and DA

- To facilitate evaluation of approaches that combine individual test methods in regulatory decision-making there is a need for consistent terminology and harmonised development
- EURL ECVAM coordinated drafting of two OECD Guidance Documents to facilitate this
- General document on definitions and principles for defined approaches to testing and assessment (DA) and IATA (nr. 255)
- Skin sensitisation specific document on DA and IATA (nr. 256)



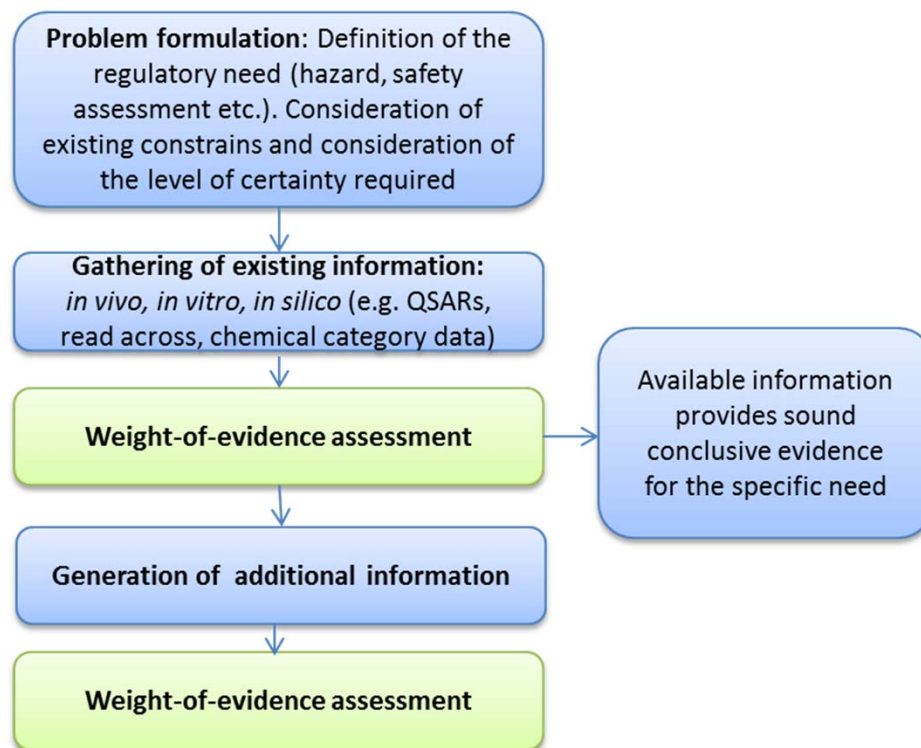
Guidance specific for skin sensitisation

- OECD Guidance Document on the reporting of defined approaches to be used within integrated approaches to testing and assessment individual information sources to be used within IATA for skin sensitisation (256) -2016
- Annex 1: Case studies on skin sensitisation DA and IATA
 - 12 case studies documented by the developers using the templates provided by the OECD
 - 11 on DA and 1 IATA for skin sensitisation
- Annex 2: Individual data sources for skin sensitisation
 - Overview of test guideline and non-guideline methods
 - Information reported according to a fixed template
 - Including known limitations and uncertainties



Integrated Approaches to Testing and Assessment

- Multiple information sources
phys-chem properties, QSARs, read-across, in chemico, in vitro, in vivo
- Integrates and weights all relevant existing evidence and guides the target generation of new data
- Involves a degree of expert judgment
- Flexibility in constructing IATA for a given chemical and regulatory need





Defined Approaches to Testing and Assessment

- Constructed using a defined set of methods / tools
- Uses a fixed data interpretation procedure (DIP) to reach a decision
- *Sequential testing strategy*
fixed stepwise approach with interim decision steps to decide if additional testing is needed
- *Integrated testing strategy*
multiple sources of data are assessed at the same time
uses specific methodologies to convert inputs from the different information sources into a prediction



Principles for Defined Approaches

To facilitate regulatory acceptance and use, DA should be associated with:

1. Defined endpoint
2. Defined purposes (i.e. regulatory application)
3. Description of the underlying rationale
4. Description of the individual information sources used
5. Description on how data from individual data sources are processed
6. Consideration of known uncertainties

