The GARD® assay -
For skin sensitization hazard identification and risk assessment

Andy Forreryd, SenzaGen AB
Genova, June 22nd, 2022

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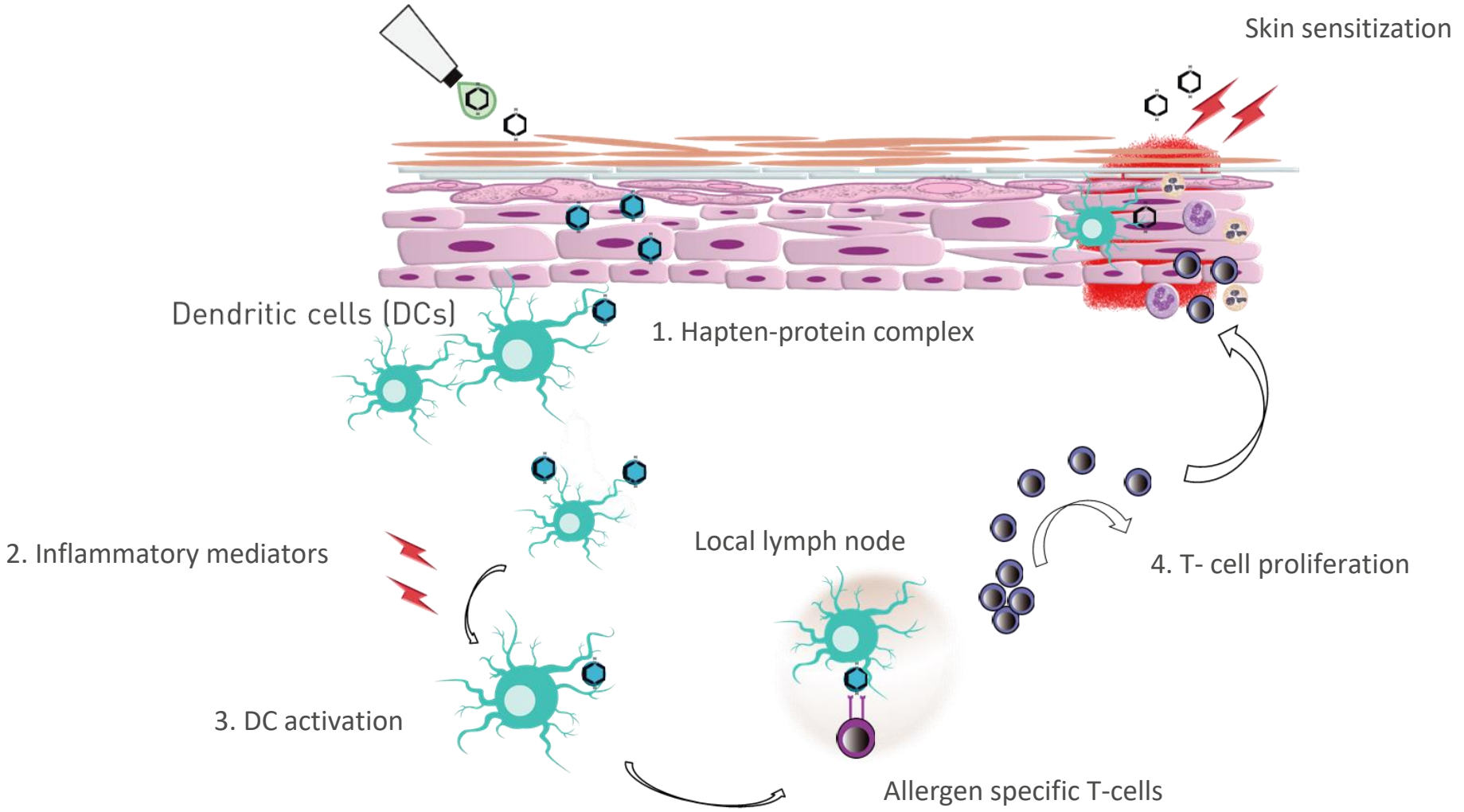
Introduction – Mechanisms of Skin Sensitization

