



Full Length Article

Assessment of metal sensitizer potency with the reconstructed human epidermis IL-18 assay



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ABSTRACT

According to the new EU Medical Devices (MDR) legislation coming into effect in 2017, manufactures will have to comply with higher standards of quality and safety for medical devices in order to meet common safety concerns regarding such products. Metal alloys are extensively used in dentistry and medicine (e.g. orthopedic surgery and cardiology) even though clinical experience suggests that many metals are sensitizers.

The aim of this study was to further test the applicability domain of the in vitro reconstructed human epidermis (RhE) IL-18 assay developed to identify contact allergens and in doing so: i) determine whether different metal salts, representing leachables from metal alloys used in medical devices, could be correctly labelled and classified; and ii) assess the ability of different salts for the same metal to penetrate the skin stratum corneum. Twenty eight chemicals including 15 metal salts were topically exposed to RhE. Nickel, chrome, gold, palladium were each tested in two different salt forms, and titanium in 4 different salt forms. Metal salts were labelled (YES/NO) as sensitizer if a threshold of more than 5 fold IL18 release was reached. The in vitro estimation of expected sensitization induction level (potency) was assessed by interpolating in vitro EC50 and IL-18 SI2 with LLNA EC3 and human NOEL values from standard reference curves generated using DNCB (extreme) and benzocaine (weak). Metal salts, in contrast to other chemical sensitizers and with the exception of potassium dichromate (VI) and cobalt (II) chloride, were not identified as contact allergens since they only induced a small or no increase in IL-18 production. This finding was not related to a lack of stratum corneum skin penetration since EC50 values (decrease in metabolic activity; MTT assay) were obtained after topical RhE exposure to 8 of the 15 metal salts. For nickel, gold and palladium salts, differences in EC50 values between two salts for the same metal could not be attributed to differences in molarity or valency. For chrome salts the difference in EC50 values may be explained by different valencies (VI vs. III), but not by molarity. In general, metal salts were classified as weaker sensitizers than was indicated from in vivo LLNA EC3 and NOEL data. Our in vitro results show that metals are problematic chemicals to test, in line with the limited number of standardized human and animal studies, which are not currently considered adequate to predict systemic hypersensitivity or autoimmunity, and despite clinical experience, which clearly shows that many metals are indeed a risk to human health.

1. Introduction

Metals have been extensively used in medical devices for many years, in particular in dentistry and orthopedic surgery. Furthermore, metals are generously incorporated into jewelry and many consumer

products e.g. sunscreens, food, paint. According to the new EU Medical Devices (MDR) legislation coming into effect in 2017, manufactures will have to comply with higher standards of quality and safety for medical devices in order to meet common safety concerns regarding such products. The current recommendations for testing of almost all

Abbreviations: EC50, chemical concentration resulting in 50% decrease in cell viability; IL-18, Interleukin 18; SI2, 2 fold stimulation index; RhE, reconstructed human epidermis; VUmc, VU university medical center

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medical devices for cytotoxicity, irritation and sensitization are described in ISO 10993-1 which describes how to set up a testing strategy for the safety evaluation of medical devices. In particular, ISO 10993-10 describes tests for irritation and sensitization. With regards to testing sensitization potential of metal salts derived from medical devices, data from animal or human studies performed under standardized conditions is limited. Metals are problematic chemicals to test in the mouse local lymph node assay (LLNA) and in human studies using eg: DSA₀₅, NOEL, LOEL (see ICCVAM database (Basketter et al., 2014; Gerberick et al., 2005; Iccvam, 2011a,b) as ionization followed by binding to a hapten is a primary condition for activation of the human immune system. The correct labeling (sensitizer or not) and classification (potency) of a chemical is important in order to determine the maximum safe concentration for human exposure and to decide whether a less potent sensitizer can replace a more potent sensitizer without effecting the function of the metal alloy.

Despite the lack of robust data from animal and human studies, clinical experience does indicate that a number of metals may be contact sensitizers and may elicit a type IV delayed hypersensitivity reaction in the form of allergic contact dermatitis. Even though metal allergy prevalence in large cohorts is generally unknown (Schedle et al., 2007), there are estimates that up to 17% of women and 3% of men have allergies to nickel and that 1–3% are allergic to cobalt and chromium (Thyssen and Menne, 2010). Mercury, gold and palladium are typical metals used in dentistry that have also been indicated as contact sensitizers with palladium cross reacting with nickel (Gawkrödger, 2005; Muris et al., 2012). Furthermore, rare adverse reactions to titanium containing implants suggests that titanium may also be a sensitizing metal (Wood and Warshaw, 2015; Fage et al., 2016). Testing of potential sensitizers and clinical diagnostic testing for suspected contact allergy is traditionally carried out by applying the metal test chemical in the form of a salt to the skin of an animal or human under standardized conditions. Preferably the salt should dissolve to form metal ions. Metal salts representative of leachables detected in blood and urine are used to apply the ionized metal to the skin of the mouse or human. However, it is generally not taken into account that a number of different salts exist for each metal with different solubility, stratum corneum penetration and cytotoxic/irritant properties which may seriously confound the interpretation of the test results by giving false negative outcomes and under-estimations

Over the last few years, considerable energy has been invested in developing human in vitro methods to identify contact sensitizers. A number of these alternative test methods, e.g. DPRA (OECD-TG 442C), KeratinoSens™ (OECD-TG 442D) and h-CLAT, when incorporated into an Integrated Testing Strategy, are now able to replace animal models such as LLNA for hazard identification (Rovida et al., 2015; Strickland et al., 2016). During the Sens-it-iv Framework 6 project, we developed a reconstructed human epidermis (RhE) in vitro assay for not just identifying contact sensitizers but also for assessing sensitizer potency (Gibbs et al., 2013). RhE consist of proliferating and differentiating keratinocytes grown at the air-liquid interface. Since RhE are cultured exposed to the air, complete epidermal differentiation takes place with the formation of a stratum corneum thus enabling topical chemical application to take place in a similar manner to animal or human testing under standardized conditions. Keratinocytes play a key role in sensitization and activation of the immune responses as described in the Adverse Outcome Pathway for sensitization (Rovida et al., 2015). The differentiated epidermis controls chemical bioavailability via the stratum corneum and the underlying viable keratinocytes trigger, via the inflammasome and NF- κ B pathway, an inflammatory response in the form of (pro-) inflammatory cytokine release (Martin, 2015a). Among the many cytokines secreted by keratinocytes, IL-18 has been shown to play a key role in induction of allergic contact dermatitis (sensitization) by influencing the migration of Langerhans cells and dendritic cells to the draining lymph nodes, and in turn the presentation of the haptenized proteins to T cells (Antonopoulos et al., 2008;

