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Research Article

Evaluation of the GARD assay in a blind Cosmetics Europe study

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Summary
Chemical hypersensitivity is an immunological response towards foreign substances, commonly referred to as sensitizers, which gives rise primarily to the clinical symptoms known as allergic contact dermatitis. For the purpose of mitigating risks associated with consumer products, chemicals are screened for sensitizing effects. Historically, such predictive screenings have been performed using animal models. However, due to industrial and regulatory demand, animal models for the purpose of sensitization assessment are being replaced by animal-free testing methods, a global trend that is spreading across industries and market segments. To meet this demand, the Genomic Allergen Rapid Detection (GARD) assay was developed. GARD is a novel, cell-based assay that utilizes the innate recognition of xenobiotic substances by dendritic cells, as measured by a multivariate readout of genomic biomarkers. Following cellular stimulation, chemicals are classified as sensitizers or non-sensitizers based on induced transcriptional profiles. Recently, a number of animal-free methods were comparatively evaluated by Cosmetic Europe, using a coherent and blinded test panel of reference chemicals with human and local lymph node assay data, comprising a wide range of sensitizers and non-sensitizers. In this paper, the outcome of the GARD assay is presented. It was demonstrated that GARD is a highly functional assay with a predictive performance of 83% in this Cosmetics Europe dataset. The average accumulated predictive accuracy of GARD across independent datasets was 86%, for skin sensitization hazard.

Keywords: GARD, sensitization, *in vitro*, predictive accuracy, alternative methods

1 Introduction

Chemical hypersensitivity is a disease state induced by the human immune system in response to chemical sensitizers, which most frequently gives rise to the clinical symptoms known as allergic contact dermatitis (ACD). The molecular and cellular mechanisms of sensitization have been extensively reviewed (Ainscough et al., 2013; Martin, 2015; Martin et al., 2011). Briefly, sensitization involves skin penetration of the sensitizing agent with a subsequent haptenisation of endogenous proteins. Protein-hapten complexes are taken up by resident dendritic cells (DCs), which upon maturation migrate to local lymph nodes where antigen presentation to naïve T cells occurs. This results in the induction of an immunologic memory towards the specific sensitizer. Upon repeated exposure, a sensitized individual will suffer from ACD-associated symptoms following the elicitation of specific Th1 and cytotoxic CD8+ T-cells.

A link has been made between the prevalence of ACD and the increased exposure to the abundance of chemical sensitizers in consumer products (Lunder and Kansky, 2000; Nguyen et al., 2008). In order to limit hazardous effects in response to chemicals, risk assessments aim at safeguarding personnel and the environment by eliminating and mitigating risks of everyday exposure. The European REACH (EU, 2006) legislation requires all manufactured substances to be safety tested in order to identify e.g. chemical sensitizers. Historically, such tests have been conducted in guinea pig (Magnusson and Kligman, 1969) and murine (Basketter et al., 2002) models. Primarily, the murine Local Lymph Node Assay (LLNA) continues to be in use today. However, the use of animals for testing purposes within the cosmetic industry has been banned in the EU since 2013 (EU, 2009), and REACH urges other industries to use animal testing only as a last resort when no relevant alternative testing methods exists, thereby clearly stating an intent to comply to the principles of the 3Rs (Russel and Burch, 1959).

As a consequence, the field of predictive toxicology has recently seen a surge in the development of novel alternative non-animal assays for assessment of chemical sensitizers. The Direct Peptide Reactivity Assay (DPRA) (Gerberick et al., 2004), KeratinoSens^{TM} (Natsch, 2010) and the Human Cell Line Activation Test (h-CLAT) (Ashikaga et

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al., 2006) have been validated by the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and have recently been accepted by the OECD as test guidelines, which demonstrates that these tests are adequately reproducible and transferable (DPRA; OECD Test No 442C, Keratinosens; OECD Test No 442D, h-CLAT; OECD Test No 442E). However, none of the aforementioned assays are thought to fully cover the complexity of the skin sensitization process as stand-alone tests. Rather, it is widely proposed that assessment of hazard and/or risk should be carried out using Integrated Testing Strategies (ITS), also referred to as Integrated Approaches to Testing and Assessment (IATA) (Jaworska and Hoffmann, 2010; Hartung et al., 2013; Rovida et al., 2015; Ezendam et al., 2016). However, the overall predictive performances of an ITS will invariably depend on the predictivity of the assay constituents. In addition, being based on a single or few biomarkers, current methods provide only limited predictive information, as well as sometimes overlapping mechanistic information. Thus, when designing ITSs, tests with high predictive performance and informational content, covering one or more of the key events of the Adverse Outcome Pathway (AOP) (OECD, 2012), would clearly be an advantageous option (Lindstedt and Borrebaeck, 2011).