Induction of an immunological memory & elicitation of clinical symptoms

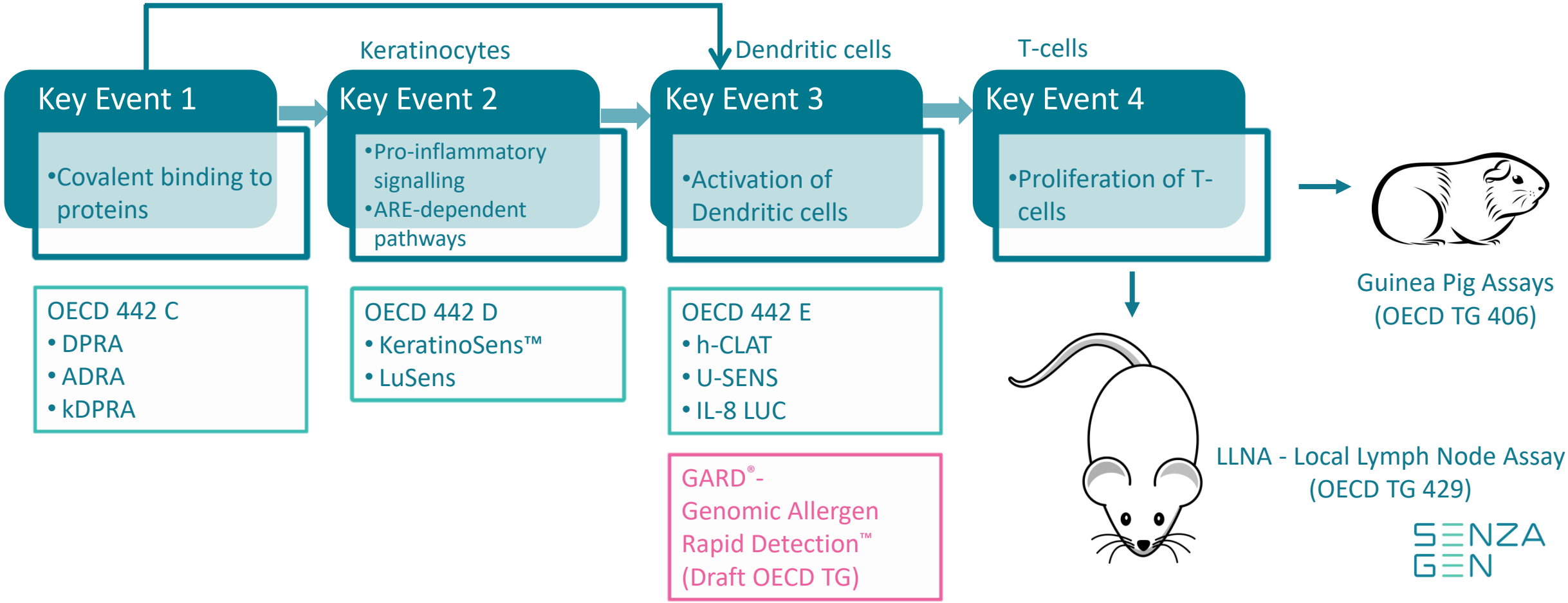


Introduction – Testing for Skin Sensitization

NAM-based OECD Test Guidelines are mapped to the AOP

AOP - Adverse Outcome Pathway

NAM - New Approach Methods target Key Event 1-3

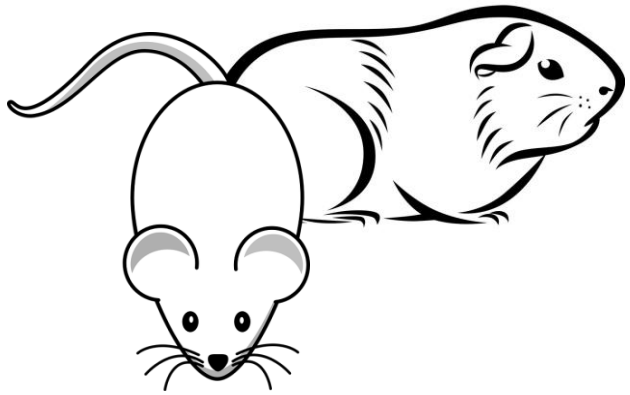


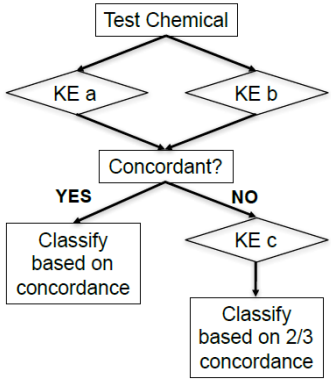
Introduction – Testing for Skin Sensitization

Defined Approaches to replace animal studies

Traditional testing:
In vivo

First generation *In vitro* assays are combined into Defined Approaches to replace animal studies.





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 (1A): 6-7
Weak (1B): 2-5
Not classified: 0-1

Local Lymph Node Assay (OECD TG 429)
Guinea Pig Assays (OECD TG 406)

OECD TG 442C
OECD 442E
OECD TG 442D

OECD TG 497 on Defined Approaches for Skin Sensitization.
Hazard: 2 out of 3
GHS potency: ITSv1, ITSv2

Remaining challenges

Data gaps and limitations to be addressed by novel *in vitro* methods

Applicability domain (AD)

- OECD TGs validated using a narrow subset of the chemical space.
- OECD TGs validated for monoconstituents. Limited data available for complex mixtures.

Case study #1: Pre/pro-haptens, hydrophobic substances & UVCBs

Case study #2: Agrochemical formulations

Quantitative assessment of relative sensitizing potency

- Quantitative assessment of skin sensitizing potency on a continuous scale for use in QRA and to establish a threshold dose.

Case study #3: Continous prediction of skin sensitization potency for use in quantitative risk assessments (QRA).

Biocompatibility testing of medical devices

- Requires assay compatibility to both polar and non-polar extraction vehicles (ISO-10993:12).
- Assay must be sensitive to detect potential sensitizers in a complex extract.

Case study #4: Testing of Medical Devices according to ISO-10993.

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GARD®-

Genomic Allergen Rapid Detection™

Key technological features – Genomics and machine learning

GARD®skin sensitization assay portfolio

From binary hazard identification to quantitative potency information on a continuous scale

GARD®skin 200 genes	GARD®potency 51 genes	GARD®skin Dose-Response 200 genes
Binary hazard identification of skin sensitizing chemicals	An add-on *in vitro* test to GARDskin for potency classification according to GHS/CLP (1A or 1B)	Quantitative assessment of skin sensitization potency on a continuous scale

GARD®skin Medical Device 200 genes
Skin sensitization testing of Medical Devices according to ISO 10993

GOOD LABORATORY PRACTICE GLP CERTIFIED

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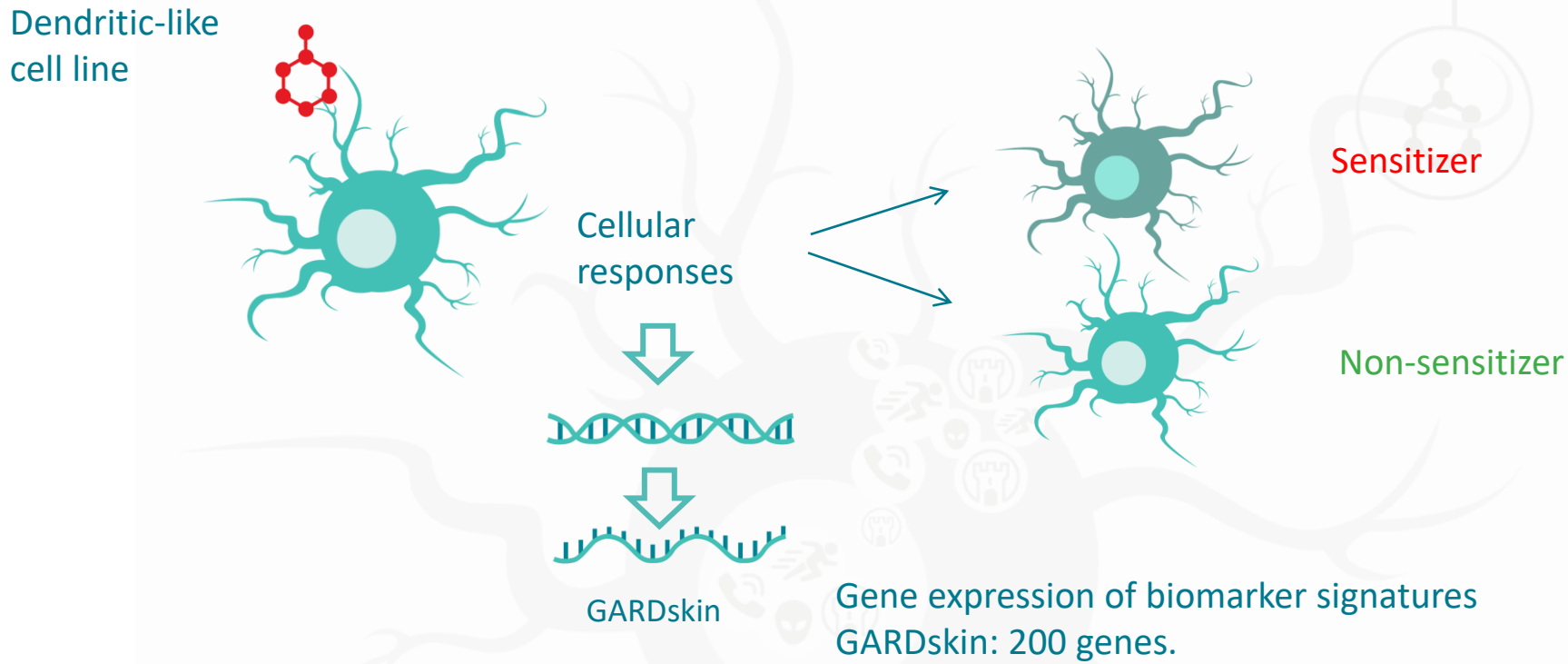
The GARD® technology platform – how it works

Transcriptomic read-out of the biological response

Biological system: Dendritic-like cell line (KE3)
Readout: Gene expression (genes and toxicity pathways)

