The GARD™skin Assay: Investigation of the Applicability Domain for Metals

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Abstract

New approach methodologies (NAMs) for hazard identification of skin sensitizing chemicals were adopted as test guidelines by the OECD during the last decade. These alternatives to animal models align to individual key events (KE) in the adverse outcome pathway (AOP) for skin sensitization for which the molecular initiating event (MIE) is covalent binding to proteins. As it currently stands, the AOP does not include mechanistic events of sensitization by metals, and limited information is available on whether NAMs accurately predict the sensitization potential of such molecules, which have been proposed to act via alternative mechanisms to organic chemicals. Methods for assessing the sensitization potential of metals would be valuable tools to support risk management within, e.g., occupational settings during production of new metal salts or within the medical device industry to evaluate leachables from metal alloys. This paper describes a systematic evaluation of the applicability domain of the GARD™skin assay for the assessment of metals. Hazard classifications were supplemented with an extended analysis of gene expression profiles induced by metal sensitizers to compare the induction of toxicity pathways between metals and organic sensitizers. Based on the results of this study, the accuracy, sensitivity, and specificity of GARD™skin for the prediction of skin sensitizing hazard were 92% (12/13), 100% (7/7), and 83% (5/6), respectively. Thus, the performance of GARD™skin for the assessment of metals was found to be similar to that observed for conventional organic substances, providing support for inclusion of metals within the applicability domain of the test method.

1 Introduction

Skin sensitizers are compounds that possess the intrinsic potential to induce an immunological hypersensitivity reaction in humans, which upon repeated topical exposure may result in the development of allergic contact dermatitis (ACD). The molecular mechanisms of skin sensitization induced by low-molecular-weight (LMW) chemicals have been reviewed in numerous publications (Kimber et al., 2011; Martin, 2015) and have been summarized in an adverse outcome pathway (AOP) by the OECD (OECD, 2014). Of particular importance, ACD develops in two phases, involving initial asymptomatic immunological priming of antigen-specific CD4+ Th1 cells and cytotoxic CD8+ T-cells resulting in the generation of an adaptive immunological memory, which upon subsequent re-exposure to the same antigen will give rise to a rapid clonal expansion and proliferation of effector cells responsible for driving the adverse skin reaction at the site of exposure (Kimber et al., 2011). Thus, in contrast to the reversible damage associated with skin irritation, which is independent of adaptive immune activation, acquired sensitization is generally irreversible and may result in elicitation of clinical symptoms upon each subsequent exposure; thus, it remains a common consumer and occupational health problem. In this context, proactive identification and evaluation of skin sensitizing potential are of central importance for safety evaluation of chemicals within the occupational setting and represents a key toxicological endpoint among regulatory authorities across multiple industries (Daniel et al., 2018; Strickland et al., 2019). Toxicological hazard assessments for skin sensitization have seen a fundamental change in direction during the last two decades, aiming to replace traditional animal models, such as the guinea pig-based assays (OECD TG 406) (OECD, 2021a) and the mouse-based local lymph node assay (LLNA) (OECD TG 429) (OECD, 2010) with mechanistically based and scientifically sound in vitro, in chemico, and in silico assays, collectively referred to as new approach methodologies (NAMs). To date, a total of nine such methods, which align with the individual key events (KE) of the AOP for skin sensitization (OECD, 2014),...
The development, validation, and inclusion of NAM-based strategies, and later also DAs, into OECD TGs was an important milestone for replacing animal models and generating the necessary trust among end-users that results will be accepted by relevant authorities. However, it is relevant to consider that the majority of all assays, both animal and non-animal based, were initially developed and validated using chemicals from a rather narrow subset of the potentially infinite chemical space (see, for example, the validation study reports for DPRA (EURL ECVAM, 2011) and h-CLAT (EURL ECVAM, 2012)). To better understand the applicability domain of individual methods, empirical data for diverse chemical classes must be generated to better understand limitations and provide guidance to end-users for the selection of the most appropriate method(s) depending on test chemical chemistry.

Of particular note, a chemical space for which little information exists to date in the scientific literature regarding the applicability of NAMs to predict the skin sensitization potential is that of inorganic molecules such as metals. The mechanisms underlying sensitization towards metals are generally not as well understood as those of organic compounds. The major immunological signals are similar and converge at T-cell activation, which occurs via interactions between T-cell receptors (TCR) and major histocompatibility complexes (MHC) in combination with the relay of secondary co-stimulatory signals (Kimber et al., 2011). However, while organic compounds form antigenic molecules by binding covalently to endogenous proteins, metals are believed to act via alternative processes that may be protein-independent (Riedel et al., 2021). For example, metals are not expected to form covalent bonds to proteins, and some compounds have been shown to possess the ability to circumvent the classical antigen processing pathways by interacting directly with peptide/MCH complexes or with TCR-peptide/MHC interfaces. The incomplete understanding of the sensitization mechanisms likely impedes inference regarding the applicability of NAMs to accurately assess such compounds. However, the limited information available for metals may be concerning, as a variety of metallic elements, including, e.g., nickel (Schuttelaar et al., 2018), cobalt (Makrilia et al., 2010), and palladium (Fauschou et al., 2011), have been associated with the potential to induce allergic reactions in humans. As metals may exist in various forms, including salts, organometallic compounds, and alloys, the potential chemical space and the risk from human exposure may be considerably underestimated.

In this work, the KE3-based Genomic Allergen Rapid Detection™ (GARD™skin) assay (Johansson et al., 2011), which was
recently adopted into OECD TG 442E as the first harmonized assay that generates and interprets genomic data for a regulatory endpoint, was utilized for assessing the skin sensitization properties of metals. For this purpose, a total of 13 compounds, including a variety of metal species and salt forms, predominantly with existing information on skin sensitization potential from human experience and/or animal testing, were evaluated. The results from this study suggest that the current applicability domain of GARD™ skin may be extended to cover metals, thus expanding the toxicologist’s toolbox to a non-animal-based approach capable of delivering reliable results for test materials associated with this chemical space.

2 Materials and methods

Chemicals

The following compounds were evaluated in the GARD™ skin assay: cisplatin, nickel (II) sulfate hexahydrate, palladium di(4-oxopent-2-en-2-oate), hydrogen hexahydroxplatinate, (trans) diamminedichloropalladium, cobalt chloride, potassium dichromate, potassium permanganate, and zinc sulfate, all of which were obtained from Sigma Aldrich (St Louis, Missouri), and diammonium hexachloroplatinate, tetraammine palladium (II) hydrogen carbonate, tetraammineplatinum (II) hydrogen carbonate, and a proprietary platinum salt, all of which were obtained from Johnson Matthey (London, UK). The proprietary platinum salt will remain coded and referred to as JM proprietary Pt salt throughout this manuscript. Table 1 provides details on the chemicals, including CAS number, molecular weight, linear formula, purity, and oxidation state.