Okamura et al., 1995). IL-18 has no apparent role in irritant contact dermatitis, indicating that the role of IL-18 in contact hypersensitivity is not simply part of a general requirement for IL-18 in skin inflammation (Antonopoulos et al., 2008). IL-18 plays a pivotal role in sensitization since it promotes a Th1- type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF α , CXCL8 and IFN γ (Okamura et al., 1995; Cumberbatch et al., 2001). Importantly, we have shown that IL-18 can now be used to identify contact sensitizers from respiratory sensitizers and non-sensitizers in an RhE in vitro assay (Gibbs et al., 2013; Andres et al., 2017; Galbiati et al., 2017). The RhE IL-18 assay could identify with 95% accuracy a panel of 17 contact sensitizers. Potency assessment correlated better with human DSA₀₅ data which assesses the induction dose per skin area that produces a positive response in 5% of the tested population than with LLNA data (Gibbs et al., 2013). The assay was extremely transferable from the in house VUmc model to commercially available RhE (Gibbs et al., 2013; Andres et al., 2017; Teunis et al., 2014). In the assay prediction model (Gibbs et al., 2013), depending on the RhE used, a chemical is labelled (YES/NO) as a sensitizer if, in 2 out of 3 independent runs, a threshold of ≥ 5 fold IL-18 release into the culture supernatant occurs in order to avoid irritants scoring as false positive. Potency, on the other hand which is related to the irritant potential of the chemical, is assessed by the chemical concentration resulting in 50% decrease in cell viability (EC50) or in 2 fold increase in IL-18 release (SI2). By definition therefore, potency is related to the irritant potential of the chemical and does not distinguish a sensitizer from a non-sensitizer: the stronger the sensitizer the lower the EC50 or IL-18 SI2 value will be (Dos Santos et al., 2011; Spiekstra et al., 2009). In line with this, the irritant capacity of chemicals has long been clinically recognized to represent an additional risk factor for sensitization induction (Agner et al., 2002; Basketter et al., 2007; Bonneville et al., 2007; Grabbe et al., 1996; Mclelland et al., 1991).

In this study, we expand on our recently published study that describes the use of the RhE assay to estimate the expected sensitization induction level by interpolating in vitro EC50 and IL-18 SI2 values to predict LLNA EC3 and/or human NOEL from standard curves generated using reference contact sensitizers (Galbiati et al., 2017). In order to test the sensitizing potential of metal salts as replacement for metal ions leaching from routinely used medical devices further, and to gain more insight into the mechanism by which different metal salts for the same metal may influence the read out of the current skin patch test, we tested a panel of 28 chemicals consisting of 17 metal salts, 8 non-metal sensitizers and 4 non-sensitizers (including zinc chloride) in the RhE IL-18 assay. For four metals (nickel, chrome, gold, palladium), the same metal was tested in two different salt forms, and titanium was tested in 4 different salt forms to investigate the influence of molarity, valency and cytotoxicity on the outcome of the assay.

2. Materials and methods

2.1. Reconstructed human epidermis

Healthy human neonatal foreskin was obtained after informed consent from patients undergoing routine surgical procedures. Skin was used anonymously and in accordance with the “Code for Proper Use of Human Tissue” as formulated by the Dutch Federation of Medical Scientific Societies (www.fmww.nl), and following procedures approved by the VU University medical center institutional review board.

VU University medical center in-house RhE (VUmc-RhE) were used in this study. RhE were constructed from human foreskin keratinocytes as described previously (Dos Santos et al., 2011; Spiekstra et al., 2009). In short, keratinocytes (passage 2) were seeded into a 12 mm diameter transwell (pore size of 0.4 mm; Corning, NY, USA) and grown submerged in medium containing DMEM/Hams F12 (3:1), 1% ultrosorG, 1 μ M hydrocortisone, 1 μ M isoproteronol, 0.1 μ M insulin and 1 ng/mL KGF for 1 week. Cultures were then lifted to the air-liquid interface and

Table 1
test chemicals and vehicles.

Chemical	CAS#	Vehicle	
Non-metal sensitizers			
Chemical tested			
Dinitrochlorobenzene	97–00-7	AOO	
4-nitrobenzylbromide	100–11-8	AOO	
Methyldibromo glutaronitrile	3591–65-7	AOO	
Cinnamaldehyde	104–55-2	AOO	
Resorcinol	108–46-3	AOO	
Eugenol	97–53-0	AOO	
Isoeugenol	97–54-1	AOO	
Benzocaine	94–09-7	AOO	
Metal sensitizers			
Chrome	Potassium dichromate (VI)	7778–50-9	Water
	Chromium (III) chloride	10025–73-7	Water
Nickel	Nickel (II) chloride hexahydrate	7791–20-0	Water
	Nickel (II) sulfate hexahydrate	10101–97-0	Water
Gold	Gold (I) chloride	10294–29-8	Water
	Sodium aurothiosulfate (I)	18497–75-1	Water
Cobalt	Cobalt (II) chloride	7646–79-9	Water
Mercury	Mercuric (II) chloride	7487–94-7	Water
Copper	Copper (II) sulfate	7758–98-7	Water
Unclassified metals			
Palladium	Sodium tetrachloropalladate (II)	13820–53-6	Water
	Palladium (II) chloride	7647–10-1	Water
Titanium	Titanium (IV) isopropoxide	546–68-9	AOO
	Titanium (IV) bis (ammonium lactato) dihydroxide solution	65104–06-5	Water
	Titanium (IV) oxide	13463–67-7	AOO
	Calcium titanate	12049–50-2	AOO
Non-sensitizers	Sodium dodecyl sulfate	151–21-3	Water
	Lactic Acid	50–21-5	Water
	Salicylic Acid	69–72-7	Water
Zinc	Zinc (II) chloride	7646–85-7	Water

The vehicles used in this study for dissolving chemicals before applying topically to RhE. AOO = acetone: olive oil (4:1); Water was distilled. Chemical suppliers were Sigma-Aldrich.

cultured for 4 days in keratinocyte culture medium (DMEM/Hams F12 (3:1), 0.2% ultrosor G, 1 μ M hydrocortisone, 1 μ M isoproterenol, 0.1 μ M insulin, 1.0×10^{-5} M l-carnitine, 1.0×10^{-2} M l-serine, and 2 ng/mL KGF). After another 4 days, 50 μ g/mL ascorbic acid was added and RhE were cultured for an additional 10 days. Unless otherwise stated, all additives were purchased from Sigma (St. Louis, MO). RhE were incubated at 37 °C, 7.5% CO₂ and medium was refreshed twice a week.

2.2. Chemicals and chemical exposure

A total of 28 chemicals were tested (Table 1). Non-metal chemicals were selected from the ICCVAM data base and consisted of 8 sensitizers of different potencies (extreme, strong, moderate, weak) and 3 non-sensitizers. To further explore the applicability domain of the RhE potency assay for testing metals used in medical devices, 5 metal sensitizers, 1 metal non-sensitizer and 2 non-classified metal sensitizers were studied. Furthermore 2 different metal salts were tested for chrome, nickel, gold and palladium, and 4 different metal salts were tested for titanium. RhE were topically exposed to chemicals as previously described (Gibbs et al., 2013). In brief, chemicals were dissolved in either acetone olive oil (AOO 4:1) or water. Finn Chamber patch test filter paper discs 11 mm (Epitest LTD Oy, Finland) were impregnated with 35 μ l of the chemical or vehicle (control) and applied topically to the RhE stratum corneum for 24 h. This method of application closely mimics diagnostic chemical administration during patch testing of patients with suspected allergy in our out-patient clinic. Chemical exposures were performed using a single dose response with 2-fold serial dilutions starting from 200 mg/ml or the maximum soluble concentration. After chemical exposure, filter paper discs were removed.