The Genomic Allergen Rapid Detection (GARD) assay is a cell-based *in vitro* assay for assessment of chemical sensitizers (Johansson et al., 2011). The readout of the assay is based on differentially regulated transcriptional changes of selected genomic biomarkers, referred to as the GARD prediction signature (GPS), induced in a myeloid dendritic cell-like cell line in response to chemical stimulation. GARD has been shown to be functional and able to accurately predict sensitizing chemicals in blind evaluations (Johansson et al., 2014) and exhibits high predictive performance in comparison with *in vitro* counterparts (Johansson and Lindstedt, 2014). Following a thorough evaluation of technological platforms (Forreryd et al., 2014), the assay was recently adapted to a medium-to-high throughput format in order to meet industrial and regulatory demands on reliability, resource effectiveness and sample capacity (Forreryd et al., 2016). Furthermore, an adaptation of GARD, using identical cellular protocols but a different biomarker signature to differentially classify respiratory sensitizers from a set of skin sensitizers and non-sensitizers has been demonstrated (Forreryd et al., 2015). This implies an unparalleled flexibility of applications provided by genomics-based platforms, due to the massive amount of information that multivariate readouts deliver.

In an attempt to evaluate the performance of currently validated assays, as well as selected assays that are currently in the validation process or considered for validation, the Cosmetic Europe Skin Tolerance Task Force (CE STTF) recently published a comparative study in which a limited set of chemicals were classified as sensitizers or non-sensitizers (Reisinger et al., 2015). Based on this study, the best-performing assays, among them GARD, were selected for a second evaluation phase comprising a larger number of blinded chemicals with human and LLNA data. Here, we present the GARD results from this exercise, and report the predictive performance on this Cosmetics Europe dataset, as well as an updated overall predictive accuracy, calculated using strictly independent sets of test chemicals.

2 Materials and Methods

Chemicals and datasets

A dataset for model training, consisting of 40 different cell stimulations in biological triplicates, has previously been defined, and dataset details are described elsewhere (Johansson et al., 2011; Forreryd et al., 2016). In this study, a total of 73 chemicals were assayed blindly, using the above-mentioned training data set. All chemicals were provided by the Cosmetic Europe Skin Tolerance Task Force (STTF), which also kept the code for the blinded chemicals. All chemicals were stored according to the recommendations of the supplier. For details of the chemicals in the test set, see Table 1. In addition to the blinded chemicals of the test set, a set of non-blind benchmark controls were included. The purpose of the benchmark controls was to calibrate the prediction model to the current batch of cells, as described (Forreryd et al., 2016), and they are listed with chemical details in Table S1 (https://doi.org/10.14573/altex.1701121s). All chemicals used as benchmark controls were purchased from Sigma Aldrich (St. Louis, MO, USA), and were stored according to instructions by the manufacturer.

Cell maintenance, chemical stimulations, phenotypic analysis and total RNA isolation

All GARD protocols for cell maintenance, cellular stimulation with chemicals, required phenotypical quality control of cells prior to chemical stimulation, and isolation of total RNA have been previously described (Johansson et al., 2013; Johansson et al., 2011; Forreryd et al., 2016) and were followed without deviations in this study. The myeloid cell line used in this study was derived from MUTZ-3 (DSMZ, Braunschweig, Germany) and is available via SenzaGen AB (SenzaGen AB, Lund, Sweden). All cellular stimulations were performed in biological triplicates, using separate cell batches for each replicate. Following chemical stimulation, cells were harvested and lysed with TRizol reagent (Thermo Scientific, Waltham, MA), and stored at -20°C until RNA extraction. Total RNA was isolated from lysed samples using Direct-zol™ RNA MiniPrep column purification kit (Zymo research, Irvine, CA) according to protocols provided by manufacturer. Total RNA concentrations and RNA integrity were assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA was stored at -80°C until NanoString nCounter analysis.