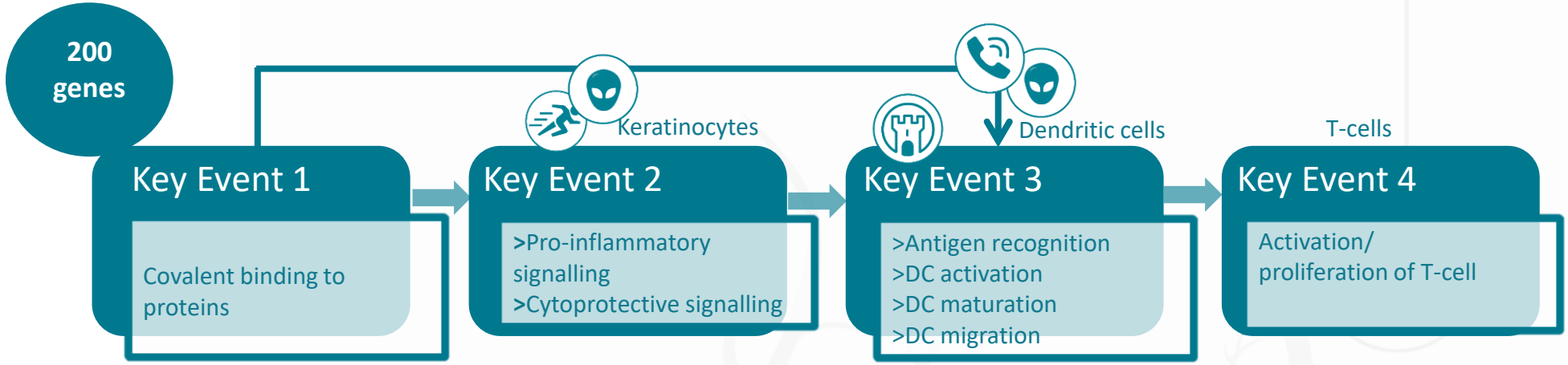


Full transparancy: Identities of genes being measured available in peer-reviewed scientific literature.

See for example: Johansson et al. (2011) A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. BMC Genomics.

The GARD® technology platform – how it works

Genes cover mechanistically relevant toxicity pathways



Captures events downstream of **KE1**

Metabolic activity & identifies pre/pro haptens
ALDH
NAT-1
CYP - Cytochrome p-450

> Keap1-Nrf2-ARE pathway & AHR signalling
NQO1
HMOX1
Thioredoxin reductase I

> Pro-Inflammatory cytokines mediating e.g. TNFα, INFγ, IL-8
FAS
MAP2KI
COX20
> Inflammasome
NLRP
PSTPIP1

> DC migration & maturation
CD86
MAPK- activation
PKA- and GPCR- mediated signalling

> Antigen recognition & Innate immune activation
TLR-4
TLR-6
RXRA – retinoic X receptor
NLRP
PSTPIP1
> Self-defence mechanisms
C3a/C5a-activation pathways

Covers the 3 Key steps for T-cell activation:
Antigen presentation
Co-stimulation
Cytokine secretion

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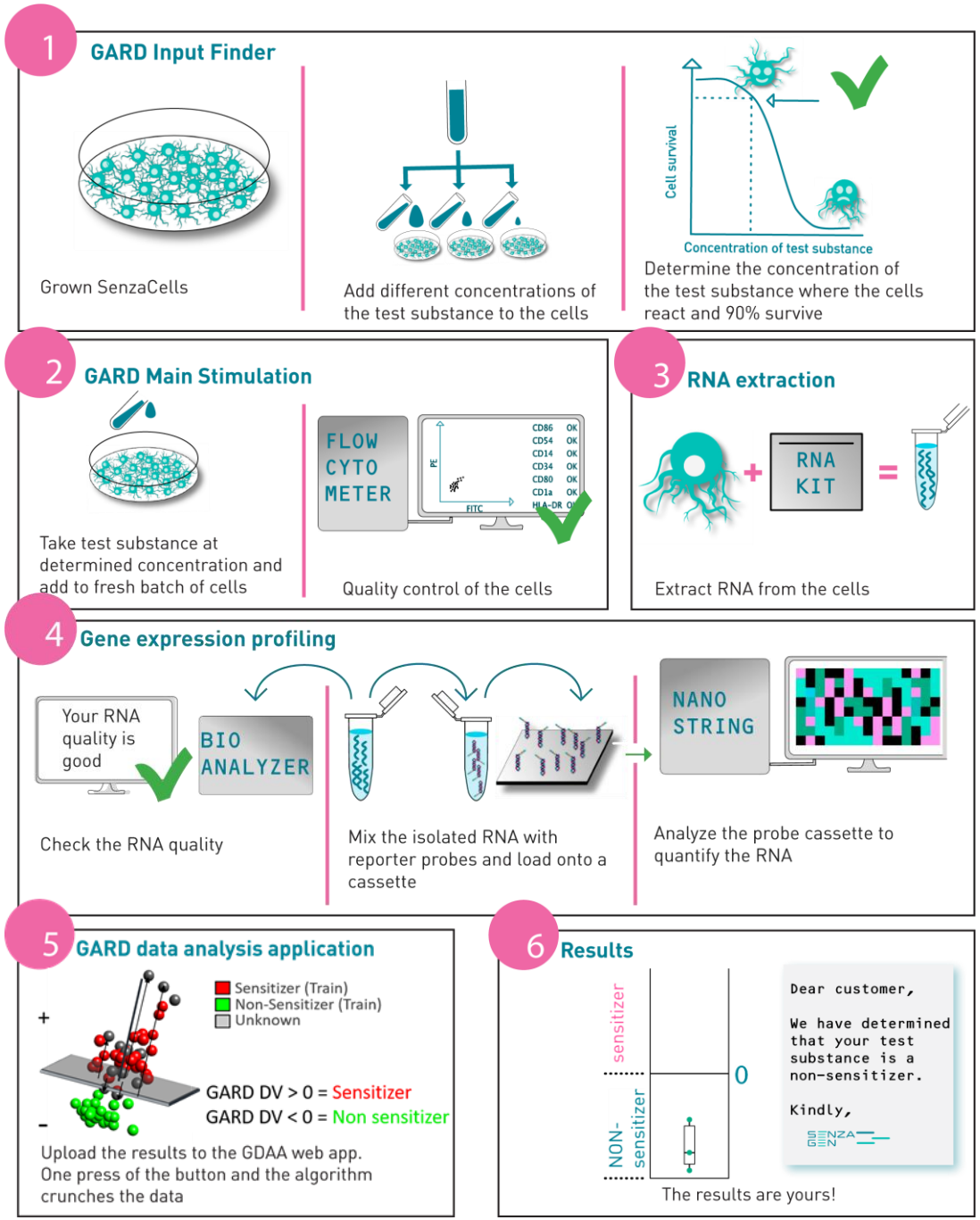
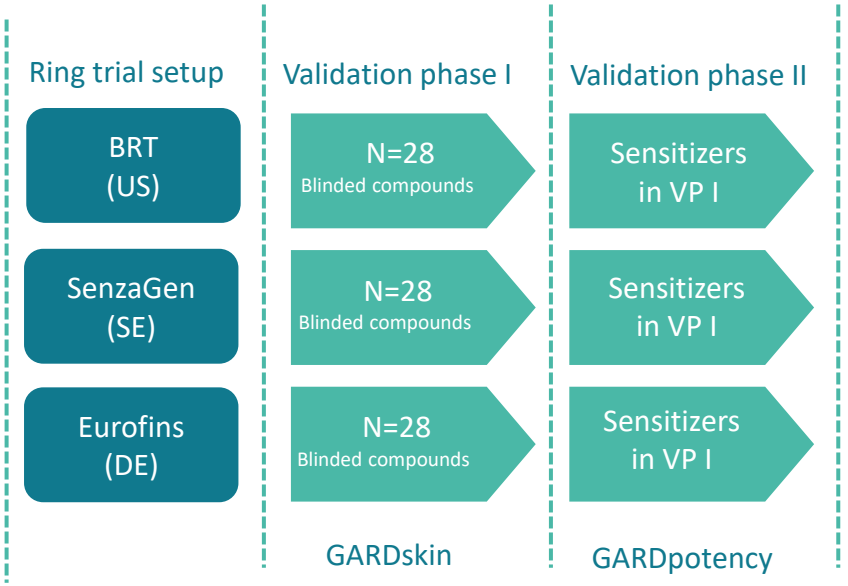
How to GARD® your products in 6 Steps