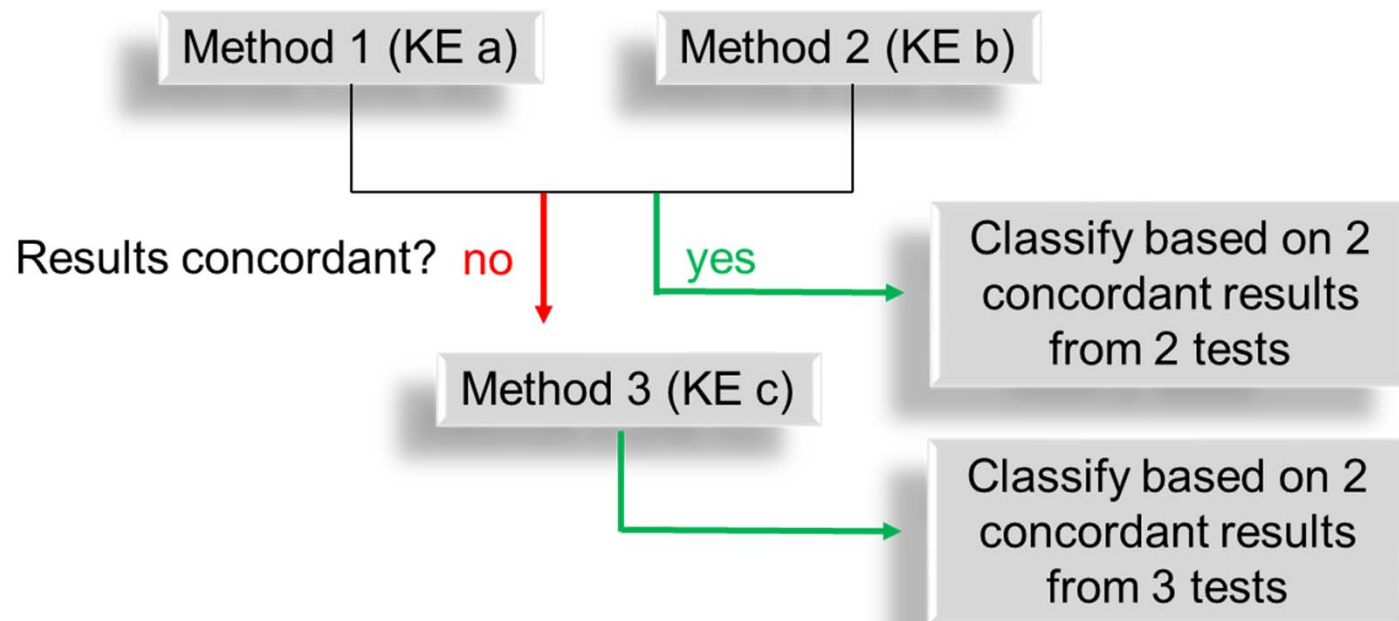


AOP-based “2 out of 3” ITS - BASF

- Rationale:
Integrate data from well-developed test methods that cover the key mechanisms involved in skin sensitization
- ITS combines multiple assays that address 3 KEs
 - MIE – protein binding DPRA
 - KE2 – keratinocyte activation Keratinosens or LuSens assay
 - KE3 – dendritic cell activation h-CLAT, U-SENS, mMUSST
- Outcome: hazard identification



Data interpretation procedure



- No predefined sequential order of testing
- Usually, DPRA and keratinocyte assays are performed first
- Dendritic Cell assays are more complex and expensive



Cooper Statistics of the "2 out of 3" ITS

Performance when OECD TGs were used in ITS (Keratinosens, DPRA, h-CLAT)

Compared to human	N	Sensitivity	Specificity	Accuracy
"2 out of 3" ITS	111	90%	90%	90%
LLNA	101	91%	64%	82%

Compared to LLNA				
"2 out of 3" ITS	180	82%	72%	79%



Bayesian Network ITS – P&G

- Rationale:
ITS structure represents the AOP
- Outcome: hazard identification and potency prediction (LLNA EC3)
- ITS integrates multiple data streams:
 - Bioavailability parameters
 - *In silico* metabolism - TIMESS
 - MIE – DPRA
 - KE2 – KeratinoSens
 - KE3 – h-CLAT
- Online tool: <http://its.douglasconnect.com>