Gene expression analysis using NanoString technology

The design of a custom NanoString CodeSet, corresponding to the GARD Prediction Signature (GPS), was recently described (Forreryd et al., 2016). All NanoString-associated protocols for gene expression analysis were performed according to instructions by the manufacturer. In short, the custom CodeSet was hybridized with 100 ng total RNA (5 µl at 20 ng/µl) and incubated at 65°C for 24h. Hybridized samples were processed in the NanoString GEN2 nCounter Prep Station 5s, using the High Sensitivity protocol, and analyzed in the NanoString Digital Analyzer 5s for digital quantification of each transcript of the GPS, using maximal resolution (555 Fields of View). All required equipment, CodeSet and master kit reagents were obtained from NanoString Technologies (NanoString Technologies, Seattle, WA).

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Data pre-processing, normalization and analysis

Raw nCounter gene expression data was imported into the R statistical environment (R Development Core Team, 2014), in which all downstream analysis was performed. Data was normalized using a Counts Per Total Counts (CPTC) algorithm, which reports normalized values for any given gene of the GPS as the ratio of digital counts for the specific gene and the total counts of all measured genes within that sample. Generation of prediction calls for each sample (sensitizer/non-sensitizers) was performed as previously described. Briefly, a support vector machine (SVM) (Cortes et al., 1995) was trained on the training dataset, and used to generate decision values (DVs) for each sample of the benchmark control dataset and test dataset, respectively. The predictive performance of the model was evaluated on the benchmark control dataset, using the additional R package ROCR (Sing et al., 2005). Observations of the Receiver Operating Characteristic (ROC) (Lasko et al., 2005) allowed the identification of the prediction model cutoff that achieves the highest accuracy of predictions of the benchmark control dataset, which was subsequently subtracted from all DVs generated from samples of the test dataset. Thus, final predictions were performed on calibrated DVs (cDVs). A specific chemical used for stimulation was classified as a sensitizer if the mean cDV from biological triplicates was greater than zero. The predictive performance of the model's classifications of the test dataset was assessed using Cooper statistics (Cooper et al., 1979).

3 Results

GARD classifications of the blinded CE-reference panel of chemicals

A set of blinded chemicals was classified as sensitizers or non-sensitizers by the GARD assay, using established protocols. GARD predictions of the chemicals used in this study are presented in Table 1. Calculations of various predictive performance parameters are presented in Table 2, based on Cooper statistics. For the purpose of binary predictions, a composite reference was defined to classify a sensitizer as a compound that is categorized as having a Human Potency (HP) (Basketter et al., 2014) of 1-4, or being categorized as HP 5, if it is also predicted as a sensitizer by the LLNA. This binary classification system perfectly correlates with the Global Harmonization System (GHS) / Classification for Labelling and Packaging (CLP) classifications. By this definition, based on the current data the accuracy, specificity and sensitivity of GARD, is 83%, 56% and 93%, respectively. Comparing GARD predictions strictly with either HP or LLNA, the concordance was estimated to be 81% and 76%, respectively. The mean magnitude of the cDVs are visualized in box-and-whisker plots in Figure 1, grouped according to their sensitizing potency as defined by the GHS/CLP system. The observed differences in mean cDVs indicate that the GARD predictions correlate with potency classifications.

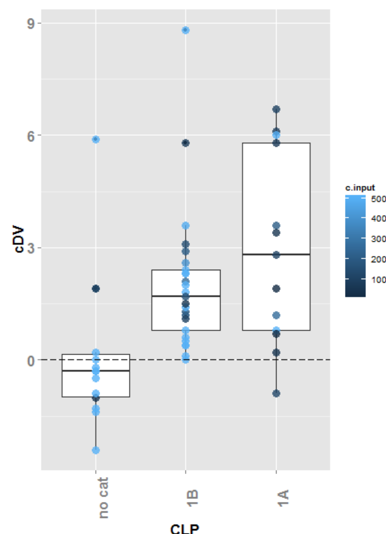

Fig. 1: GARD predictions correlate with potency classifications
Box-and-whisker plots of mean GARD cDVs, grouped by sensitizing potency as defined by the GHS/CLP classification system. Only test substances for which such classifications are available are included, see Table 1 (n chemicals = 52). The color of each data point is mapped to the GARD input concentration (µM) used for that test substance.