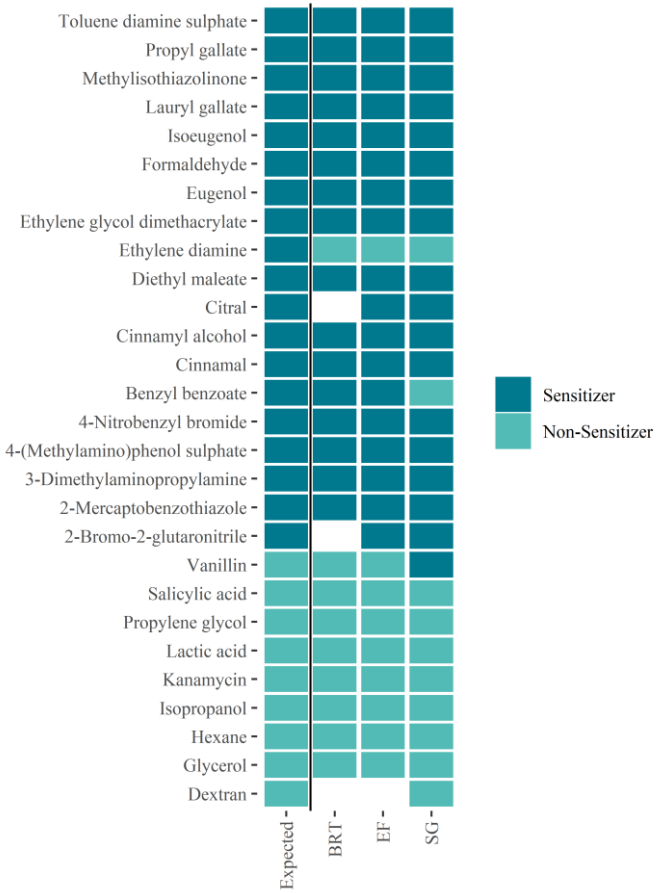


The GARD® technology platform

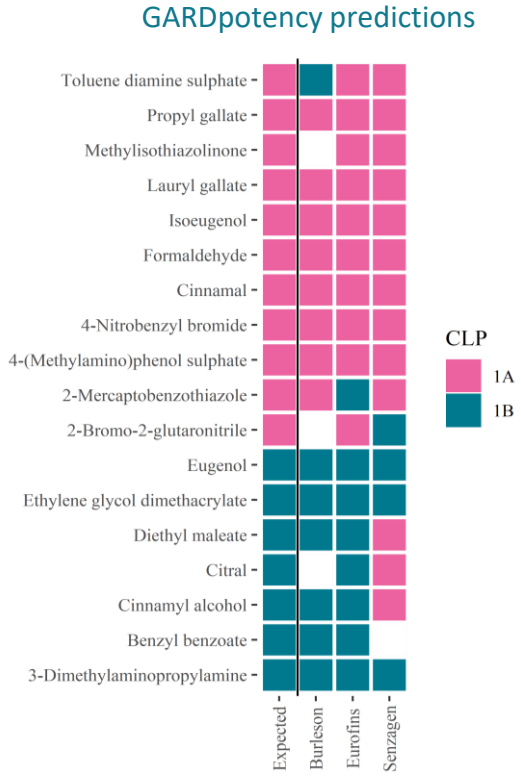
Machine learning and omics arrive in the field of regulatory toxicology



GARDskin predictions



GARDpotency predictions



Validation studies published in peer-reviewed scientific journals:

GARDskin: Published in Johansson et al. (2019), Validation of the GARD™skin assay for assessment of chemical skin sensitizers - ring trial results of predictive performance and reproducibility. ***Toxicological Sciences.***

GARDpotency: Published in Gradin et al. (2020), The GARD™potency Assay for Potency-Associated Subclassification of Chemical Skin Sensitizers - Rationale, Method Development and Ring Trial Results of Predictive Performance and Reproducibility. ***Toxicological Sciences.***

Performance statistics:

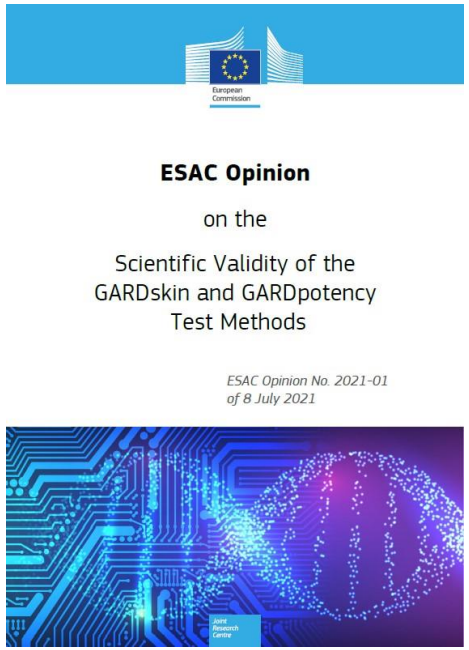
GARDskin accuracy: **94%**

GARDpotency accuracy: **88%**

GARD Defined Approach: **86%**

The GARD® technology platform

Machine learning and omics arrive in the field of regulatory toxicology



- The EURL ECVAM Scientific Advisory Committee (ESAC) conducted an independent review of the scientific validity of GARD.
- First time a machine-learning algorithm has been reviewed for use within the field of regulatory toxicology.
- Draft OECD TG available.

"ESAC considers that GARDskin is ready to progress to further consideration by the OECD for Test Guideline development"

- ESAC Opinion

Case study #1 & 2: Applicability Domain (AD)

In vitro skin sensitization testing of challenging substances

Applicability domain (AD)

- OECD TGs validated using a narrow subset of the chemical space.
- OECD TGs validated for monoconstituents. Limited data available for complex mixtures.

ECHA
EUROPEAN CHEMICALS AGENCY

"To use the results of in chemico, in vitro or in silico tools, the substance must fit into the applicability domain of a given method or tool."

Limitations of individual assays are specified in the individual OECD Test Guideline (as far as they have been identified).