GARD™ skin assay protocol and experimental conditions

All testing was performed at SenzaGen’s GLP-compliant laboratory (Lund, Sweden) and performed under GLP-like conditions. Experimental procedures were conducted according to the GARD™ skin assay protocol (EURL ECVAM, 2021) and in compliance with the GARD™ skin method of the OECD TG 442E for testing of single substances (OECD, 2022), with a minor adaptation considering the selection of vehicle for the cellular exposure experiments. For the purpose of this study, dimethylsulfoxide (DMSO) was not considered an appropriate vehicle due to concerns regarding the specific solvation chemistry, particularly with platinum group metal compounds. Platinum has a high affinity for sulfur, and solvation in DMSO results in ligand displacement and changes to the structure of the complex, potentially interfering with the toxicity profile of the parent molecule (Hall et al., 2014; IPA, 2017). Instead, alternative (inorganic) solvents such as Dulbecco’s phosphate buffered saline (DPBS, Cytiva), distilled water, and the cell culture medium (MEM-alpha, Active) were prioritized and evaluated. The solubility of each material was initially assessed by preparing a 10x stock-solution in cell culture medium, and or a 1000x stock solution in water or DPBS that was further diluted in cell culture medium to a default maximum in-well concentration of 500 μM. For test chemicals that were not soluble in any of the above solvents, less polar solvents not considered to have an impact on the chemical speciation, such as dimethyl formamide (DMF), were explored. A 1000x stock solution was prepared prior to dilution to a final in-well concentration of 0.1%. Table 2 provides a summary of the selected vehicles for each chemical in the study. The experimental vehicles were included as additional negative controls at corresponding in-well concentrations.

Following selection of appropriate vehicle, experimental procedures were performed as described in the GARD™ skin assay protocol (EURL ECVAM, 2021). In short, the human myeloid dendritic-like cell line SenzaCells™ (available from DSMZ, ACC 295) was exposed to a test chemical at a single concentration, referred to as the GARD input concentration, which was established based on the solubility or cytotoxicity profiles of the individual chemicals. Cells were exposed in three individual experiments to generate three independent biological replicate samples for each test chemical. Following 24 h incubation, cells were harvested and RNA isolated and quality controlled. Transcriptomic levels of the genes in the GARD Prediction Signature (GPS), whose identity has been transparently disclosed in several publications (see, e.g., Forreryd et al., 2016), were measured using NanoString nCounter technology. For final classifications, a support vector machine (SVM) algorithm (Cortes and Vapnik, 1995), trained and frozen during assay development as described in (Forreryd et al., 2016), was used to assign a decision value (DV) to each individual biological replicate of each test chemical. Final assignment of a test chemical as a skin sensitizer or non-sensitizer was strictly based on the mean DV from the biological replicate measurements, where a mean DV < 0 is classified as a non-sensitizer (UN GHS no category), and a mean DV ≥ 0 is classified as a skin sensitizer (UN GHS Category 1), without acknowledging borderline ranges, in full compliance with the GARD™ skin protocol applied during the formal validation study.

Exploratory data analysis

The induced gene expression profiles were explored using differential expression analysis, visualization of pairwise associations, and principal component analysis (PCA). Differential expression analysis was performed using limma version 3.52.0 (Ritchie et al., 2015; Phipson et al., 2016), which is a statistical framework for differential gene expression analysis based on linear regression models. It uses the empirical Bayes method for borrowing information across genes to stabilize performances when sample sizes are small. Designs were defined to model chemical-specific effects (each compound compared with unstimulated controls; n = 3 per group), and to model the effects induced by metal sensitizers and organic sensitizers (each group was compared with non-sensitizers; 7 metal sensitizers, 18 organic sensitizers, and 21 non-sensitizers). Genes identified with a false discovery rate below 0.05 were considered significantly differentially expressed between compared conditions.

For the correlation-based analysis, chemical-specific log fold changes were extracted from the differential expression analysis and Spearman correlation coefficients were estimated between every compound pair. The correlation coefficients were visualized using a heatmap created using the R-package pheatmap, version 1.0.12, where the visualized dendrograms were created using complete linkage clustering on Euclidean distances. Finally, the PCA was created on centered and scaled (mean = 0, standard deviation = 1) log fold changes. Matrix factorization was performed
3 Results

3.1 Assay compatibility for testing of metals

A pre-validation study was performed prior to initiation of the main GARD™skin study to evaluate the solubility and cytotoxicity profiles of the metal salts to ensure compatibility of the test materials with assay components. Two materials, palladium di(4-octopept-2-en-2-oate) and hydrogen hexahydroxy platinate, were insoluble in the stock solutions in the preferred vehicles in this study (cell culture medium and water). Alternative vehicles were explored to further increase final in-well concentrations for these materials. Based on results from the extended solubility testing, DMF was deemed the most appropriate solvent for palladium di(4-octopept-2-en-2-oate), resulting in a maximum in-well concentration of 60 µM. Hydrogen hexahydroxy Platinate was insoluble in the majority of evaluated solvents at the maximum tested concentration of 500 mM, including ethanol, acetone, and using singular value decomposition, and the principal components were calculated by multiplying the standardized variables by the right singular vectors.

| Chemical                                               | Reference classification | Reference source | Vehicle | In-well concentrations (µM) | GARD™skin classifications |
|--------------------------------------------------------|--------------------------|------------------|---------|----------------------------|----------------------------|
| Cisplatin                                              | S                        | Human           | Media   | 7                          | S                          |
| Cobalt chloride                                        | S                        | WoE             | Water   | 500                        | S                          |
| Diammonium hexachloroplatinate                         | S                        | WoE             | Media   | 80                         | S                          |
| Nickel (II) sulfate hexahydrate                        | S                        | Human           | Water   | 500                        | S                          |
| Palladium di(4-octopept-2-en-2-oate)                   | S                        | LLNA            | DMF     | 2                          | S                          |
| Potassium dichromate                                   | S                        | LLNA            | Water   | 1.5                        | S                          |
| Tetraammine palladium (II) hydrogen carbonate          | S                        | GPMT            | Media   | 450                        | S                          |
| (trans) Diamminedichloropalladium                      | NS                       | LLNA            | Media   | 50                         | S                          |
| Hydrogen hexahydroxy platinate                         | NS                       | WoE             | DPBS    | 100                        | NS                         |
| JM proprietary Pt salt                                | NS                       | LLNA            | Media   | 500                        | NS                         |
| Potassium permanganate                                 | NS                       | GPMT            | Water   | 500                        | NS                         |
| Tetraammineplatinum (II) hydrogen carbonate            | NS                       | GPMT            | Media   | 500                        | NS                         |
| Zinc sulfate                                           | NS                       | LLNA            | Water   | 500                        | NS                         |

Tab. 2: Summary of reference classifications and obtained GARD™skin classifications

Abbreviations: GARD™skin, Graphical Assessment of Respiration, Toxicity, and Irritation; LLNA, Local Lymph Node Assay; GPMT, guinea pig maximization test; S, skin sensitizer; NS, non-sensitizer; LC, local contact sensitization; SNIH, Skin irritation; SI, sensitization index; V, vehicle; PMF, percent maximal flare; ELISA, enzyme-linked immunosorbent assay; DPBS, Dulbecco’s phosphate-buffered saline; WoE, Where’s the Evidence; NICEATM, The NIEHS-NTP Center for the Evaluation of Chemicals in the Environment; OECD, Organization for Economic Co-operation and Development; GLP, good laboratory practice; DLC, dorsal application; WOE, weight of evidence.