Metabolic activity was determined by MTT assay and culture supernatants were stored at –20 °C for analysis by ELISA.

2.3. Determination of RhE viability

The MTT assay measures mitochondrial activity, which is representative of cell viability. The MTT analysis was performed in 12-well plates, exactly as described in detail previously (Gibbs et al., 2013). In short, RhE were placed on top of 0.5 ml MTT (Sigma) dissolved in PBS (5 mg/ml) in a 12 well plate for 2–3 h. RhE were then transferred to a new 12-well plate and incubated overnight in the dark, at room temperature with 0.5 ml isopropanol (Merck). Next day, absorbance was measured at 570 nm with a Mithras LB 940 spectrophotometer. Results are expressed relative to vehicle.

2.4. Determination of IL-18 production

The amount of IL-18 present in culture supernatants was quantified using a commercially available sandwich ELISA according to the supplier's instructions (MBL, Nagoya, Japan) and exactly as described previously (Gibbs et al., 2013). Results are calculated in pg/mL from a standard curve and then converted to a Stimulation Index (SI) compared to vehicle exposed RhE.

2.5. Determination of interference of chemical with MTT assay or IL-18 ELISA

Chemicals which interfere with the readout of the MTT assay or IL-18 were excluded since they fall outside of the applicability domain of the assay.

MTT assay: the highest soluble chemical concentration was tested in the absence of RhE. If a colour change was observed then the chemical was excluded. In this way, calcium titanate was excluded from MTT/viability analysis.

IL-18 ELISA: a known amount of human recombinant IL-18 (500 pg/ml) was used to spike directly culture supernatant from RhE exposed to 200 mg/ml of the metals (the highest salt concentration used in the assay). If the salt penetrated the RhE reaching the culture supernatant and interfered with the IL-18 ELISA then the calculated IL-18 value obtained from the ELISA would differ from the spiked 500 pg/ml value indicating that the metal interfered with the ELISA and had to be excluded. None of the tested metals interfered with the ELISA and therefore all data was included.

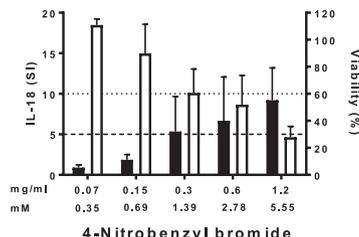
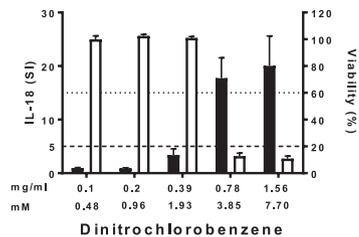
2.6. Data analysis and prediction model

Data analysis and prediction model were performed according to SOP (Gibbs et al., 2013; Teunis et al., 2014). Different RhE batches were used in each experimental run. Data represent at least two independent experiments. As results are incorporated into prediction models it is not feasible nor necessary to run statistics on each experiment. The following readouts were used:

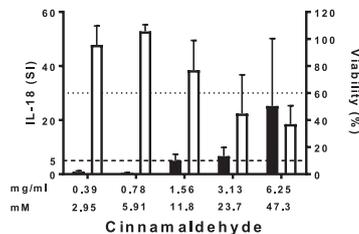
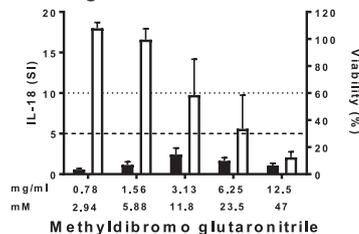
- Sensitizer prediction model (YES/NO): Chemicals were labelled as sensitizers according to the prediction model described previously which avoids irritants scoring as false positives: if 2/3 independent runs results in ≥ 5 fold increase in IL-18 secretion at RhE viability $\leq 40\%$ (60% cytotoxicity) compared to vehicle then the chemical scores as a sensitizer (H317) (Gibbs et al., 2013). (Cytotoxicity/membrane permeability is required in order to release intracellular IL-18 into the culture supernatant, thus making it not necessary to perform an additional tissue dissociation and extraction step). Note, the chemical concentration which results in ≥ 5 fold increase in IL-18 secretion may be different between runs due to batch and donor variation in response to the chemical.
- Sensitizer potency assessment, which correlates to irritant potential

Non-metal sensitizers

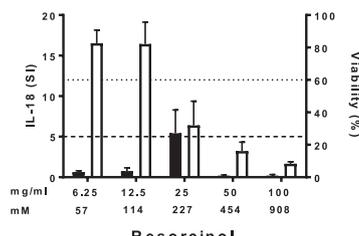
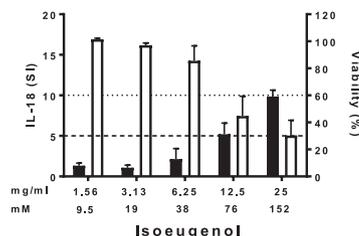
Extreme



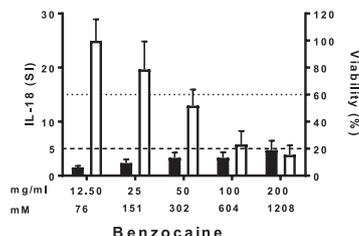
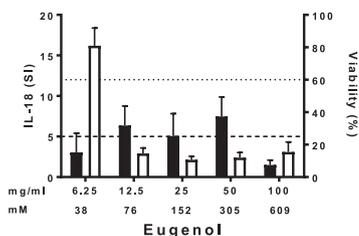
Strong



Moderate



Moderate / weak



Non sensitizers

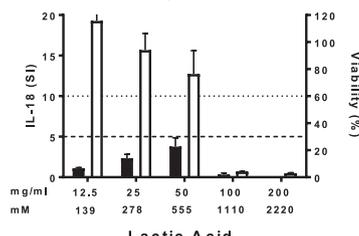
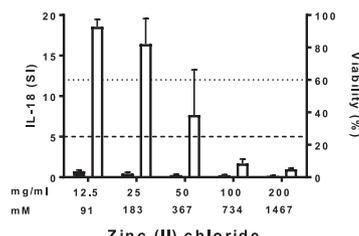
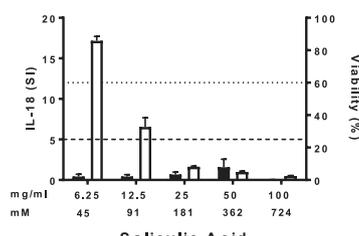
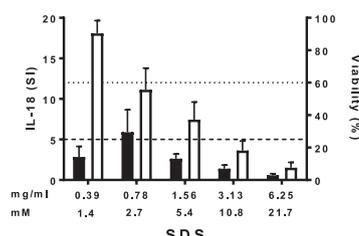


Fig. 1. Dose response effects of non metal sensitizers and non sensitizers on IL-18 release and cell viability in the VUmc in house RhE model. RhE were exposed to chemicals as described in Materials and Methods and Table 1. IL-18 (filled bars) was assessed by ELISA and results are expressed as stimulation index (SI) compared to vehicle alone. Cell viability (open bars) was assessed by MTT reduction assay and is expressed relative to vehicle. Results are expressed as average \pm SD of 3 independent runs. Both dotted line = 60% viability cut-off and dashed line = IL-18 SI5 cut-off are used in the chemical labeling (YES/NO) prediction model shown in Table 2 and described in Materials and Methods, section 2.6. Chemical classification is derived from Tables 1 and 3.

of the chemical: i) EC50 value is the chemical concentration required to reduce metabolic activity (corresponding to cell viability) to 50% of the value obtained by the vehicle (water or acetone/olive

oil 4:1). Values were obtained by linear regression analysis based on changes in metabolic activity (MTT). ii) IL-18 SI2 values were obtained by linear regression analysis based on the chemical

Table 2
Prediction model for chemical labeling.