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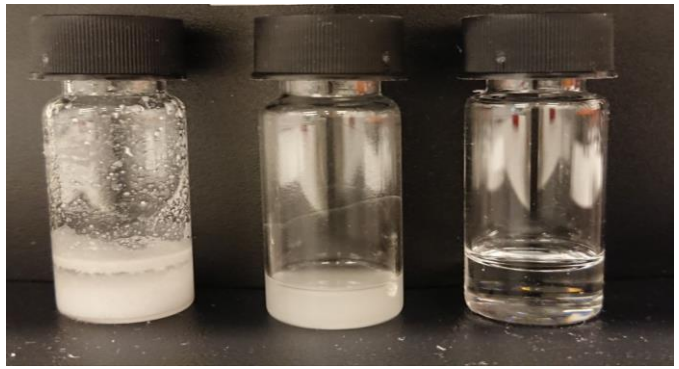
Case Study #1: Applicability Domain (AD)

In vitro skin sensitization hazard assessment of challenging substances

Certain chemicals are challenging to assess in OECD TGs

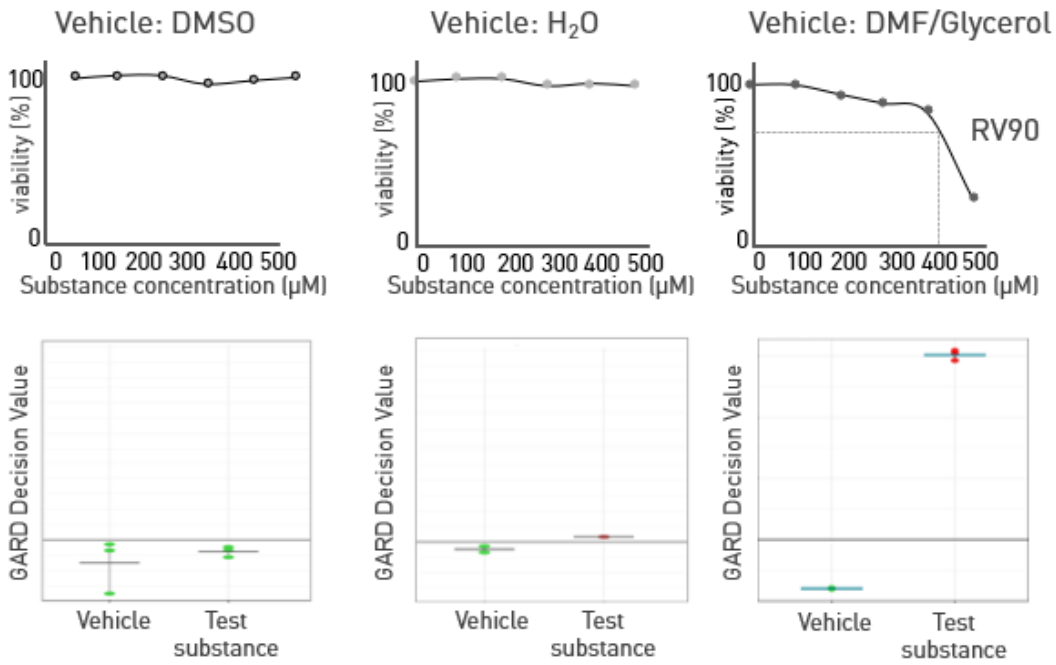
- **Pre/pro haptens:** indirect acting haptens require activation to become protein reactive.
- **Hydrophobic substances:** Solubility limitations may prevent testing at sufficiently high concentrations.
- **UVCBs*:** No specified Molecular weight (Mw) – difficult to establish relevant molar concentration for testing.



Biphasic immiscible liquids | Homogenous suspension | True solution

Solubility issues:

Can (at least partly) be addressed by a panel of alternative solvents.



*UVCBs: Unknown or Variable Composition, Complex Reaction Products and Biological Materials.

Case Study #1: Applicability Domain (AD)

In vitro skin sensitization hazard assessment of challenging substances

Background

- Generation of experimental data to support inclusion of "challenging" substances into the AD of GARDskin.
- A large dataset was evaluated:
  - Indirect acting haptens (n=25)
  - Hydrophobic items, (n =25, log P_{ow} > 3.5)
  - UVCBs (n=7) (provided by Lubrizol Inc).

Results

- GARDskin classified indirectly acting haptens and hydrophobic items with a similar accuracy as reported for other subsets.
- As evident from results from OECD TG, the subset of chemicals were indeed "challenging" to test.
- Results from testing of the UVCBs provides an indication on the capacity to test very hydrophobic test items.
- GARDskin can contribute to fill data gaps where other OECD TGs has reported technical limitations.

Dataset	Indirectly acting haptens		Hydrophobic test Items	
Reference	LLNA	Human	LLNA	Human
GARD	92.4%	87.5%	85.7%	80.8%
DPRA	56.0%	50.0%	48.0%	69.2%
KeratinoSens	72.0%	62.5%	60.0%	61.5%
h-CLAT	88.0%	87.5%	72.0%	76.9%

Substance	Log P_{ow}	S/NS	GARD
LUB 1	12	S	S
LUB 2	3	S	S
LUB 3	5	S	S
LUB 4	11	NS	NS
LUB 5	11	S	S
LUB 6	4	S	S
LUB 7	4	NS	S

Published in Forreryd et al. (2022), Exploration of the GARDskin Applicability Domain: Indirectly Acting Haptens, Hydrophobic Substances and UVCBs. ***ALTEX***

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Case Study #2: Applicability Domain (AD)

In vitro skin sensitization hazard and potency assessment of agrochemical formulations

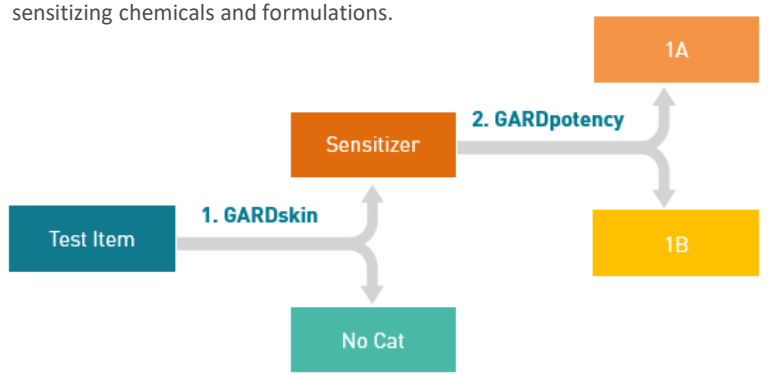
Background

- OECD TGs validated for mono-constituents: limited data available in literature on applicability to (agrochemical) formulations.

Methods

- 20 liquid-based agrochemical formulations (11 water based / 9 organic solvent based), with reliable *in vivo* data (mostly LLNA).
- Mw not available and SOP had to be modified: MW for formulations approximated to **400g/mol.**

The GARD defined approach for identification and subcategorization of skin sensitizing chemicals and formulations.



	TOTAL	Type	Not Classified	Cat 1B	Cat 1A
Water based	11	SL	4	2	-
		SC	5	-	-
Solvent based	9	EC	1	1	1
		EW	1	2	
		OD	-	2	-
		ME	-	1	-
TOTAL	20		11	8	1

Corvaro, M. et al. (2022) GARD™skin and GARD™potency: a proof-of-concept study to investigate the applicability domain for agrochemical formulations. Manuscript submitted.