a Human evidence associated with both skin sensitization (Type IV) and respiratory sensitization (Type I) mechanisms has been reported (Makrilia et al., 2010). Positive results reported in LLNA (Dearman et al., 2013). b Three studies reported in NICEATM LLNA Database (https://ntp.niehs.nih.gov/whatwestudy/niceatm/test-method-evaluations/skin-sens/llna/index.html). Two reported clearly positive classifications, with EC3 values of 0.4 and 0.8, respectively (Basketter and Scholes, 1992; Ikarashi et al., 1992), and one study reported a borderline positive result at 5% (SI = 2.8%; Mandervelt et al., 1997). Cobalt chloride is reported as a skin sensitizer in humans (Bauch et al., 2012). c Data from animal models and human experience available in the ECHA registration dossier suggest that daimonium hexachloroplatinate is a skin and respiratory sensitizer (ECHA, 2018). This is in line with available evidence indicating that chloroplatinates, such as hexachloroplatinate, are potent sensitizers in humans (Cleare et al., 1976). d Nickel (II) sulfate hexahydrate is a frequent sensitizer in humans and included in the European baseline series (EBS) of contact allergens, see for example (Schutteelaar et al., 2018; Uter et al., 2020). e No human data available. Classified as a skin sensitizer in a LLNA study performed in accordance with OECD TG 429 (ECHA, 2017a). No EC3 was calculated. Initial data were indicative of strong sensitizer. Full LLNA was not performed. f Classified as a skin sensitizer in LLNA (Basketter and Scholes, 1992) and a skin sensitizer in humans (human NOEL 111, Basketter et al., 2014). g No human data available. In the guinea pig maximization test (GPMT), conducted under GLP according to OECD TG 406, tetraammine palladium (II) hydrogen carbonate was classified as a skin sensitizer (ECHA, 2017b). h No human data available. Classified as a non-sensitizer in an LLNA study conducted under GLP according to OECD TG 429 when tested up to 25% (ECHA, 2017c). i No human data available. A modified LLNA study based on the protocols described in (Dearman et al., 2002) did not indicate any sensitization potential when tested at 40% and 80% (ECHA, 2017d). This is in line with an absence of protein reactivity reported in a study using a slightly modified protocol of the DPRA assay (Hemming et al., 2019). j JM proprietary Pt salt is classified as a non-sensitizer in an LLNA study performed in accordance with OECD TG 429 (personal communication with JM). k Classified as a non-sensitizer based on data from a GPMT study performed under GLP according to OECD TG 406 as reported in (ECHA, 2010), induction 1% intradermal injection, induction 10% topical application, and 0.1% for challenge. l Classified as a non-sensitizer based on literature assessment and absence of protein reactivity reported in a study using a slightly modified protocol of the DPRRA assay (Hemming et al., 2018). Classified as a non-sensitizer in Buehler and GPMT (ECHA, 2017e); challenged with 50% following a 2-week induction period involving three 6-h epicutaneous occlusive applications at 50%; challenged with 75% following a two-stage induction with 25% by intradermal injection and 75% by topical application. m Classified as a non-sensitizer in LLNA (Basketter et al., 1999). Tested up to 25%.
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Diamminedichloropalladium, and cobalt chloride, demonstrated low cytotoxicity, rendering RV90 values between 450-500 µM. Overall, based on the results from the pre-validation study, all test materials, except for hydrogen hexahydroxy platinate, were either freely soluble at the maximum default concentration or induced cytotoxicity at lower concentrations, complying with all acceptance criteria specified in the GARD™skin assay protocol. For hydrogen hexahydroxy platinate, the material was soluble at a maximum in-well concentration of 100 µM and did not induce cytotoxicity at any of the assessed concentrations. Nevertheless, previous studies in GARD™skin have demonstrated that most sensitizers, irrespective of their sensitizing potential, are detected at concentrations below 100 µM (Gradin et al., 2021). Therefore, despite lower solubility and no apparent cytotoxicity, hydrogen hexahydroxy platinate was also considered acceptable.

To conclude, based on the above presented results, evaluated metals were considered as compatible with the already established chemical preparation and cellular exposure protocols for organic chemicals and did not pose any apparent issues from a technical perspective.

3.2 GARD™skin classifications

Following the completion of the pre-validation study, which resulted in the selection of appropriate vehicles and the establishment of material-specific GARD input concentrations, downstream testing in the GARD™skin assay was performed in full compliance with

![Cellular cytotoxicity profiles induced by the test materials](image)

Fig. 1: Cellular cytotoxicity profiles induced by the test materials

All test materials were screened for cytotoxicity by exposing the SenzaCell cell line to a titrated range of concentrations, starting from the default concentration of 500 µM or the highest soluble concentration. The GARD input concentration for the main stimulations was determined based on the RV90 concentration, i.e., the concentration of a test material inducing 10% cell death compared to unstimulated control. The x-axis is in logarithmic scale.

DMF (data not shown). Instead, a less concentrated stock solution of 10 mM in DPBS was prepared, rendering a stable dispersion that could be diluted further in cell culture medium to a final in-well concentration of the test material of 100 µM (10% w/v DBPS). For all remaining test materials, no solubility issues were reported using the solvents indicated in Table 2, and they were fully soluble at a maximum in-well concentration of 500 µM, thereby adhering to standard GARD™skin protocols.

Following the solubility testing, cytotoxicity profiles were generated by examining the relative viability of cells exposed to the test materials within a titrated range of different concentrations, starting from the default maximum concentration of 500 µM or the highest soluble concentration. Results are illustrated in Figure 1, and the derived RV90 concentrations for the cytotoxic materials are summarized in Table 2. As shown in the figure, five of the materials, including JM proprietary Pt salt 1, tetraammineplatinum (II) hydrogen carbonate, hydrogen hexahydroxy platinate, potassium permanganate and zinc sulfate, did not induce sufficient cytotoxicity at tested concentrations to derive an RV90 concentration. Four of the test materials, including diammonium hexachloroplatinate, cisplatin, palladium di(4-oxopent-2-en-2-oate), and potassium dichromate were highly cytotoxic, rendering RV90 concentrations of 80 µM, 7 µM, 2 µM, and 1.5 µM, respectively. Remaining test materials, including tetraammine palladium (II) hydrogen carbonate, nickel (II) sulfate hexahydrate, (trans) diamminedichloropalladium, and cobalt chloride, demonstrated low cytotoxicity, rendering RV90 values between 450-500 µM.

Overall, based on the results from the pre-validation study, all test materials, except for hydrogen hexahydroxy platinate, were either freely soluble at the maximum default concentration or induced cytotoxicity at lower concentrations, complying with all acceptance criteria specified in the GARD™skin assay protocol. For hydrogen hexahydroxy platinate, the material was soluble at a maximum in-well concentration of 100 µM and did not induce cytotoxicity at any of the assessed concentrations. Nevertheless, previous studies in GARD™skin have demonstrated that most sensitizers, irrespective of their sensitizing potential, are detected at concentrations below 100 µM (Gradin et al., 2021). Therefore, despite lower solubility and no apparent cytotoxicity, hydrogen hexahydroxy platinate was also considered acceptable. To conclude, based on the above presented results, evaluated metals were considered as compatible with the already established chemical preparation and cellular exposure protocols for organic chemicals and did not pose any apparent issues from a technical perspective.
were 92% (12/13), 100% (7/7), and 83% (5/6), respectively, when compared with available reference data. The 95% confidence interval (95% CI) for the accuracy in this study, calculated with the Clopper-Pearson method, was (64.0%, 99.8%). Further, the p-value for evaluating the null hypothesis of an accuracy of 50% (H0: 0.5) was 0.0034, demonstrating statistically significant results.