Chemical	IL-18 SI ≥ 5 at \leq EC40			Positive repetitions	Classification	
	Repetition 1	Repetition 2	Repetition 3			
Non-metal sensitizers						
Dinitrochlorobenzene	10.2	28.27	22.41	3/3	Sensitizer	
4-Nitrobenzylbromide	17.47	1.79	13.01	2/3	Sensitizer	
Methyldibromo gluaronitrile	2.79	3.58	1.52	0/3	Non Sensitizer	
Cinnamaldehyde	12.55	6.02	22.81	3/3	Sensitizer	
Resorcinol	2.56	8.3	0.04	1/3	Non Sensitizer	
Eugenol	12.25	15.95	4.28	2/3	Sensitizer	
Isoeugenol	9.74	11.25	8.83	3/3	Sensitizer	
Benzocaine	3.32	9.76	6.10	2/3	Sensitizer	
Metal sensitizers						
Chrome	Potassium dichromate (VI)	10.9	9.56	6.67	3/3	Sensitizer
	Chromium (III) chloride	1.63	–	1.39	0/3	Non Sensitizer
Nickel	Nickel (II) chloride hexahydrate	2.98	3.39	4.61	0/3	Non Sensitizer
	Nickel (II) sulfate hexahydrate	–	–	–	0/3	Non Sensitizer
Gold	Gold (I) chloride	0.25	1.76	–	0/3	Non Sensitizer
	Sodium aurothiosulfate (I)	–	–	–	0/3	Non Sensitizer
Cobalt	Cobalt (II) chloride	5.63	8.6	6.38	3/3	Sensitizer
Mercury	Mercuric (II) chloride	–	–	–	0/3	Non Sensitizer
Copper	Copper (II) sulfate	–	–	nd	0/2	Non Sensitizer
Unclassified metals						
Palladium	Sodium tetrachloropalladate (II)	–	1.31	1.29	0/3	Non Sensitizer
	Palladium(II) chloride	–	–	–	0/3	Non Sensitizer
Titanium	Titanium (IV) isopropoxide	–	–	–	0/3	Non Sensitizer
	Titanium (IV) bis(ammonium lactato) dihydroxide solution	–	–	–	0/3	Non Sensitizer
	Titanium (IV) oxide	–	–	–	0/3	Non Sensitizer
	Calcium titanate	nd	nd	nd	0/0	Not classified
Non-sensitizers						
Zinc chloride (II)	0.15	0.45	0.18	0/3	Non Sensitizer	
Sodium dodecyl sulfate	2.90	11.36	2.28	1/3	Non Sensitizer	
Lactic Acid	0.67	–	0.29	0/3	Non Sensitizer	
Salicylic Acid	3.45	0.10	1.18	0/3	Non Sensitizer	

The prediction model states that if 2/3 independent runs results in ≥ 5 fold increase in IL-18 secretion at RhE viability $\leq 40\%$ compared to vehicle then the chemical scores as a sensitizer. The maximum IL-18 SI observed in the dose response at a cell viability $\leq 40\%$ relative to the vehicle is shown. Note the chemical concentration at which the maximum IL-18 SI occurs may differ between independent runs due to batch and donor variation in RhE (see detailed prediction model in Materials and Methods). Chemical concentrations in the dose response were 2 fold serial dilutions with highest concentration being 200 mg/ml (see Fig. 1). Correctly labelled chemicals are shown bold underlined.

(–) No values obtained at cell viability $\leq 40\%$ relative to the vehicle and/or IL-18 was below the detection limit of the ELISA. (nd) Not done.

concentration resulting in a 2 fold release of IL-18 into culture supernatants.

Interpolation prediction model: LLNA EC3 (%) and NOEL ($\mu\text{g}/\text{mL}$) were predicted from in vitro EC50 and IL-18 SI2 values using the slope of the standard curve obtained from reference chemicals DNCB (Basketter et al., 1997) and benzocaine (Van Och et al., 2000) and the following equation: $Y = \text{slope} \times X + \text{intercept}$ where $Y =$ predicted in vivo value and $X =$ in vitro EC50 or IL-18 SI2 value.

A binary prediction model was used in which EC50 or IL-18 SI2 $\geq 0.7\%$ = weak to moderate sensitizer and EC50 or IL-18 SI2 $< 0.7\%$ = strong to extreme sensitizer. Note, potency assessment does not distinguish a sensitizer from a non sensitizer since i) EC50 value is related to the irritant/cytotoxic potential of the chemical and ii) IL-18 SI2 value is below the threshold for labeling a chemical.

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA) and Microsoft excel.

3. Results

3.1. Chemical labeling (YES/NO)

Non-metal chemicals: dose response results used in the prediction model for the 8 non-metal sensitizers and 4 non-sensitizers are shown in Fig. 1 and values obtained for the 3 independent runs are shown in Table 2. All chemicals resulted in a dose dependent decrease in RhE

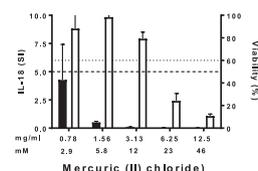
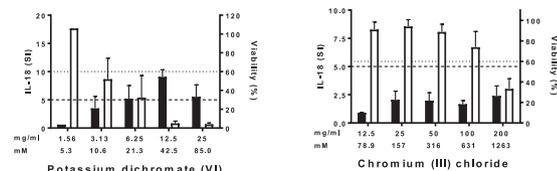
viability within the range of \leq EC40 thus enabling the YES/NO IL-18 SI5 prediction model to be implemented as previously described (Gibbs et al., 2013). Of the 8 non-metal sensitizers tested, 6 correctly scored as sensitizers showing a good correlation with the VUmC RhE and commercially available epiCS and EpiDerm models (Gibbs et al., 2013; Galbiati et al., 2017). Methyldibromo glutaronitrile and resorcinol, both chemicals not tested in the RhE assay before, scored false negatively as non-sensitizers. All 4 non-sensitizers were scored correctly, including the metal zinc chloride.