Tab. 1: Dataset details and test results

Chemical identifiers		**References[a]**			**Assay parameters[b]**				**GARD output**		
Substance ID	**CAS**	**LLNA**	**HP**	**GHS/CLP**	**vehicle**	**c.max**	**c.rv90**	**c.input**	**cDV (±std)**		**Prediction**
Sensitizers											
1,4-phenylenediamine	106-50-3	strong	1	1A	DMSO	500	70	70	5.8	± 0.6	sensitizer
Tetrachlorosalicylanilide	1154-59-2	extreme	1	-	DMSO	500	20	20	3.3	± 0.2	sensitizer
Dimethyl fumarate	624-49-7	strong	1	-	DMSO	500	-	90	5.9	± 0.4	sensitizer
2-aminophenol	95-55-6	strong	2	1A	DMSO	500	80	80	6.1	± 1.6	sensitizer
2-Nitro-1,4-phenylenediamine	5307-14-2	moderate	2	1A	DMSO	500	200	200	3.6	± 0.5	sensitizer
Formaldehyde (act.. 37%)	50-00-0	strong	2	1A	DMSO	500	260	260	1.2	± 0.5	sensitizer
Glutaraldehyde (act. 50%)	111-30-8	extreme	2	1A	DMSO	500	100	100	2.8	± 2.2	sensitizer
Methyl heptine carbonate	111-12-6	strong	2	1A	DMSO	500	50	50	0.2	± 0.8	sensitizer
Propyl gallate	121-79-9	strong	2	1A	DMSO	500	100	100	6.7	± 1.9	sensitizer
Toluene diamine sulphate	615-50-9	strong	2	-	DMSO	500	100	100	1.9	± 1	sensitizer
Glyoxal (act. 40%)	107-22-2	strong	2	1A	DMSO	500	-	500	0.8	± 1.1	sensitizer
Isoeugenol	97-54-1	moderate	2	1A	DMSO	500	500	500	6	± 0.4	sensitizer
1,2-Benzisothiazolin-3-one	2634-33-5	moderate	2	-	DMSO	500	12.5	12.5	1.6	± 0.7	sensitizer
3-dimethylaminopropylamine	109-55-7	moderate	2	-	DMSO	500	-	500	0.3	± 0.2	sensitizer
Thioglycerol	96-27-5	moderate	2	-	DMSO	500	-	500	-0.8	± 0.5	NS
Lyral	31906-04-4	weak	2	1B	DMSO	400	200	200	2.9	± 0.6	sensitizer
Chlorpromazine	50-53-3	moderate	3	1A	DMSO	100	10	10	1.9	± 0.9	sensitizer
Benzoyl peroxide	94-36-0	extreme	3	-	DMSO	500	85	85	-1.1	± 0.4	NS
Bisphenol A-diglycidyl ether	1675-54-3	moderate	3	1A	DMSO	200	50	50	3.4	± 1.9	sensitizer
Ethylene diamine	107-15-3	moderate	3	1B	DMSO	500	-	500	1.4	± 2.5	sensitizer
Glyceryl monothioglycolate	30618-84-9	moderate	3	1B	DMSO	500	200	200	1.2	± 1.5	sensitizer
Farnesol	4602-84-0	moderate	3	-	DMSO	500	-	500	2.1	± 0.9	sensitizer
Abietic acid	514-10-3	weak	3	1B	DMSO	200	-	200	1.3	± 1.3	sensitizer