A single non-concordant result was reported for the test material (trans) diamminedichloropalladium, which was classified as false positive in GARD™skin when compared with LLNA data as reported in an EU REACH registration dossier where it was tested at a maximum concentration of 25%. Furthermore, for the JM proprietary Pt salt, GARD™skin results are indicative of a borderline classification, with DVs on both sides of the binary classification threshold (DV: -1.8, 0.01, 0.59), albeit the final classification (based on mean DV) was negative. Available LLNA data (unpublished) for this compound was difficult to interpret, as a decrease in response with increasing dose over the whole dose range tested was observed. However, this Pt salt was found to elicit a cytokine fingerprinting profile consistent with respiratory sensitizing activity (selective Th2-type cytokine secretion) corresponding to that described previously as associated with several respiratory allergens (Dearman and Kimber, 2001). The only definitive conclusion to be drawn from the LLNA was

3.3 Comparison of GARD™skin classifications with available reference data

To determine the predictive value of GARD™skin for the subset of investigated metals, the binary hazard classifications from the assay were compared with existing reference data on skin sensitization potential, predominantly based on human experience but also results from animal testing or both. The concordance between GARD™skin classifications and reference data is summarized in Table 3, and further detailed for the individual test materials in Table 2, together with justifications and sources for the reference classifications. In total, based on the 13 metal-containing compounds evaluated in this study, the accuracy, sensitivity, and specificity of GARD™skin for prediction of skin sensitizing hazard were 92% (12/13), 100% (7/7), and 83% (5/6), respectively, when compared with available reference data. The 95% confidence interval (95% CI) for the accuracy in this study, calculated with the Clopper-Pearson method, was (64.0%, 99.8%). Further, the p-value for evaluating the null hypothesis of an accuracy of 50% (H0: 0.5) was 0.0034, demonstrating statistically significant results.

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that JM proprietary Pt salt is not a potent contact sensitizer in this test system, which corresponds well with the absence of allergic dermatitis cases in humans. Thus, based on the WoE, this substance was not classified as a skin sensitizer (JM internal data). The negative, albeit borderline, classification in GARD™skin aligns with this conclusion.

Based on available data, it was concluded that GARD™skin provided highly concordant classifications to the reference data, supporting the inclusion of metals into the applicability domain of the method.

3.4 Exploratory data analysis and mapping of toxicity pathways activated by metals

The transcripts in the biomarker signature have been associated with a variety of pathways of relevance to the sensitization process, such as innate immune recognition, oxidative stress, and dendritic cell activation (Johansson et al., 2011), and are thus “in line with mechanisms described under key events of the skin sensitization AOP” (Corsini et al., 2021). To gain further insight into possible similarities and differences in molecular mechanisms and toxicity pathways induced by metal sensitizers in comparison to organic sensitizers, the gene expression profiles of the subset of metals evaluated in this study were compared with gene expression profiles from the training dataset of GARD™skin, comprising mainly LMW organic chemicals (18 sensitizers, 20 non-sensitizers). Figure 3 shows a heatmap of Spearman correlation coefficients of pairwise comparisons between log fold changes for the metals investigated in this study compared to unstimulated control samples side-by-side with historical data from the GARD™skin training dataset. As expected, overall highest correlations were observed among samples with similar GARD™skin classifications, i.e., samples classified as either skin sensitizers or non-sensitizers, indicating a high similarity in the gene expression profiles driving classification outcomes. For the metals, some of the tested materials induced very similar gene expression profiles. Of special interest, (trans) diaminedichloropalladium, which was classified as a GARD™skin false positive in this study, demonstrated an overall high similarity to the sensitizers in the dataset, particularly to tetraammine palladium (II) hydrogen carbonate (Spearman = 0.93). Moreover, among the non-sensitizers, weak metal-specific correlation structures associated with the platinum species could also be observed, as evident by the slightly higher Spearman correlation coefficients for the pairwise comparisons of the JM proprietary Pt salt, hydrogen hexahydroxy platinate, and tetraammineplatinum (II) hydrogen carbonate, which indicate a slightly different gene expression activation pattern for these metals without affecting their overall correct classifications as non-sensitizers. Overall, comparing the induced gene expression profiles of the metals classified as skin sensitizers and the GARD™skin training data, no unique expression structures associated with the metals could be observed, indicating an overall similarity in the pathways driving the classifications as skin sensitizers for the metals and the organic chemicals for the investigated dataset. Interestingly, however, among the classified sensitizers, the metal sensitizers demonstrated highest concordance with the extreme or strong organic sensitizers in the GARD™skin training dataset.

To further investigate the gene expression induced by the metals in comparison with organic chemicals, a PCA analysis was performed. Figure 4A and B illustrate a PCA plot of the two first components for the metals in the GARD™skin training data and the metals analyzed in this study, using the gene expression from the GPS as variable input (n = 196). The metal sensitizer cobalt chloride gave rise to particularly strong effects on the genes, as evident by its distant position compared with other compounds in the figure. For the remaining compounds, a separation between sensitizers and non-sensitizers was observed along the first and the second component for both the training data and the metals. Metals classified as non-sensitizers clearly overlap with non-sensitizers in the training dataset, while metals classified as skin sensitizers, with the exception of the above-mentioned test material cobalt chloride, occupied a similar space as the training dataset, again demonstrating a high similarity in the gene expression profiles of metals and organic chemicals in this dataset.

Finally, a more detailed analysis of genes responsible for driving classifications as skin sensitizers was performed by comparing the subset of differentially expressed genes (DEGs) for the metal sensitizers and the organic sensitizers, respectively, versus non-sensitizers. Based on this analysis, no genes with completely different expression patterns were identified (i.e., up and down regulations). Furthermore, genes and signaling pathways responsible for driving the positive classifications of metal sensitizers overlapped with those previously demonstrated to be activated by organic chemical sensitizers, including nuclear factor erythroid 2-related factor (Nrf2) pathway activation (HMOX1, NQO1, TNFRD1, GSR, SLC3A2, RXRA, MGST3), upregulation of co-stimulatory molecules in DCs (CD86), regulation of toll-like receptor signaling pathway (TLR6, LY96, CD86, MAP2K1, MAPK13, TLR9), and activation of intracellular proinflammatory signaling pathways, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). A list of differentially expressed genes for the investigated metal sensitizers is available in Table S1.

![Image](https://example.com/image.png)

**Figure 4.**

**Legend:**

- PCA plot of the two first components for the metals in the GARD™skin training data and the metals analyzed in this study, using the gene expression from the GPS as variable input (n = 196).