3.1.1. Metal sensitizers

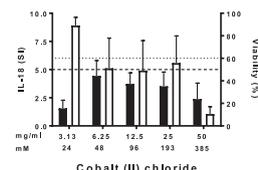
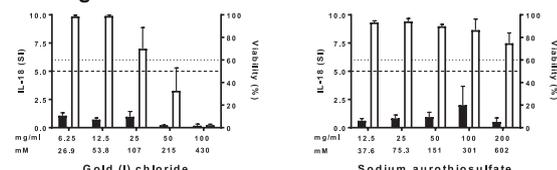
With regards to RhE viability, 6 of the 14 metal salts did not result in a decrease in RhE viability $\leq 40\%$ compared to vehicle exposed cultures and therefore did not fulfil the requirements for the prediction model (Fig. 2; Table 2). Calcium titanate interfered with the MTT assay and therefore was also excluded from the viability analyses. Therefore, a total of 7 metal salts could not be labelled since it was questionable whether they penetrated the stratum corneum and also cytotoxicity/membrane porosity is required for the release of intracellular IL-18 into culture supernatants. Indeed, the IL-18 SI5 was not reached for these 7 metals. Of the 8 remaining metal salts which did fulfil the requirements for the prediction model, very surprisingly only 2 resulted in ≥ 5 fold increase in IL-18 in 2/3 independent runs, thus scoring positive as sensitizers (Table 2). These metals were cobalt (II) chloride (run 1: 25 mg/ml, IL-18 SI = 5.63; run 2: 100 mg/ml, IL-18 SI = 8.6; run 3: 6.25 mg/ml, IL-18 SI = 6.38) and potassium dichromate (VI) (run 1: 12.5 mg/ml, IL-18 SI = 10.9; run 2: 12.5 mg/ml, IL-18 SI = 9.56; run

Metal sensitizers

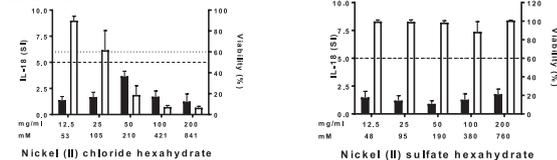
Extreme



Strong



Moderate



Unclassified

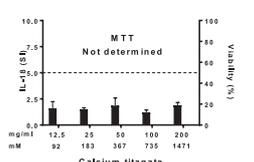
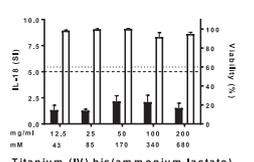
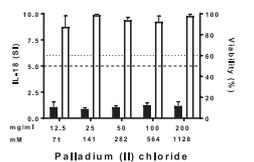
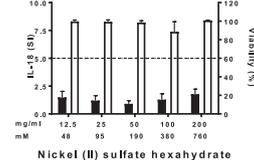
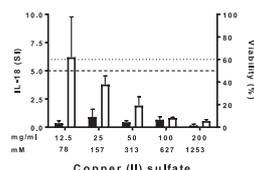
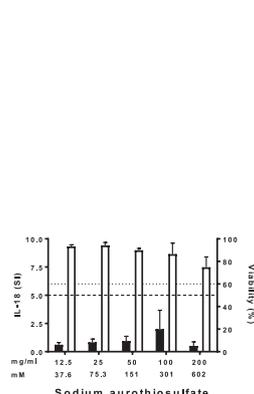
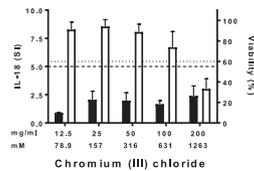
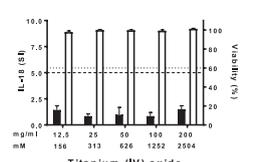
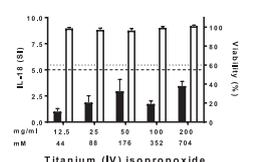
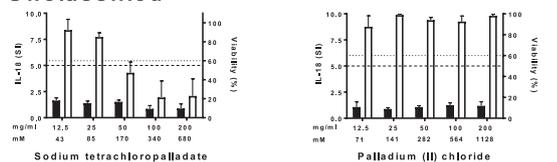


Fig. 2. Dose response effects of metal sensitizers and unclassified metal salts on IL-18 release and cell viability in the VUmc in house RhE model. RhE were exposed to chemicals as described in Materials and Methods and Table 1. IL-18 (filled bars) was assessed by ELISA and results are expressed as stimulation index (SI) compared to vehicle alone. Cell viability (open bars) was assessed by MTT reduction assay and is expressed relative to vehicle. Results are expressed as average \pm SD of 3 independent runs. Both dotted line = 60% viability cut-off and dashed line = IL-18 SI5 cut-off are used in the chemical labeling (YES/NO) prediction model shown in Table 2 and described in Materials and Methods, section 2.6. Chemical classification is derived from Tables 1 and 3.

3: 12.5 mg/ml, IL-18 SI = 6.67). Of note, chemical concentrations resulting in maximum IL-18 SI may differ between runs due to RhE batch and donor variation, and explains why the average of the values obtained for Cobalt (II) chloride in the 3 independent runs, when taken together, falls below SI5 as observed in Fig. 2. Notably, the other chrome salt tested in the assay, chromium (III) chloride, scored negative even though RhE viability decreased to \leq 40%. In summary, with the exception of cobalt (II) chloride and potassium dichromate (VI), IL-18 was detected consistently at low levels (no SI5 reached) in RhE culture supernatants exposed to metals resulting in a consistent negative score as non-sensitizer or not labelled (0/3 positive repetitions; Table 2).

Since very few metals resulted in an IL-18 SI5 from RhE required to give a label (YES/NO), it was next determined whether metal exposure could result in an IL-18 SI2 from RhE since this would enable sensitizer potency to be determined even if the label could not be established for metals in this assay (Gibbs et al., 2013). Note, an IL-18 SI2 in the VUmc RhE model will not distinguish a sensitizer for an irritant as this low threshold will result in false positives in line with sensitizer potency correlating to irritant potential of a chemical. For the non-metal sensitizers, all 8 chemicals resulted in an IL-18 SI2 in line with our previous publication. For the metals, only 5/16 metals salts (including zinc chloride) resulted in an IL-18 SI2 value in at least 2/3 runs.

3.2. Determination of chemical concentration which results in 50% decrease in RhE viability (EC50 value)

Previously we have shown that non-metal sensitizer potency could be accurately determined from the EC50 value obtained after chemical exposure (Gibbs et al., 2013; Teunis et al., 2014). For all 11 non-metal chemicals (sensitizers and non-sensitizers), an EC50 value could be obtained (Table 3). However, with regards to the metals, an EC50 value could only be obtained for 9/16 metal salts (including the non-sensitizer zinc chloride) (Fig. 2, Table 3). No EC50 value was obtained within the tested range of < 200 mg/ml for nickel (II) sulfate hexahydrate, sodium aurothiosulfate (I), palladium (II) chloride, or the titanium salts.