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Butyl glycidyl ether	2426-08-6	weak	3	1B	DMSO	500	480	480	3.6	± 2.1	sensitizer
Cinnamic alcohol	104-54-1	weak	3	1B	DMSO	500	-	500	8.8	± 1	sensitizer
Citral	5392-40-5	moderate	3	1B	DMSO	500	80	80	5.8	± 0.7	sensitizer
Eugenol	97-53-0	weak	3	1B	DMSO	500	400	400	2.6	± 0.4	sensitizer
Imidazolidinyl urea	39236-46-9	weak	3	1B	dH2O	500	50	50	1.5	± 2.4	sensitizer
Penicillin G	61-33-6	weak	3	-	DMSO	500	-	500	-0.8	± 0.9	NS
5-methyl-2,3-hexanedione	13706-86-0	weak	3	-	DMSO	500	-	500	3.1	± 0.9	sensitizer
Coumarin	91-64-5	NS	3	-	DMSO	500	-	500	0.3	± 0.8	sensitizer
Hexyl salicylate	6259-76-3	strong	4	1A	DMSO	500	120	120	-0.9	± 0.1	NS
Iodopropynyl butylcarbamate	55406-53-6	strong	4	1A	DMSO	500	10	10	0.7	± 1.5	sensitizer
Neomycin sulphate	1405-10-3	NS	4	-	dH2O	500	-	500	0.7	± 2	sensitizer
Resorcinol	108-46-3	moderate	4	1B	dH2O	500	-	500	2	± 1.1	sensitizer
Amylcinnamyl alcohol	101-85-9	NS	4	1B	DMSO	500	260	260	2.1	± 1.3	sensitizer
Aniline	62-53-3	weak	4	1B	DMSO	500	-	500	0.4	± 2.2	sensitizer
Benzocaine	94-09-7	NS	4	1B	DMSO	500	-	500	0.8	± 1.7	sensitizer
Geraniol	106-24-1	weak	4	1B	DMSO	500	-	500	2.4	± 1.7	sensitizer
Lillial	80-54-6	weak	4	1B	DMSO	500	160	160	1.7	± 0.5	sensitizer
Linalool	78-70-6	weak	4	1B	DMSO	500	-	500	0.6	± 0.8	sensitizer
Amyl cinnamic aldehyde	122-40-7	weak	4	-	DMSO	500	110	110	5.3	± 1.2	sensitizer
Carvone	6485-40-1	weak	4	-	DMSO	500	-	500	2.3	± 0.7	sensitizer
Kanamycin	70560-51-9	NS	4	-	dH2O	200	-	200	0.2	± 1.2	sensitizer
Anethole	104-46-1	moderate	5	1B	DMSO	500	-	500	2.3	± 1.5	sensitizer
Anisyl alcohol	105-13-5	moderate	5	1B	DMSO	500	-	500	0.1	± 1.5	sensitizer
Benzyl salicylate	118-58-1	moderate	5	-	DMSO	500	200	200	0.6	± 1.4	sensitizer
Limonene	5989-27-5	weak	5	1B	DMSO	500	-	500	0	± 0.4	sensitizer
Hexyl cinnamic aldehyde	101-86-0	weak	5	1B	DMSO	500	100	100	1.1	± 0.8	sensitizer
Benzyl benzoate	120-51-4	weak	5	1B	DMSO	500	500	500	2.3	± 1.8	sensitizer
Citronellol	106-22-9	weak	5	1B	DMSO	500	-	500	1.8	± 0.7	sensitizer

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Diethanolamine	111-42-2	weak	5	1B	DMSO	500	-	500	0.5	± 0	sensitizer
Pentachlorophenol	87-86-5	weak	5	1B	DMSO	200	150	150	3.1	± 0.8	sensitizer
Pyridine	110-86-1	weak	5	1B	DMSO	500	-	500	0.4	± 0.2	sensitizer
Non-sensitizers											
Hydrocortisone	50-23-7	NS	5	no cat	DMSO	500	-	500	5.9	± 0.1	sensitizer
Isopropanol	67-63-0	NS	5	no cat	DMSO	500	-	500	-0.9	± 0.8	NS
Methyl salicylate	119-36-8	NS	5	no cat	DMSO	500	-	500	0.2	± 2.4	sensitizer
Phenoxyethanol	122-99-6	NS	5	no cat	DMSO	500	-	500	-0.3	± 1.3	NS
Propylene glycol	57-55-6	NS	5	no cat	DMSO	500	-	500	-1.3	± 0.7	NS
Triethanolamine	102-71-6	NS	5	-	DMSO	500	-	500	0.2	± 2.4	sensitizer
4-aminobenzoic acid	150-13-0	NS	5	no cat	DMSO	500	-	500	-1.4	± 0.8	NS
Benzaldehyde	100-52-7	NS	5	no cat	DMSO	500	-	500	0	± 1.2	NS
Propyl paraben	94-13-3	NS	5	-	DMSO	500	-	500	5.3	± 0.1	sensitizer
Vanillin	121-33-5	NS	5	no cat	DMSO	500	-	500	-2.4	± 0.9	NS
Dextran	9004-54-0	NS	6	no cat	DMSO	40	-	40	-1	± 0.5	NS
Glycerol/Glycerin	56-81-5	NS	6	no cat	DMSO	500	-	500	-0.5	± 0.8	NS
Octanoic acid	124-07-2	NS	6	no cat	DMSO	500	-	500	-0.2	± 1.1	NS
Phenol	108-95-2	NS	6	no cat	DMSO	500	-	500	-0.3	± 1.9	NS
Tocopherol	59-02-9	moderate	6	-	DMSO	100	-	100	0.7	± 1.7	sensitizer
Diethyl phthalate	84-66-2	NS	6	no cat	DMSO	500	-	500	1.9	± 1	sensitizer
Diethyl toluamide	134-62-3	NS	6	-	DMSO	500	-	500	1.3	± 0.4	sensitizer
Tween 80	9005-65-6	NS	6	no cat	DMSO	500	13	13	1.9	± 1.4	sensitizer