**Table 3: Summary of GARD™skin assay concordance to reference data**

| Reference classifications | NS (6) | S (7) |
|--------------------------|--------|-------|
| GARD™skin classifications | NS      | 5     |
|                          | S       | 1     |
| Accuracy                 |         | 92.3% |
| Sensitivity              |         | 100%  |
| Specificity              |         | 83.3% |

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of information or be more informative than the LLNA. OECD TG 497 describes DAs for binary hazard classification and potency classification. They are deemed suitable for replacing currently used in vivo methods within a variety of chemical sectors and geographical regions (for an overview of the regulatory landscape, see Daniel et al., 2018 and Strickland et al., 2019). However, due to properties inherent to a specific test system, or the biomarkers governing the classifications, not all assays may be equally applicable for a specific chemistry. Thus, to provide con-

4 Discussion

During the last decade, several mechanistically based NAMs targeting individual KEs in the skin sensitization AOP were developed and incorporated into OECD TG 442C, D, and E for the prediction and classification of chemical skin sensitizers. Results from these assays, when combined into a WoE approach or a DA, can overcome some of the limitations associated with the individual assays and have been demonstrated to provide a similar level of information or be more informative than the LLNA. OECD TG 497 describes DAs for binary hazard classification and potency classification. They are deemed suitable for replacing currently used in vivo methods within a variety of chemical sectors and geographical regions (for an overview of the regulatory landscape, see Daniel et al., 2018 and Strickland et al., 2019). However, due to properties inherent to a specific test system, or the biomarkers governing the classifications, not all assays may be equally applicable for a specific chemistry. Thus, to provide con-

Fig. 3: Correlation heatmap comparing gene expression profiles induced by the metals in this study to historical GARD™ skin training data

The heatmap displays Spearman correlation coefficients for pairwise comparisons between induced log fold changes of the genes in the GPS (n = 196) for evaluated metals and historical data from the GARD™ skin training dataset, which comprises mainly LMW organic chemicals (18 sensitizers and 20 non-sensitizers). Materials are labeled based on dataset (metals in green, training set in orange) and sensitizing potential according to reference data (non-sensitizer in green, sensitizer in orange). The color of a tile reflects the Spearman correlation coefficient. The order of the samples was based on hierarchical clustering, as described by the dendrogram.
ception of nickel, which is a well-characterized false negative in the assay (Basketter et al., 1999; ICCV AM, 2010).

In the current study, the applicability of the GARD™ skin assay for hazard assessment of metal contact allergens was investigated by comparing binary hazard predictions with existing reference data on skin sensitization potential, predominantly based on human experience, results from animal testing, or both. In this context, it should be acknowledged that some commentators have highlighted the importance of applying a so-called triangular approach for establishing the predictive performance of NAM-based methods, including a comparison to both human and animal reference data, as well as a head-to-head comparison between human and animal data (Natsch et al., 2021). This strategy is indeed valid and provides a comprehensive assessment of the predictivity of the evaluated method while accounting for potentially conflicting reference data between humans and animals. However, it is imperative to consider that such a strategy is dependent on the availability of well-curated reference data from both human and animal tests. For example, for ethical reasons, human skin sensitization data obtained from human repeated insult patch testing (HRIPT) are rarely available for substances not explicitly designed for use in products in direct contact with human skin (e.g., cosmetics). For this reason, human data were available in the literature only for a limited number of the substances in this study (n = 5). The limited availability of human reference data is far from unique to the present study, and a recent example includes the assessment of agrochemical formu-

fidence in classification outcomes, careful characterization of the applicability domain of a given method is critical.

While certain subsets of the chemical space, such as hydrophobic substances and indirectly acting haptens, have been recognized to be difficult to accurately assess in one or several NAM-based methods (Mehling et al., 2019; Bergal et al., 2020; Forreryd et al., 2023), the lack of systematic evaluations and the limited availability of data for the testing of metals, metal salts, and organometallic compounds, has made it difficult to arrive at a solid conclusion on whether such compounds fall within the applicability of these methods.

The lack of data for metals may partly be explained by the perceived inability of the current NAM-based strategies to accurately assess such compounds, since metals “are known to react with proteins with mechanisms other than covalent binding” (OECD, 2014) and thus do not perfectly align to the current AOP-based testing paradigm for organic molecules for which the molecular initiating event (MIE) is covalent binding to endogenous proteins (OECD, 2014; Sullivan et al., 2017). In this regard, OECD TG 442C clearly states that methods under this TG are not applicable for the testing of metal compounds, since they are known to react with mechanisms other than covalent binding to proteins, while OECD TG 442D and 442E state that methods have been shown to be applicable to test chemicals covering a variety of organic functional groups but provide no specific guidance for metals or other inorganic molecules. In contrast, in vivo models such as the LLNA provide acceptable performance for metals, with the exception of nickel, which is a well-characterized false negative in the assay (Basketter et al., 1999; ICCV AM, 2010).

In the current study, the applicability of the GARD™ skin assay for hazard assessment of metal contact allergens was investigated by comparing binary hazard predictions with existing reference data on skin sensitization potential, predominantly based on human experience, results from animal testing, or both. In this context, it should be acknowledged that some commentators have highlighted the importance of applying a so-called triangular approach for establishing the predictive performance of NAM-based methods, including a comparison to both human and animal reference data, as well as a head-to-head comparison between human and animal data (Natsch et al., 2021). This strategy is indeed valid and provides a comprehensive assessment of the predictivity of the evaluated method while accounting for potentially conflicting reference data between humans and animals. However, it is imperative to consider that such a strategy is dependent on the availability of well-curated reference data from both human and animal tests. For example, for ethical reasons, human skin sensitization data obtained from human repeated insult patch testing (HRIPT) are rarely available for substances not explicitly designed for use in products in direct contact with human skin (e.g., cosmetics). For this reason, human data were available in the literature only for a limited number of the substances in this study (n = 5). The limited availability of human reference data is far from unique to the present study, and a recent example includes the assessment of agrochemical formu-

![Fig. 4: Gene expression induced by metals compared to LMW organic chemicals](image)

Principal component analysis (PCA) was used to compare gene expression profiles induced by the subset of metals to historical gene expression profiles from the training dataset of GARD™ skin, comprising mainly LMW organic chemicals (18 sensitizers and 20 non-sensitizers), using the genes in the GPS (n = 196) as variable input. Materials are colored according to sensitizing potential based on reference classifications (Sensitizers in orange, non-sensitizers in green). The metals evaluated in this study are encircled and labeled. (A) The Euclidean space has been zoomed to facilitate the visualization of the training data, excluding the test material cobalt chloride, which demonstrated a unique expression profile compared to other materials. (B) Visualization of the complete Euclidean space including the test material cobalt chloride.
The accuracy, sensitivity, and specificity of GARD™skin for the prediction of skin sensitizing hazard were 92% (12/13), 100% (7/7), and 83% (5/6), respectively, when compared with available reference data. Thus, the overall predictive performance of GARD™skin for hazard assessment of metals was similar to previously reported estimations of predictive performance for organic LMW chemicals, providing support for the inclusion of metals into the applicability domain of the test method. The reported performance metrics for GARD™skin can be considered in the context of results from other predictive tests. For the LLNA, a systematic evaluation of 13 metal salts demonstrated a balanced accuracy of 87%, with a false negative and a false positive classification reported for nickel chloride and copper chloride, respectively (Basketter et al., 1999). Furthermore, so zinc sulfate, which was correctly labeled as a non-sensitizer in this study, has been reported as a false positive in the LLNA (Gradin et al., 2021). Thus, based on the lack of systematic evaluations and the inconsistency in the reported results, currently available experimental data are not sufficient to arrive at a firm conclusion on whether inorganic molecules or metals can be included in the applicability domain of the above-mentioned assays.