3.3. Comparison of different salts for same metal

When comparing salts for the same metal more closely it was observed that potassium dichromate (VI) and nickel (II) chloride had a lower EC50 and IL-18 SI2 than chrome (II) chloride and nickel (II) sulfate respectively; and sodium tetrachloropalladate (II) and gold (I) chloride had a lower EC50 value than their respective salts palladium (II) chloride, sodium aurothiosulfate (I) (Table 3). For nickel, gold and palladium, these differences could not be attributed to differences in molarity or valency since both salts for the same metal were of the same valency and one metal salt was clearly more cytotoxic than the other metal salt at a lower (rather than higher) molarity (Fig. 2). For chrome salts the difference may possibly be explained by the different valencies (VI vs. II), but not by molarity.

Table 3
Comparison of in vivo data with RhE EC₅₀ and IL-18 SI₂.

Chemical classification according to LLNA	Human Category scale	LLNA-EC3 (%) prediction			Human NOEL (µg/cm ²) prediction			EE EC50		EE IL-18 _{SI2}	
		In vivo	EC50	IL-18	In vivo	EC50	IL-18	%	µg/cm ²	%	µg/cm ²
Extreme < 0.1											
Dinitrochlorobenzene	1	0.006–0.13	0.08	0.06	8.8	8.8	8.8	0.06 ± 0.001	27.6 ± 0.45	0.03 ± 0.007	13.4 ± 3.12
4-Nitrobenzylbromide	ND	0.05	0.05	0.06	ND	8.8	8.8	0.05 ± 0.05	22.3 ± 22.3	0.03 ± 0.03	13.4 ± 13.4
Strong > 0.1 < 1											
Methyl dibromo gluaronitrile	2	0.9	1.42	1.60	ND	131	149	0.54 ± 0.37	241 ± 165	0.21 ± 0.05	93.6 ± 23.3
Cinnamaldehyde	2	0.2–3.1	1.00	1.00	200, 400, 591 ⁺	93	94	0.39 ± 0.34	174 ± 152	0.14 ± 0.15	62.4 ± 66.9
Moderate > 1 < 10											
Isoeugenol	2	0.5–5.0	3.62	6.31	69, 250	330	576	1.32 ± 0.55	589 ± 245	0.76 ± 0.05	339 ± 22.3
Resorcinol	4	5.5–6.3	6.24	7.59	ND	568	693	2.25 ± 0.99	1003 ± 441	0.91 ± 1.27	406 ± 566
Moderate - Weak > 1 < 100											
Eugenol	3	4.9–40.9	2.52	2.45	1938, 3200	231	226	0.93 ± 0.70	415 ± 312	0.31 ± 0.20	138 ± 89.2
Benzocaine	4	18,22,37	22	22	2000	2000	2000	7.85 ± 3.27	3500 ± 1458	2.59 ± 1.38	1155 ± 615
Metal sensitizers											
Extreme < 0.1											
Potassium dichromate (VI)	1	0.01–0.33	1.17	2.88	111	107	266	0.45 ± 0.38	199 ± 171	0.36 ± 0.34	161 ± 151
Mercuric (II) chloride	1	0.39	1.26	0.75	924	115	67	0.48 ± 0.05	214 ± 24.1	0.11 ± 0.08	46.8 ± 33.7
Strong > 0.1 < 1											
Gold (I) chloride	2	0.48	11.56	–	65	1053	–	4.15 ± 2.29	1850 ± 1022	NR	NR
Cobalt (II) chloride	2	0.4–0.8	4.69	5.12	ND	428	477	1.70 ± 2.01	758 ± 896	0.63 ± 0.34	282 ± 152
Copper (II) sulfate	ND	0.4 [#]	4.2	–	ND	389	–	1.55 ± 1.19	690 ± 532	NR	NR
Moderate > 1 < 10											
Nickel (II) sulfate hexahydrate	2	4.8–5.5	26.5	–	ND	2409	–	> 20.0	> 9000	NR	NR
Nickel (II) chloride hexahydrate	2	5.5	8.4	21.9	154	768	1991	3.03 ± 1.24	1352 ± 551	2.58 ± 1.21	1150 ± 539
Unclassified metal salts											
Sodium aurothiosulfate (I)	ND	ND	> 55	–	ND	> 500-0	–	> 20.0	> 9000	NR	NR
Chromium (III) chloride	ND	ND	33	–	ND	3057	–	11.99 ± 3.93	5344 ± 1754	NR	NR
Sodium tetrachloropalladate (II)	ND	ND	8.58	–	ND	780	–	3.08 ± 1.45	1374 ± 648	NR	NR
Palladium (II) chloride	ND	ND	> 55	–	ND	> 500-0	–	> 20.0	> 9000	NR	NR
Titanium (IV) isopropoxide	ND	ND	> 55	18	ND	> 500-0	1658	> 20.0	> 8917	2.15 ± 0.06	959 ± 25.0
Titanium (IV) bis(ammonium lactato) dihydroxide solution	ND	ND	> 55	–	ND	> 500-0	–	> 20.0	> 8917	NR	NR
Titanium (IV) oxide	ND	ND	> 55	–	ND	> 500-0	–	> 20.0	> 8917	NR	NR
Calcium titanate	ND	ND	–	17	ND	–	1520	nd	nd	1.97 ± 1.82	880 ± 811
Non sensitizers											
Zinc chloride (II)	6 [§]	NS			NS§			4.65 ± 2.5	2073 ± 1115	NR	NR
Sodium dodecyl sulfate	6	NS			NS			0.13 ± 0.06	58.0 ± 26.7	0.08 ± 0.04	35.7 ± 17.8
Lactic Acid	6	NS			NS			6.76 ± 1.14	3014 ± 508	2.39 ± 0.76	1066 ± 339
Salicylic Acid	6	NS			NS			1.06 ± 0.08	473 ± 35.7	NR	NR

The LLNA EC3 values are expressed as % and relative potency classification is reported. Potency classification is based on the mathematical estimation of the EC3 value (%) > 10 to < 100 are classified as weak, > 1 to < 10 moderate, > 0.1 to < 1 strong, < 0.1 extreme. For LLNA in vivo data, a range of values is shown which was obtained from the ICCVAM database and Gerberick et al., 2005 (2, 4).

Human Category Scale: 1 = Extensive evidence of contact allergy in relation to degree of exposure and size of exposed population; 2 = A frequent cause of contact allergy, but of less significance compared with induction of skin sensitization in a HRIPT category 1; 3 = A common cause of contact allergy, perhaps requiring higher exposure compared with category 2; 4 = Infrequent cause of contact allergy in relation to level of exposure; 5 = A rare cause of contact allergy except perhaps in special circumstances (1).

For NOEL in vivo data, references were obtained from Basketter et al., 2014 (1) and ICCVAM data base (4).

In vitro EC50 and IL-18 SI₂ values are the arithmetic means obtained from at least 2 independent experiments, and were calculated from RhE exposed to the chemicals as described in the Materials and Methods section. EC50 and IL-18 SI₂ values were converted from mg/ml to % and µg/cm² for comparisons with in vivo data. µg/cm² conversion: µg = 35 µl chemical x µg/1 µl; cm² = pi x r² = pi x 0.25 cm² = 0.785 (example DNCB EC50 (0.62 x 35)/0.785 = 27.6 µg/cm²). These values are used to predict in vitro EC50 and IL-18 SI₂ values correlating to NOEL and LLNA-EC3 values using reference chemicals Benzocaine (29) and DNCB (28) and regression analysis derived from Figure 3.