[a]LLNA- Local Lymph Node Assay (as listed in the CE STTF database). HP – Human Potency (as listed in Basketter et al., 2014). GHS/CLP – Global Harmonization System / Classification for Labelling and Packaging (as listed in Piroird et al., 2015).
[b]c.max – Maximum concentration of titration range (µM). rv90 – In-well concentration yielding 90% relative viability (µM). c.input – Concentration used for cell stimulation, derived from c.max and c.rv90 (µM). For details, see Johansson et al., 2013.

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Table 2. Cooper statistics of current data.

Characteristic	LLNA	HP[a]	Composite
Accuracy (%)	76	81	83
Sensitivity (%)	90	84	93
Specificity (%)	45	50	56

[a]Human Potency

Table 3. Accumulated predictive performance.

Dataset	Sensitivity		Specificity		Accuracy		Source
GARD in-house validation	89%	(17/19)	86%	(6/7)	88%	(23/26)	Johansson, 2014
Technology transfer and method optimization	94%	(16/17)	83%	(10/12)	90%	(26/29)	Forreryd, 2016
Current study	93%	(50/54)	56%	(10/18)	83%	(60/72)	-
Accumulated predictive performance	92%	(83/90)	70%	(26/37)	86%	(109/127)	-

Accumulated GARD performance parameters across historical datasets

In order to relate the current results to previously published figures of predictive performance, an update of accumulated Cooper statistics for independent GARD assessments across various datasets are presented in Table 3. Combined, the accuracy of GARD was calculated to 86%, including datasets that taken together comprises 127 chemicals.

4 Discussion

In the last decade, substantial efforts have been made to develop and validate alternative non-animal assays for assessment of chemical sensitizers, in order to meet changing regulatory and industrial demands. The current leading opinion is that no single assay is likely to provide sufficient information for accurate safety assessment of chemicals as a stand-alone test. This is a notion supported by the data generated by currently validated tests and the subsequent recommendations given by EURL ECVAM (EC, 2013, 2014, 2015). For this reason, it is of great importance to continuously compare and evaluate novel and already established test methods using coherent reference chemical panels, in order to prioritize assays that display superior functionality and predictivity, when designing IATAs, or in the quest for stand-alone tests.

In this report, we present novel data regarding the functionality and predictive performance of GARD, generated in a blind study performed in association with the CE STTF. In this independent dataset, GARD accurately classifies 83% out of a total of 72 chemicals for skin sensitization hazard. Adding this figure to previously published data from independent evaluation studies, GARD display an accumulated accuracy of 86%, based on classifications of 127 chemicals in total.

It is appropriate at this point to consider the gold standard of sensitization assessment, i.e. the reference by which such performance estimations are calculated. In this report, comparisons have been made to both LLNA classifications, and Human Potency (HP), as defined by Basketter et al. The concordances of GARD to these metrics were 76% and 81%, respectively. Of note, the concordance between LLNA and HP within the same data is 78%, clearly demonstrating that perfect correlation with either metric is mutually exclusive. In particular, the HP category 5 includes numerous compounds that have historically been classified as both sensitizers (e.g. hexyl cinnamic aldehyde) and non-sensitizers (e.g. isopropanol). For this reason, a composite reference was proposed for binary classifications, in which a sensitizer was defined to include HP categories 1-4, together with chemicals of the HP category 5, for which the LLNA classification was positive. Still, looking at the present data, we find that GARD misclassifications are overrepresented in HP category 5. Considering chemicals assigned within the HP categories 1-4, GARD accurately predicts 91% as sensitizers, while the corresponding accuracy within category 5 is 80%. Based on the reasoning above, it is logical to assume that the annotations provided as a reference may include errors based on flawed conclusions, as discussed (Basketter et al., 2014).