Case Study #2: Applicability Domain (AD)

In vitro skin sensitization hazard and potency assessment of agrochemical formulations

GARDskin classifications:

Accuracy:	75%	(15/20)
Sensitivity:	89%	(8/9)
Specificity:	64%	(7/11)

GARDpotency classifications:

GHS 1B:	75 %	(6/8)

The only GHS 1A was underpredicted as 1B

Conclusions

- GARDskin & GARDpotency showed promising concordance to available reference data for assessment of complex agrochemical formulations.
- Mispredictions generally attributed to borderline *in vivo* results.

Code	GHS Cat.	Test	Max SI[a]	EC3	GARD Prediction	Predictivity Outcome
COR-4	1A	LLNA	-	1	Sensitizer	True Positive
COR-3	1B	LLNA	-	12.8	Sensitizer	True Positive
COR-8	1B	LLNA	-	17.24	Sensitizer	True Positive
COR-10	1B	LLNA	-	25.27	Non-Sensitizer	False Negative
COR-16	1B	LLNA	-	29	Sensitizer	True Positive
COR-9	1B	LLNA	-	38.6	Sensitizer	True Positive
COR-7	1B	LLNA	-	42.3	Sensitizer	True Positive
COR-31	1B	LLNA	-	52.3	Sensitizer	True Positive
COR-34	1B	BT	-	-	Sensitizer	True Positive
COR-13	NC	LLNA	2.8	-	Sensitizer	False Positive
COR-17	NC	LLNA	2.3	-	Sensitizer	False Positive
COR-12	NC	LLNA	2.1	-	Sensitizer	False Positive
COR-2	NC	LLNA	1.8	-	Non-Sensitizer	True Negative
COR-15	NC	LLNA	1.7	-	Sensitizer	False Positive
COR-11	NC	LLNA	1.7	-	Non-Sensitizer	True Negative
COR-5	NC	LLNA	1.3	-	Non-Sensitizer	True Negative
COR-6	NC	LLNA	1.3	-	Non-Sensitizer	True Negative
COR-14	NC	LLNA	1.2	-	Non-Sensitizer	True Negative
COR-18	NC	LLNA	0.7	-	Non-Sensitizer	True Negative
COR-1	NC	LLNA	0.9	-	Non-Sensitizer	True Negative

Corvaro, M. et al. (2022) GARD™skin and GARD™potency: a proof-of-concept study to investigate the applicability domain for agrochemical formulations. Manuscript submitted.

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Case Study #3: Quantitative potency assesments

In vitro quantitative assessment of skin sensitizing potency

Quantitative assessment of relative sensitizing potency

- Quantitative assessment of skin sensitizing potency on a continuous scale for use in QRA and to establish a threshold dose.

Regulatory Toxicology and Pharmacology 118 (2020) 104805

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

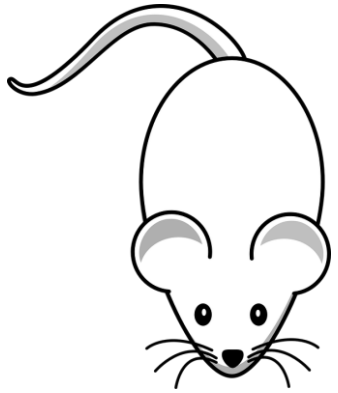
journal homepage: www.elsevier.com/locate/yrtph

Updating exposure assessment for skin sensitization quantitative risk assessment for fragrance materials

Anne Marie Api [a,*], David Basketter [b], James Bridges [c], Peter Cadby [d], Graham Ellis [e], Nicola Gilmour [f], Helmut Greim [g], Peter Griem [h], Petra Kern [i], Alain Khaiat [j], John O'Brien [k], Thomas Rustemeyer [l], Cindy Ryan [m], Bob Safford [n], Benjamin Smith [o,p], Matthias Vey [q], Ian R. White [r]

Identified skin sensitizers can be safely formulated into consumer products, guided by the Quantitative Risk Assessment (QRA) framework.

- Skin sensitization is a threshold phenomenon, and a maximum acceptable concentration for each material can be determined.
- Local Lymph Node Assay (LLNA) provides a continous prediction of skin sensitizing potency and can (often) be used as a surrogate value for the human NOEL (no-observed effect level).



Local Lymph Node Assay
(OECD TG 429)

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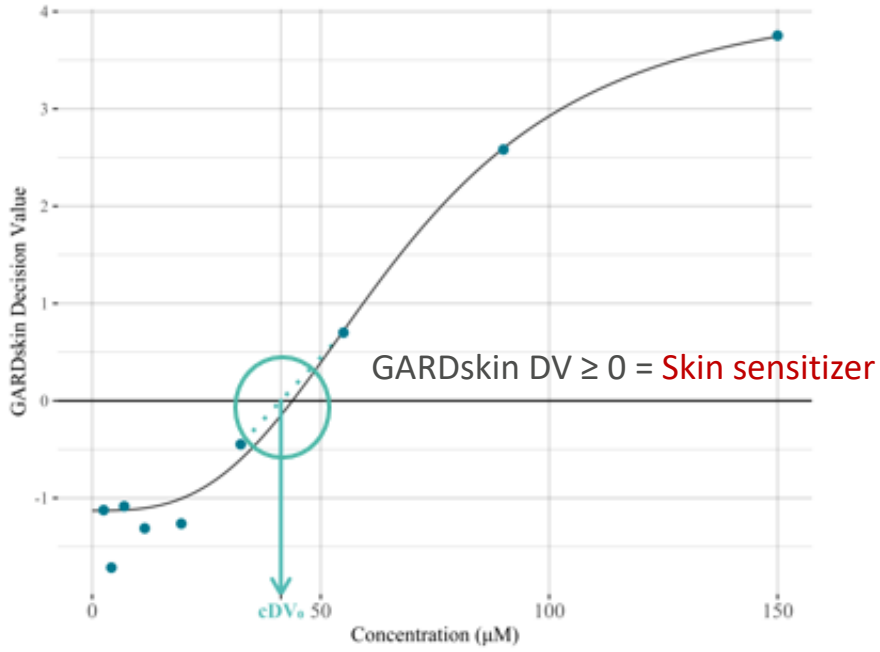
Case Study #3: Quantitative potency assesments

In vitro quantitative assessment of skin sensitizing potency

- Perform the GARDskin assay in a titrated range of concentrations (n ≥ 6).
- Apply standard GARDskin protocol to generate a decision value (DV) for each concentration.
- Visual inspection of dose-response curve possible by plotting DVs vs input concentrations.
- Estimate cDV_0: lowest concentration required to induce a positive classification (DV≥ 0).