ND indicates no data available; NS non-sensitizer; NR not reached in at least 2 runs; * cobalt sulfate data presented; § zinc oxide data presented; # copper chloride presented.

3.4. Chemical potency: prediction of human NOEL and LLNA EC3 from in vitro EC50 value and IL-18 SI₂ values

Next the LLNA EC3 and human NOEL values were predicted with

the simple approach we recently described to estimate the in vivo sensitization induction level (Gibbs et al., 2013; Galbiati et al., 2017). Correlation curves were created with reference chemicals extreme strong DNCB and weak benzocaine using in vivo and in vitro data

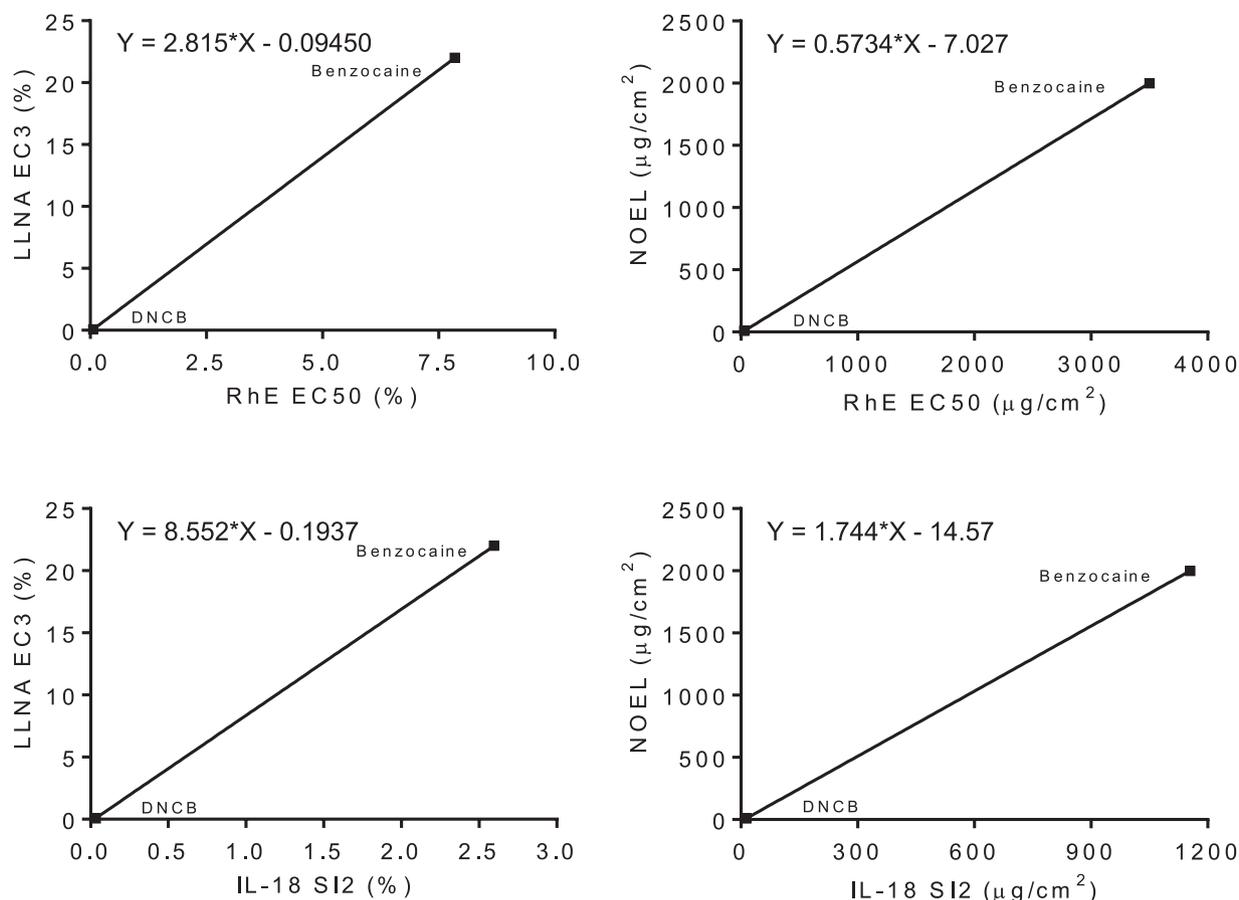


Fig. 3. Linear regression curves of RhE assay created with data from reference chemicals benzocaine and DNCB. Murine LLNA EC3 and human NOEL are plotted against in vitro RhE EC50 and IL-18 SI2 values using data for these 2 chemicals from Table 3. The regression curves were used to predict LLNA EC3 and NOEL (Y) values from in vitro (X) testing of chemicals shown in Table 3 using the equation $Y = \text{slope} \times X - \text{intercept}$ (shown top left of each graph).

(Fig. 3; Table 3). The predicted LLNA EC3 (%) and NOEL ($\mu\text{g}/\text{cm}^2$) values of the remaining 26 chemicals were then calculated from the linear regression curves using the corresponding EC50 and IL-18 SI2 values.

For the non metal sensitizers, the predicted LLNA EC3 and NOEL values were very close to actual values. Only methyldibromo guaronitrile fell slightly outside of its LLNA EC3 class but was within its human NOEL class. Of note, methyldibromo gluaronitrile LLNA EC3 classification was based on a single study reported in the literature (1). Also, eugenol fell outside of its NOEL class by being predicted as a stronger sensitizer than implied by actual NOEL data. However, it fell correctly into the lower range of available LLNA EC3 data predicting it as a moderate sensitizer. The human category score proposed by (Basketter et al., 2014) also correlated well with the non metal LLNA EC3, NOEL, EC50 and IL-18 SI2 data.

With regards to the metal salts, it should be emphasized that in vivo data was extremely scarce and often a single value from a single study is all that is reported (Table 3). In our study using the RhE prediction model, in general the known metal sensitizing salts were predicted as being weaker sensitizers than actual in vivo data indicated. Only predicted NOEL values for potassium dichromate correlated to actual values. Furthermore, all unclassified metal salts were predicted as extreme weak contact sensitizers.

3.5. Chemical potency according to RhE EC50 value $\geq 0.7\%$ representing a weak/moderate sensitizer and $\leq 0.7\%$ representing extreme/strong sensitizer

Having observed that the metals predicted a lower LLNA EC3 and

NOEL potency than the actual in vivo values we next determined the accuracy of predicting in vitro potency according to the prediction model: RhE EC50 value $\geq 0.7\%$ representing a weak/moderate sensitizer and $\leq 0.7\%$ representing extreme/strong sensitizer (Teunis et al., 2014). Chromium (III) chloride, sodium aurothiosulfate (I), the palladium salts and the titanium salts were not included in the following analysis since no LLNA data was available. EC50 and IL-18 SI2 values were obtained from 15 and 13 known sensitizing salts respectively and showed an overall accuracy of 83% and 94% respectively with the prediction model. With regards to the 8 non-metal sensitizers, the EC50 value had 100% accuracy and the IL-18 SI2 had 87.5% accuracy (outlier = eugenol) showing an extremely good correlation with LLNA data. The metal sensitizers also showed good correlation with LLNA data with EC50 and IL-18 SI-2 accuracy being 70% and 100% respectively (outliers = gold (I) chloride, cobalt (II) chloride and copper (II) sulfate). It was found that specificity (prediction of moderate/weak sensitizers) was higher than sensitivity (prediction of extreme/strong sensitizers) for the metal salts indicating again that metals, in general, were predicted with lower potency in the human RhE assay than in LLNA. The concordancy (same result scoring per chemical) between the EC50 and IL-18 SI2 prediction models was 85%.