While the work presented in this paper provides empirical evidence of accurate predictions of the GARD™skin assay for a variety of metals, it is important to proactively consider potential restrictions, if any, for the inclusion of metals into the applicability domain of the GARD™skin in a regulatory context. Firstly, from a technical perspective, the tested metals were directly compatible with assay components, enabling testing in accordance with the validated GARD™skin protocol, with only a minor adaptation considering the specific solvent used to dissolve the materials prior to cellular exposure. Precise understanding of metal solubility/bioelution and bioavailability is key to the potential for metal ions to have sensitizing hazard (IPA, 2017). In this context, it should be stressed that the use of alternative solvents to DMSO in this study was a deliberate choice based on the chemistry of the test materials. Platinum, for example, has a high affinity for sulfur and when dissolved in DMSO will result in ligand displacement and changes to the structure of the complex, potentially interfering with the toxicity profile of the parent molecule (Hall et al., 2014; IPA, 2017). Instead, water and cell culture medium were successfully used to dissolve the test materials to the maximum default concentration for all test materials, except for hydrogen hexahydroxy platinate and palladium di(4-oxopent-2-en-2-oate), where alternative approaches were required. Importantly, the highest applied concentration was limited by solubility for only a single test material, hydrogen hexahydroxy platinate. However, using an alternative vehicle (DPBS), a final in-well concentration of 100 μM could be obtained, which is within the concentration range where a signal from a potential sensitizer would be detected in the assay based on previously reported data (Gradin et al., 2021). Thus, using a combination of carefully selected standard and alternative vehicles, appropriate measures could be taken to not alter the chemical speciation or
the test materials while increasing the test concentrations above the limit of detection of the assay. In the context of the above discussion, it should be considered that OECD TG 442C, D, and E, support the use of alternative solvents/vehicles to those specified in the guideline when scientifically justified and encourage consideration of chemical stability of the test material in the selected solvent. Thus, the herein reported use of alternative solvents should not per se constitute an obstacle for direct inclusion of metals in the applicability domain of GARD™skin.

Secondly, it is relevant also to consider if the subset of metals in this study is sufficiently representative of the metal space. The rationale for the selection of test materials in this study was to include a variety of different metals for which there were available reference data in the literature for evaluation of assay performance. Details on references used for sensitization categorization of each material are summarized in Table 2. Nickel was a special case since it is a frequent cause of ACD in humans but still fails to induce a response in the LLNA, and the molecular mechanisms responsible for the observed discrepancy between the animal and human data are discussed in detail below. Furthermore, the study also included a variety platinum and palladium salts. These comprised a useful subset of compounds to investigate, since they include both skin sensitizers and non-sensitizers. Amongst the evaluated platinum compounds, diammonium hexachloroplatinate and cisplatin are recognized as skin sensitizers, based mainly on urticaria in workers and non-contact urticaria in patients, respectively, while hydrogen hexahydroxy platinate and JM proprietary Pt salt are considered non-sensitizers. All these compounds were correctly classified by GARD™skin. Similarly, palladium, tetraamine palladium (II) hydrogen carbonate, and palladium (I)(4-oxopent-2-en-2-oate) were correctly classified as skin sensitizers by GARD™skin, while the third palladium salt, (trans) diaminodichloropalladium, was non-concordant with available reference data, indicating a potential false positive response. Reasons for this single discrepancy in the dataset are currently unknown, however differences in dissolution of the metal ion in the GARD™skin assay medium versus the vehicles used in the LLNA may play a part. In summary, the results demonstrate the applicability of the GARD™skin assay to a variety of clinically important metals, such as nickel and cobalt, where the latter is a common constituent in a wide range of medical devices (Eichenbaum et al., 2021). Furthermore, the results demonstrate that the assay can provide valuable data to support risk management in the occupational setting during the production of new platinum compounds for use within the electronics or medical device industry and during manufacturing of platinum-based catalysts, as well as defining limitations on the product application areas. The number of test materials in the present study is similar to the 14 metals that were present in the LLNA database when ICCVAM published the LLNA Applicability Domain Evaluation Report in 2010, resulting in a recommendation to support the inclusion of metals, with the exception of nickel, in the applicability domain of LLNA (ICCVAM, 2010). The reported accuracy from that study, although excluding nickel from the calculations, was lower than the herein reported performance for GARD™skin. Therefore, the predictive performance, as well as the composition and size of the herein reported GARD™skin dataset may be considered sufficient for inclusion of metals into the applicability domain of the assay. The inclusion of metals into the applicability domain of GARD™skin may thus contribute to reducing the need for animal testing, serving as a scientifically justified stand-alone approach for non-regulatory testing during research projects and product development, but also as an information source within a WoE approach to comply with regulatory requirements. In such a regulatory context, GARD™skin was recently adopted into OECD TG 442E following the positive recommendation from the EURL ECVAM Scientific Advisory Committee (ESAC) (Corsini et al., 2021). Thus, depending on the regulatory context, positive classifications may be used as a stand-alone information source to identify skin sensitizers, while negative results have to be considered together with additional evidence. For example, to comply with information requirements in REACH, the latter can be accomplished either directly by providing supporting data from other KE in the AOP in a WoE approach, or by the subsequent adoption of the assay into any of the DAs described in the OECD TG 497 (ECHA, 2021).

Furthermore, in this context, it is also imperative to mention that the current protocol of the GARD™skin assay, similar to many other in vitro assays, is dependent on animal-derived serum (fetal calf serum, FCS) for cultivation of cells, but work is currently ongoing to adapt the assay to animal component-free cultivation protocols (manuscript in preparation).