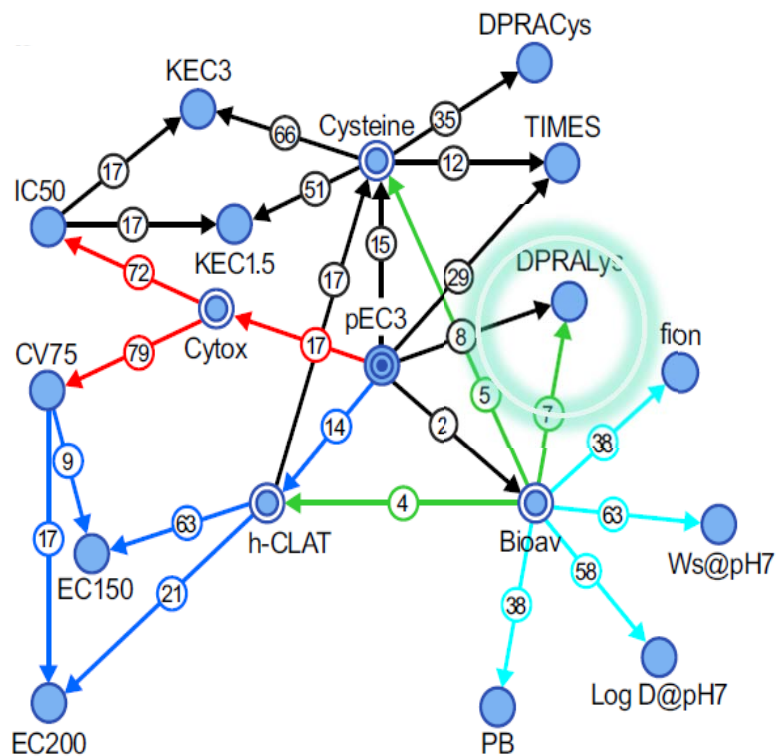


Bayesian Network and DIP

- BN formulates a probabilistic hypothesis about a target variable → LLNA EC3 (potency)

Bayesian Network ITS

- Each node represents one of the input variables
- Bayesian statistics:
 - Quantifies uncertainty in a prediction
 - Provides insight in the strenght of evidence
 - Provides leads if additional inputs/info is needed



LLNA pEC3 probability distribution

4 potency classes:

- Non-sensitizers
- Weak sensitizers
- Moderate sensitizers
- Strong and extreme sensitizers

ITS was trained with training set of 147 chemicals



Evaluation performance of Bayesian Network ITS

- ITS was evaluated with test set (n=60): 46 skin sensitizers and 14 non-sensitizers
- Accuracy to predict human skin sensitizers not provided

Hazard identification compared to LLNA

	N	Sensitivity	Specificity	Accuracy
Bayesian ITS	60	100%	100%	100%

Category	Accuracy	N
Overall:		
-GHS C&L (1A, 1B)	96%	60
-four categories	89%	60



Defined approaches for skin sensitisation

- All defined approaches show improved predictivity compared to the individual information sources
- Predictive performance is similar or slightly better than the LLNA, despite the fact that not all KEs of the AOP are covered
- Predictive performance for hazard id is quite similar between DAs
 - construction seems not to have a major impact on the predictivity of a strategy
- Limitations and uncertainties mostly refer to limitations of the individual data sources not on DA



Future perspectives

- Ongoing initiatives to further evaluate DAs for skin sensitisation
- Cosmetics Europe has analysed performance of six DA for skin sensitisation safety assessment of cosmetic ingredients
 - Same dataset of 128 substances, enables comparison of performance
 - Publication expected this year
- EURL-ECVAM is developing a performance-based test guideline on defined approaches for skin sensitisation
 - Establish assessment criteria for DA
 - Equal footing with a test guideline - Mutual Acceptance of Data
 - OECD meeting Dec 2017 planned



Conclusions

- Much progress on the level of individual test method acceptance and use, but:
too much options for DA, this may hamper their regulatory use
- Key priority should be facilitation of the regulatory use of DAs:
 - ❖ Increase understanding applicability domain and technical limitations of individual test methods by broadening chemical space
 - ❖ Better understanding of impact of design and interpretation procedures on the performance of a DA
 - ❖ Evaluate approaches that provide potency estimation with priority
 - Include the known variability and uncertainty related to the LLNA in this evaluation




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REVIEW ARTICLE

State of the art in non-animal approaches for skin sensitization testing: from individual test methods towards testing strategies

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