	GARD	LLNA
Response value	DV	SI
Binary Threshold	DV = 0	SI = 3
Readout	cDV_0 (DV_0 Concentration)	EC3 Concentration



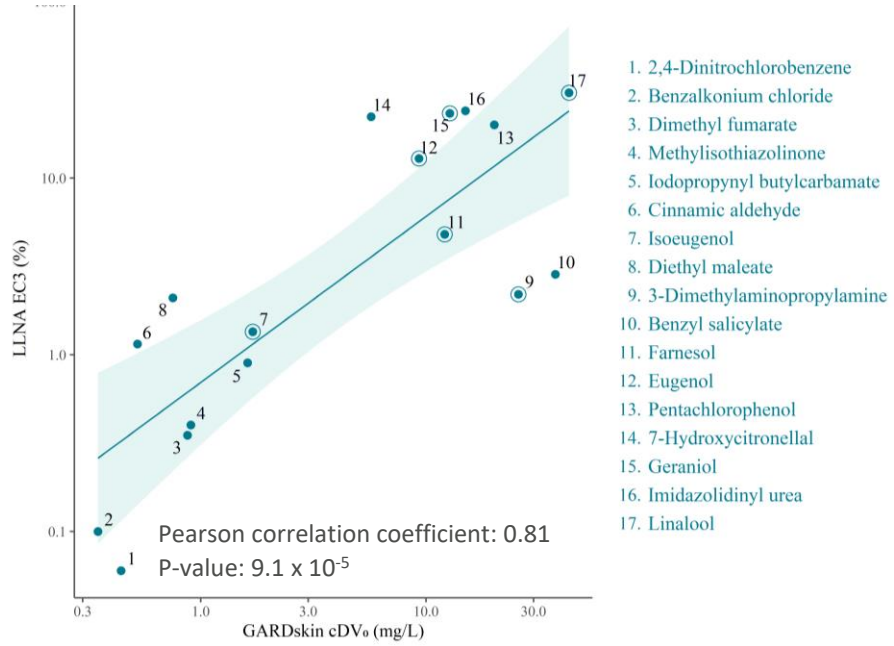
Gradin, R., Forreryd, A., Mattson, U., Jerre, A., Johansson, H. (2021) Quantitative assessment of sensitizing potency using a dose-response adaptation of GARDskin. Nature Scientific Reports

Case Study #3: Quantitative potency assesments

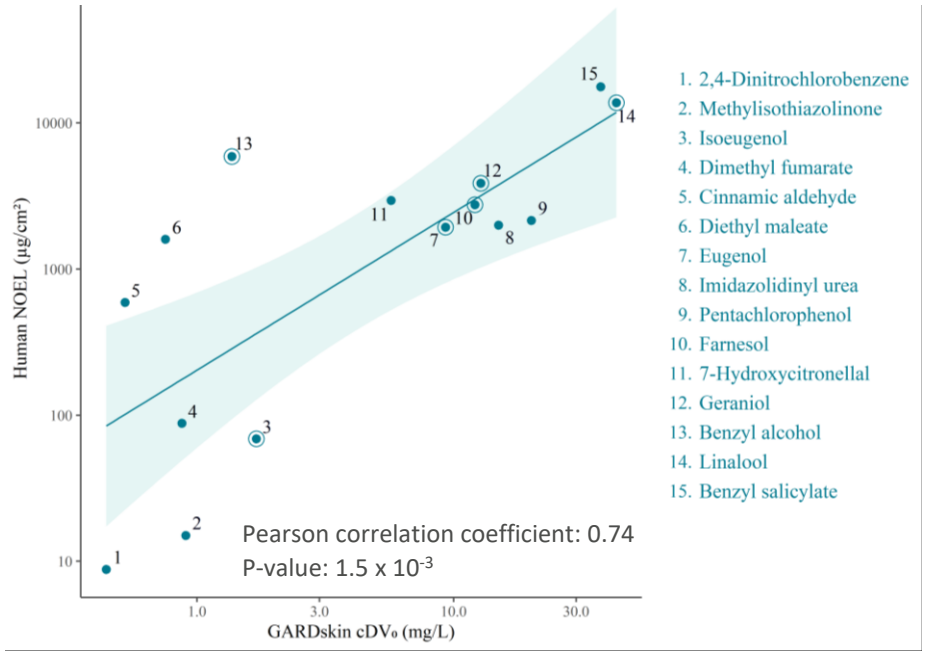
In vitro quantitative assessment of skin sensitizing potency

Experimentally derived cDV_0 concentrations correlates strongly and significantly to LLNA EC3 values and Human NOEL values.

LLNA EC3 vs GARDskin Dose-Response



Human NOEL vs GARDskin Dose-Response



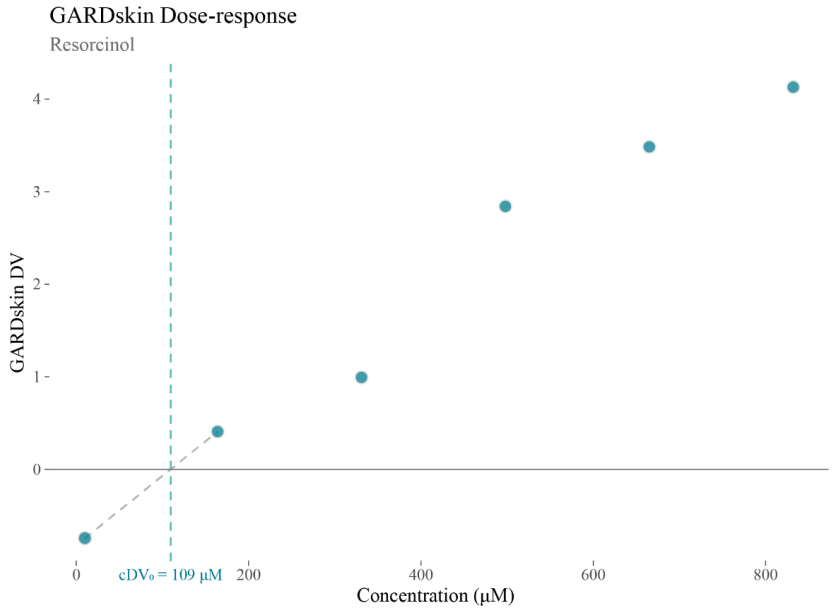
Gradin, R., Forreryd, A., Mattson, U., Jerre, A., Johansson, H. (2021) Quantitative assessment of sensitizing potency using a dose-response adaptation of GARDskin. Nature Scientific Reports

Case Study #3: Quantitative potency assesments

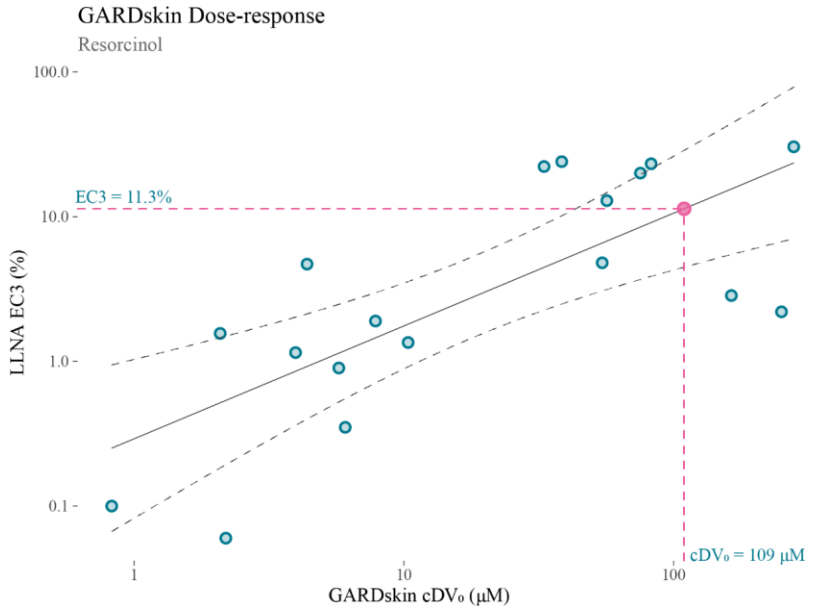
In vitro quantitative assessment of skin sensitizing potency

Predictive models based on linear regression can exploit the relationship by using experimentally derived cDV_0 values to derive a continous prediction of skin sensitizing potency.