Thirdly, in a regulatory context, available OECD TGs are KE-based and strictly align to the AOP for skin sensitization, which is currently considered to be applicable only to organic molecules, for which the molecular initiating event (MIE) is covalent binding to endogenous proteins (OECD, 2014). In this context, metals have largely been excluded from the applicability domain since they are considered to act via alternative mechanisms. For example, following the validation of the DPRA assay, on the basis of assumed understanding of the metal mechanisms, EURL ECVAM concluded that the assay was not designed for the identification of metal allergens (Casati et al., 2013; EURL ECVAM, 2012), which was later also incorporated in the OECD TG as a known limitation of the assay (OECD, 2021a). However, as described above, when challenged with a dataset of metals, a slightly modified version of the assay showed promising results (see Hemming et al., 2019), highlighting that inference on applicability domains should ultimately be based on a scientific method involving empirical data collection and hypothesis testing. Nonetheless, although the AOP, as it currently stands, is not assumed to fully reflect the skin’s immune response towards metals, some mechanisms may still overlap those initiated by organic chemicals, and thus it may still serve as a viable framework for designing testing strategies. In this context, it is interesting to recapitulate the current science by which sensitizing metals are known to induce allergic hypersensitivity reactions in humans, which has been excellently reviewed in (Riedel et al., 2021). First, the pathomechanism of metal allergy, similar to all adaptive immune responses, is dependent on at least two main signals, including both cell surface antigen presentation by DCs in the context of major histocompatibility complexes (MHC) to naïve allergen specific T-cells, as well as a co-stimulatory signal, or so-called danger signal (Matzinger, 1998; Schmidt et al., 2010). Considering the former, LMW chemicals must first bind to
endogenous proteins to generate an immunogenic hapten-protein complex (Landsteiner and Jacobs, 1936). The polarized nature of metals enables the acceptance of electrons from donor atoms such as amino acid side chains of appropriate proteins in the skin. However, in contrast to classical haptons, which bind covalently to proteins, the associated coordinate bond between the metal and the protein need not be irreversible, and it has been hypothesized that such bonds, in contrast to covalent bonds, may not be sufficiently stable to survive the intracellular processing mechanisms required for surface expression on MHC complexes (Chipinda et al., 2011). Interestingly, although the exact molecular mechanisms of the haptenation remain unknown for most metals, it has been demonstrated that nickel may bypass the conventional antigen processing pathways by direct binding to peptides loaded on the MHC complex from the extracellular space, or to conserved residues at the T-cell receptor – peptide-MHC interface to activate the T-cells in a protein independent manner (Riedel et al., 2021). Furthermore, for nickel, the co-stimulatory signal is delivered by direct interaction between the nickel ion and non-conserved histidine residues on human toll-like receptor 4 (TLR4) on DCs, resulting in the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and production of inflammatory cytokines (Schmidt et al., 2010). These non-conserved histidine residues are species-specific and not present on the equivalent receptor in mice, which effectively explains why nickel fails to provoke the necessary inflammatory signals to initiate and sustain an adaptive immune response in mice and subsequently gives rise to a false negative classification in the LLNA. Moreover, recent data has also indicated a similar TLR4 dependent activation for cobalt and platinum (Rachmawati et al., 2013). Although exact mechanisms remain to be explored for palladium, mono-sensitization is uncommon, and hypersensitivity to palladium is often observed together with nickel allergy, indicating cross reactivity and overlapping mechanisms (Faurschou et al., 2011). Altogether, this is interesting since it would suggest that sensitization to metals may be acquired also independently of the conventional protein binding mechanisms and that the direct interaction between metal ions and DCs may be sufficient to activate the inflammatory response driving the induction of downstream sensitization reactions. In this context, considering the central role for DCs in the activation of the immune sensitization cascades required for sensitization to metals, the relevance of DC-based methods for evaluation of the skin sensitization potential of metals is supported by available science on the molecular mechanisms behind metal allergy. This is further substantiated by the GARD™skin results in this study, which suggest that the response patterns for the metal compounds do not differ significantly from the response patterns of organic compounds, potentially allowing for accurate discrimination between sensitizers and non-sensitizers also for metals.

In conclusion, the GARD™skin assay was shown to be applicable for the successful hazard assessment of skin sensitizers in the chemical domain of metals. The evaluated metals were compatible with the assay protocols, and only a minor adaptation associated with the selection of vehicle was introduced to ensure sample stability. Furthermore, the predictive performance, the number of tested metals, and the current understanding of the pathomechanisms responsible for metal allergy together provide support for the inclusion of metals into the applicability domain of the GARD™skin assay. Regulatory acceptance and inclusion of metals into the OECD TG will be an important step to support risk management within occupational settings to define limitations on the product application areas for new metal salts as well as for the medical device industry, significantly reducing the need for animal testing within a variety of industries.

References
Basketter, D.A. and Scholes, E. W. (1992). Comparison of the local lymph node assay with the guinea-pig maximization test for the detection of a range of contact allergens. Food Chem Toxicol 30, 65-69. doi:10.1016/0278-6915(92)90138-b
Basketter, D. A., Lea, L. J., Cooper, K. J. et al. (1999). Identification of metal allergens in the local lymph node assay. Am J Contact Dermat 10, 207-212. doi:10.1053/ajcd1000207
Basketter, D. A., Alépée, N., Ashikaga, T. et al. (2014). Categorization of chemicals according to their relative human skin sensitizing potency. Dermatitis 25, 11-21. doi:10.1097/der.0000000000000003
Bauch, C., Kolle, S. N., Ramirez, T. et al. (2012). Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials. Regul Toxicol Pharmacol 63, 489-504. doi:10.1016/j.yrtph.2012.05.013
Bergal, M., Puginier, M., Gerbeix, C. et al. (2020). In vitro testing strategy for assessing the skin sensitizing potential of “difficult to test” cosmetic ingredients. Toxicol In Vitro 65, 104781. doi:10.1016/j.tiv.2020.104781
Casati, S., Griesinger, C. and Whelan, M. (2013). EURL EC- VAM Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing. EUR 26383. Luxembourg (Luxembourg): Publications Office of the European Union. JRC5936. doi:10.2788/48229
Chipinda, I., Hettick, J. M. and Siegel, P. D. (2011). Haptenation: Chemical reactivity and protein binding. J Allergy (Cairo) 2011, 839682. doi:10.1155/2011/839682
Cleare, M. J., Hughes, E. G., Jacoby, B. et al. (1976). Immediate (type I) allergic responses to platinum compounds. Clin Allergy 6, 183-195. doi:10.1111/j.1365-2222.1976.tb01897.x
Corsini, E., Clewell, R., Cotgreave, I. et al. (2021). ESAC Opinion on the Scientific Validity of the GARDskin and GARDpotency Test Methods. Asturiol Boill, D., Casati, S. and Viegas Barroso, J.F. (eds), Publications Office of the European Union, Luxembourg. ISBN 978-92-76-40345-6. doi:10.2760/626728
Cortes, C. and Vapnik, V. (1995). Support-vector networks. Mach Learn 20, 273-297. doi:10.1023/A:1002627411411
Daniel, A. B., Strickland, J., Allen, D. et al. (2018). International regulatory requirements for skin sensitization testing. Regul Toxicol Pharmacol 95, 52-65. doi:10.1016/j.yrtph.2018.03.003
Dearman, R. J. and Kimber, I. (2001). Cytokine fingerprinting and hazard assessment of chemical respiratory allergy. J Appl Toxicol 21, 153-163. doi:10.1002/jat.743
Dearman, R. J., Warbrick, E. V., Skinner, R. et al. (2002). Cytokine fingerprinting of chemical allergens: Species comparisons and statistical analyses. Food Chem Toxicol 40, 1881-1892. doi:10.1016/s0278-6915(02)00179-5
Dearman, R. J., Basketter, D. A. and Kimber, I. (2013). Inter-relationships between different classes of chemical allergens. J Appl Toxicol 33, 558-565. doi:10.1002/jat.1758

Dumont, C., Barroso, J., Matys, I. et al. (2016). 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. doi:10.1016/j.tiv.2016.04.008

ECHA – European Chemicals Agency (2010). Registration dossier Potassium permanganate. https://echa.europa.eu/de/registration-dossier/-/registered-dossier/14531/7/5/1 (accessed 13.02.2022)

ECHA (2017a). Registration dossier Palladium (II) di(4-oxopent-2-ene-2-oate). https://echa.europa.eu/de/registration-dossier/-/registered-dossier/18885/1/2 (accessed 13.02.2022)