On a chemical by chemical basis, false GARD classifications were obtained for thioglycerol, benzoyl peroxide, penicillin G, hexyl salicylate (false negatives, HP category 1-4), hydrocortisone, methyl salicylate, triethanolamine, propyl paraben (false positives, HP category 5) and tocopherol, diethyl phthalate, diethyl toluamide and Tween 80 (false positives, HP category 6).

For the false negatives, the obvious common denominator is that a majority fails to induce any cytotoxic effect in the present cellular system. It should be noted, however, that reaching cytotoxic effects is not a requirement for a successful assessment of a sensitizer. Indeed, numerous examples of correctly classified sensitizers that do not induce cytotoxicity are available within this dataset. Correspondingly, toxic effects are not exclusively induced by sensitizers. It has previously been observed that non-toxic compounds are overrepresented among false negatives (Johansson et al., 2014). Furthermore, the connection between toxic or irritating effects and induction of sensitization has previously been discussed by other authors (Nukada et al., 2011). Thus, the overrepresentation of misclassifications among non-toxic sensitizers is a

problem shared with many cell-based assays. For false negatives that do induce cytotoxicity, no apparent explanation is available at this point.

The false positives among HP category 5 is, as discussed above, likely related to the ambiguous annotations provided by current gold standards. Indeed, the fact that they are listed within HP category 5 separates them from true non-sensitizers, at least by one metric, suggesting that observed LLNA classifications are non-concordant with the effects observed in the clinic. As an example, clinical cases of sensitization towards hydrocortisone are indeed not infrequent (Burden and Beck, 1992). Thus, the correctness of calling such substances non-sensitizers, and thereby concluding that GARD produces misclassifications, is certainly controversial.

Finally, false positives within HP category 6 include tocopherol, which is classified as a moderate sensitizer by the LLNA. Furthermore, diethyl phthalate and diethyl toluamide are both frequently classified as positives in cell-based assays (Ashikaga et al., 2010; Piroird et al., 2015), while Tween 80 is consistently classified as a sensitizer in numerous assays (Emter et al., 2013; Piroird et al., 2015; Ramirez et al., 2014). Indeed, the sensitizing capacity of Tween 80 has been closely examined and confirmed to be evident both before and after oxidation (Bergh et al., 1997). Consequently, the inherent difficulty to accurately assess these compounds should rather be regarded as general. Naturally, these aspects were a contributing factor to include such compounds in the blinded dataset used in this study, likely skewing the estimated specificity within the dataset towards lower figures compared to what would be expected in broader chemical domains.

During GARD development, it was observed that the relative magnitude of the GARD decision values correlates with sensitizing potency (Johansson et al., 2011), a hypothesis that has been maintained since. In light of the above discussed ambiguities regarding sensitizing potency, as estimated by current gold standards, GARD development towards potency assessment focuses on the distinction between strong and weak sensitizers in accordance with the GHS/CLP classification system. In Figure 1, the cDVs of the test substances are grouped according to this system. From the current data, it is clear that the hypothesis based on earlier observations prevails, since strong sensitizers (1A) on average generates higher DVs compared to weak sensitizers (1B). Furthermore, it is evident that the cytotoxicity of a chemical is also related to the sensitizing potency. In current GARD protocols, cytotoxic compounds are used at concentrations that induce 90% relative cell viability. From figure 1, it is evident that strong sensitizers (1A) are on average assayed at lower concentrations compared to weak sensitizers (1B), due to their higher levels of cytotoxic effects. While the GARD platform indeed holds information regarding sensitizing potency, there is an overlap between the different categories, which presently hampers its utilization for accurate potency assessment. However, the harnessing of accurate potency information is currently being refined for accurate sub-categorization (manuscript in preparation).

In conclusion, we here report data of GARD performance on an extended blinded set of chemicals. Taken together, GARD is consistently functional across datasets, with a predictive accuracy of 83% in this Cosmetics Europe dataset and average predictive accuracy of 86% in a combined dataset of 127 chemicals for skin sensitization hazard.

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Conflict of interest statement
The authors are employed or collaborate with SenzaGen, a company which commercializes the GARD test.

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