Step 1: Dose-response to identify cDV_0



Step 2: Regression model to predict LLNA EC3/human NOEL.



Gradin, R., Forreryd, A., Mattson, U., Jerre, A., Johansson, H. (2021) Quantitative assessment of sensitizing potency using a dose-response adaptation of GARDskin. Nature Scientific Reports

Case Study #3: Quantitative potency assesments

In vitro quantitative assessment of skin sensitizing potency

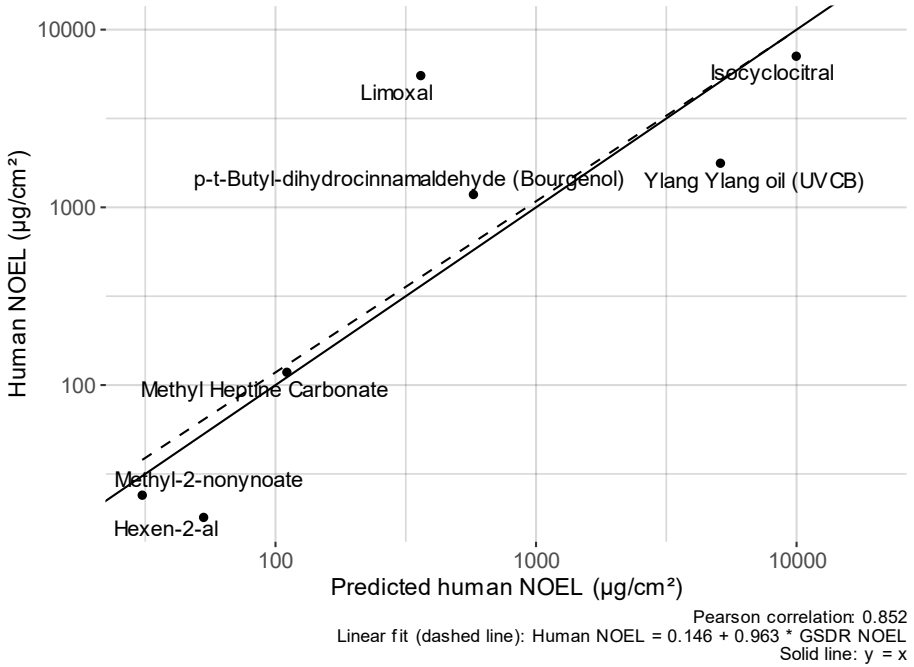
Background

- Blinded testing of 12 materials (incl. a UVCB and a multiconstituent).
- GARDskin Dose-response cDV_0 values used to predict Human NOELs.

Results

- GARDskin Dose-Response predicted Human NOEL values correlated extremely well with reference data.

- NESIL – No Expected Sensitization Induction Level is the point of departure for QRA.



Case Study #4: Medical device testing

In vitro skin sensitization of medical devices and solid material according to ISO-10993

Biocompatibility testing of medical devices

- Requires assay compatibility to both polar and non-polar extraction vehicles (ISO-10993:12).
- Assay must be sensitive to detect potential sensitizers in a complex extract.

Adaption of protocols

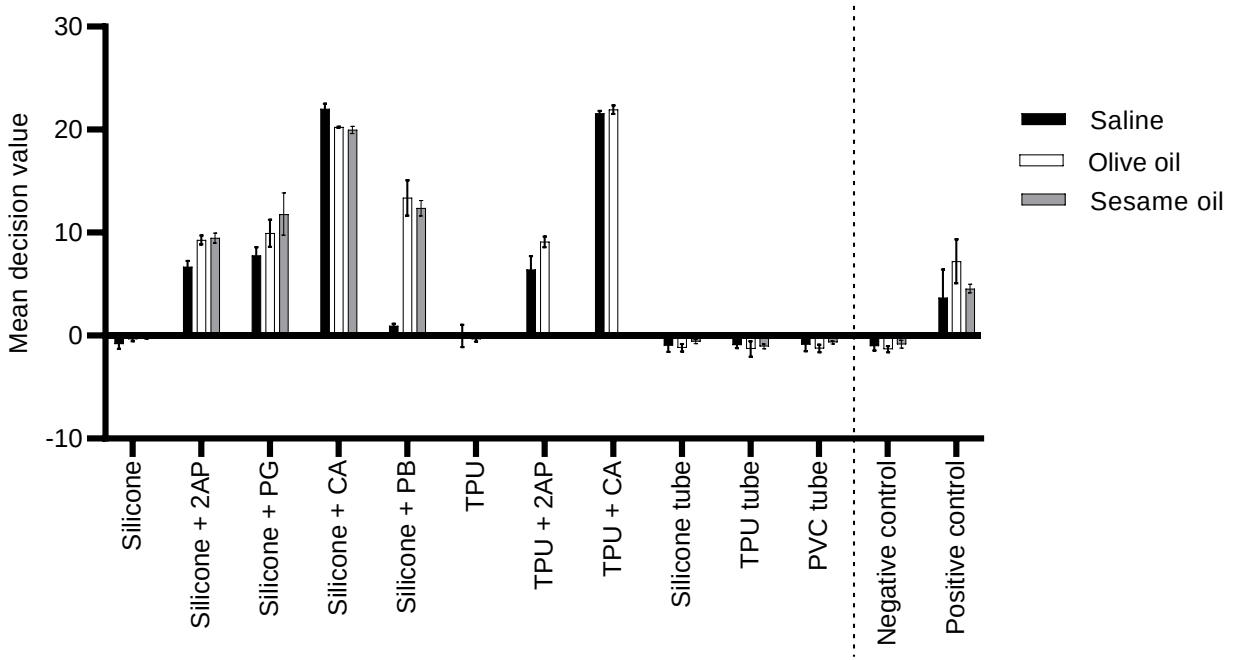
- Protocols adapted to polar and non-polar solvents.
- OECD TGs not compatible with non-polar vehicles.

Proof of concept study

- Polymers (Silicon/TPU) spiked with sensitizers.
- Tubes (Silicone, TPU and PVC) – neg controls.
- Extractions in saline, olive oil and sesame oil.

Results

- Protocols adapted for testing in polar/non-polar vehicles. All materials correctly classified.



Jenvert R, et et al. (2022) Evaluation of the Applicability of GARDskin to Predict Skin Sensitizers in Leachables from Medical Device Materials. Manuscript in preparation.

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Summary and conclusions

1. The current NAM-based OECD TGs for skin sensitization are useful in many situations and their validation has been an important milestone for replacement of animal studies.
2. This presentation shows that emerging technologies are useful to fill data gaps where traditional methods have shown technical limitations.
3. Let’s not forget that limitations apply to any test method. Empirical evidence must guide the selection of most appropriate assay.

Andy Forreryd, PhD
Scientific Liaison Manager
SenzaGen AB

Thank you for listening!