ECHA (2017b). Registration dossier Tetraammine palladium (II) hydrogen carbonate. https://echa.europa.eu/sv/registration-dossier/-/registered-dossier/19204/7/5/1 (accessed 13.02.2022)

ECHA (2017c). Registration dossier Diamminedichloropalladium. https://echa.europa.eu/de/registration-dossier/-/registered-dossier/18770/7/5/1 (accessed 13.02.2022)

ECHA (2017d). Registration dossier Hydrogen hexahydroxy platinum. https://echa.europa.eu/sv/registration-dossier/-/registered-dossier/19739/7/5/1 (accessed 13.02.2022)

ECHA (2017e). Registration dossier Tetraammine platinum (II) hydrogen carbonate. https://echa.europa.eu/sv/registration-dossier/-/registered-dossier/20065/1/2 (accessed 13.02.2022)

ECHA (2018). Registration dossier Diammonium hexachloroplatinate. https://echa.europa.eu/sv/registration-dossier/-/registered-dossier/21776/7/5/1 (accessed 13.02.2022)

ECHA (2021). OECD Test guidelines skin sensitization. https://bit.ly/3uvyOts (accessed 01.06.2022)

Eichenbaum, G., Wilsey, J. T., Fessel, G. et al. (2018). Assessment of metal sensitizer potency with the reconstructed human epidermis IL-18 assay. Toxicology 393, 62-72. doi:10.1016/j.tox.2017.10.014

Gradin, R., Forreryd, A., Mattson, U. et al. (2021). Quantitative assessment of sensitizing potency using a dose-response adaptation of GARDskin. Sci Rep 11, 18904. doi:10.1038/s41598-021-98247-7

Hall, M. D., Telma, K. A., Chang, K. E. et al. (2014). Say no to DMSO: Dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res 74, 3913-3922. doi: 10.1158/0008-5472.can-14-0247

Hemming, J. D. C., Hosford, M. and Shafer, M. M. (2019). Application of the direct peptide reactivity assay (DPRA) to inorganic compounds: A case study of platinum species. Toxicol Res (Camb) 8, 802-814. doi:10.1039/c9tx00242a

ICCVAM (2010). ICCVAM Test Method Evaluation Report on Using the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products. N. I. o. E. H. Sciences. NIH Publication No. 10-7512. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Ikarashi, Y., Ohno, K., Tsuchiya, T. et al. (1992). Differences of draining lymph node cell proliferation among mice, rats and guinea pigs following exposure to metal allergens. Toxicology 76, 283-292. doi:10.1016/0300-483x(92)90196-I

IPA – International Platinum Group Metals Association (2017). Safe Use of Platinum Group Metals in the Workplace. https://ipa-news.de/assets/sustainability/IPA_Guidance/Chapter%201_PGM_Guide.pdf

Johansson, H., Lindstedt, M., Albrect, A. S. et al. (2011). A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. BMC Genomics 12, 399. doi:10.1186/1471-2164-12-399

Kimber, I., Basketter, D. A., Gerberick, G. F. et al. (2011). Chemical allergy: Translating biology into hazard characterization. Toxicol Sci 120, S238-268. doi:10.1093/toxsci/kfq346

Landsteiner, K. and Jacobs, J. (1936). Studies on the sensitization in medical devices: Implications for regulatory requirements in the European Union. Regul Toxicol Pharmacol 125, 105004. doi:10.1016/j.yrtph.2021.105004

Mehling, A., Adriaens. E., Casati, S. et al. (2019). In vitro RHE
skin sensitisation assays: Applicability to challenging substances. *Regul Toxicol Pharmacol* 108, 104473. doi:10.1016/j.yrtph.2019.104473

Natsch, A., Landsiedel, R. and Kolle, S. N. (2021). A triangular approach for the validation of new approach methods for skin sensitization. *ALTEX 38*, 669-677. doi:10.14573/altex.2105111

OECD (2010). Test No. 429: Skin Sensitisation: Local Lymph Node Assay. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264071100-en

OECD (2014). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. *OECD Series on Testing and Assessment, No. 168*. OECD Publishing, Paris. doi:10.1787/9789264221444-en

OECD (2018a). Test No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264229822-en

OECD (2021a). Test No. 406: Skin Sensitisation. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/97892640470660-en

OECD (2021b). Test No. 442C: In Chemico Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264229709-en

OECD (2021c). Guideline No. 497: Defined Approaches on Skin Sensitisation. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264264359-en

OECD (2022). Test No. 442E: In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264264359-en

Phipson, B., Lee, S., Majewski, I. J. et al. (2016). Robust hyperparameter estimation protects against hyervariable genes and improves power to detect differential expression. *Ann Appl Stat* 10, 946-963. doi:10.1214/16-AOAS920

Rachmawati, D., Bontkes, H. J., Verstege, M. I. et al. (2013). Transition metal sensing by Toll-like receptor-4: Next to nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact Dermatitis* 68, 331-338. doi:10.1111/cod.12042

Riedel, F., Aparicio-Soto, M., Curato, C. et al. (2021). Immunological mechanisms of metal allergies and the nickel-specific TCR-pMHC interface. *Int J Environ Res Public Health* 18, 10867. doi:10.3390/ijerph182010867

Ritchie, M. E., Phipson, B., Wu, D. et al. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47. doi:10.1093/nar/gkv007

Schmidt, M., Raghavan, B., Müller, V. et al. (2010). Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol* 11, 814-819. doi:10.1038/ni.1919

Schutteelar, M. L. A., Ofenloch, R. F., Bruze, M. et al. (2018). Prevalence of contact allergy to metals in the European general population with a focus on nickel and piercings: The EDEN fragrance study. *Contact Derm* 79, 1-9. doi:10.1111/cod.12983

Strickland, J., Daniel, A. B., Allen, D. et al. (2019). Skin sensitization testing needs and data uses by US regulatory and research agencies. *Arch Toxicol* 93, 273-291. doi:10.1007/s00204-018-2341-6

Strickland, J., Truax, J., Corvaro, M. et al. (2022). Application of defined approaches for skin sensitization to agrochemical products. *Front Toxicol* 4, 852856. doi:10.3389/fox.tox.2022.852856

Sullivan, K., Enoch, S., Ezendam, J. et al. (2017). An adverse outcome pathway for sensitization of the respiratory tract by low-molecular-weight chemicals: Building evidence to support the utility of in vitro and in silico methods in a regulatory context. *Appl In Vitro Toxicol* 3, 213-226. doi:10.1089/aivt.2017.0010

Urbisch, D., Mehling, A., Guth, K. et al. (2015). Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul Toxicol Pharmacol* 71, 337-351. doi:10.1016/j.yrtph.2014.12.008

Uter, W., Werfel, T., Lepoittevin, J. P. et al. (2020). Contact allergy-emerging allergens and public health impact. *Int J Environ Res Public Health* 17, 2404. doi:10.3390/ijerph17072404

Conflict of interest

Johnson Matthey authors have no financial interest regarding the GARD™ platform assays and their development or execution. SenzaGen AB is the method developer of GARD™ skin.

Data availability

Raw data were generated at the SenzaGen testing facility. Derived data supporting the findings of this study are available from the corresponding author AF on request. The identity of the proprietary Pt salt will not be disclosed.

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