Next, we proceeded to predict the potency of the unclassified metal salts (Table 4). Both palladium salts and all 4 titanium salts scored as extreme weak sensitizers/irritants (no YES/NO label; see Table 2). Furthermore, chromium (III) chloride and sodium aurothiosulphate (I), chemicals for which no LLNA data was available, scored as moderate/weak and extreme weak sensitizers respectively. Notably, when the alternative salts for these two metals were analyzed it was found that potassium dichromate (VI) scored as extreme/strong in line with LLNA

Table 4

Predictive capacity of RhE potency based on cut-off value for EC50 and/or IL-18 SI2 at < 0.7% for strong/extreme sensitizers and ≥ 0.7% for weak/moderate sensitizers.

Reference result for chemical according to LLNA	EE-EC50 prediction		IL-18 SI2 prediction	
	E/S < 0.7	M/W ≥ 0.7	E/S < 0.7	M/W ≥ 0.7
9 strong/extreme chemicals	6	3	7	0
5 metals	2	3	3	0
4 non metals	4	0	4	0
5 weak/moderate chemicals	0	6	1	5
2 metals	0	2	0	2
3 non metals	0	4	1	3
total number of chemicals	15		13	
metals	7		5	
non metals	8		8	
Sensitivity (%)	66.7		100	
Metals only	40		100	
non metals only	100		100	
Specificity (%)	100		87.5	
Metals only	100		100	
non metals only	100		75	
Accuracy (%)	83		93.7	
Metals only	70		100	
non metals only	100		87.5	
Concordance between EC50 and IL-18 SI2 values	11 of 13 chemicals tested = 85 %			
Metals only	4 of 5 chemicals tested = 80 %			
non metals only	7 of 8 chemicals tested = 88 %			

Prediction model for unclassified metal salts

Unclassified metal salts	EE-EC50 %	IL-18 SI2 %	E/S < 0.7	M/W ≥ 0.7	LLNA EC ₃ prediction
Chromium (III) chloride	> 20	NR	No	Yes	Extreme weak
Sodium aurothiosulfate (I)	12 ± 4	NR	No	Yes	Moderate/weak
Sodium tetrachloropalladate (II)	3.1 ± 1.5	NR	No	Yes	Moderate/weak
Palladium (II) chloride	> 20	NR	No	Yes	Extreme weak
Titanium (IV) isopropoxide	> 20	2.2 ± 0.6	No	Yes	Extreme weak
Titanium (IV) bis(ammonium lactato) dihydroxide solution	> 20	NR	No	Yes	Extreme weak
Titanium (IV) oxide	> 20	NR	No	Yes	Extreme weak
Calcium titanate	nd	2.0 ± 1.8	No	yes	Moderate/weak

Data are based on results obtained for the total number of chemicals sensitizers tested from which an EC50 value (15 chemicals) or IL-18 SI2 value (13 chemicals) could be obtained (see Table 3). No IL-18 SI2 value was obtained for gold (II) chloride or copper (II) sulfate and therefore the prediction model used 13 rather than 15 chemicals in the analysis.

E/S < 0.7 = extreme/strong sensitizer with EC50 cut-off value < 0.7 mg/ml M/W ≥ 0.7 = moderate/weak sensitizer with EC50 cut-off value of ≥ 0.7 mg/ml. Sensitivity = percentage of correctly identified strong/extreme sensitizers; specificity = percentage of correctly identified weak/moderate sensitizers; accuracy = average of sensitivity and specificity. Concordance is shown for 13 chemicals where both an EC50 value or IL-18 SI2 value was obtained. In brackets results are shown without inclusion of metals. For the non labelled metals (no human NOEL, DSA₀₅ or LLNA_{EC3} data available), potency prediction correlates to irritant or sensitizer potency. NR = IL-18 SI2 not reached in 2/3 runs. EC50 > 20% = extreme weak.

data, whereas gold (I) chloride scored as a moderate/weak sensitizer in contrast to LLNA data which scores gold as a strong sensitizer (data from one LLNA study only, see Table 3).

4. Discussion

Metal alloys are extensively used in dentistry and orthopedic surgery. This study had two aims: i) test the skin sensitizing potency of metals used in medical devices; and ii) gain insight into how different metal salts may influence the readout of the diagnostic patch test assay. Our results clearly show that metal ions entering the blood and urine which form from metal ions leaching from routinely used in medical devices are problematic chemicals to test in vitro, in line with the limited number of standardized human and animal studies described in the literature and despite clinical experience which clearly shows that many metals are indeed a risk to human health. Currently used animal models cannot reliably predict systemic hypersensitivity or autoimmunity (Descotes, 2006). Where, with systemic hypersensitivity reactions, we refer to the immune-mediated reactions that involve the entire body, and distinguish them from local (i.e. contact dermatitis) reactions. In line with this it is important to remember that the RhE IL-

18 assay only identifies a type IV hypersensitivity reaction and chemicals triggering humoral hypersensitivity responses (types II and III) are likely to be negative (Cumberbatch et al., 2001). Notably, metals (nickel, cobalt, chromium, palladium, gold) have been reported to induce a mixed Th1 and Th2-type cytokine response indicating that the immunological mechanism involved is mixed and not a true type IV response (Minang et al., 2006).

Skin irritation is the clinical result of an inflammatory response resulting mainly from the release of pro-inflammatory cytokines from epidermal cells (keratinocytes) in response to a chemical stimulus. The pathophysiological changes associated with skin irritation include the disruption of skin barrier, modification of skin cells, cytokine release and nerve ending changes. In order for a chemical to induce skin irritation, the chemical must reach the viable epidermis, therefore, penetration is needed. The in vitro skin irritation test using RhE is based precisely on this mechanism: absorption and damage (cytotoxicity) of keratinocytes, which parallel the release of pro-inflammatory mediators (i.e. IL-1 α) (see OECD test number 439). Intracellular accumulation of IL-18 after classical sensitizer exposure is a result of neosynthesis of pro-IL-18 via TLR activation and the NF- κ B pathway followed by posttranslational cleavage of pro IL-18 to IL-18 via the inflammasome

and caspase 1 (Martin, 2015a, 2015b; Galbiati et al., 2014). In our assay, IL-18 is then released when the cell membrane becomes porous due to cytotoxicity. Besides the low spontaneous release of IL-18 measurable in RhE, only contact sensitizers can induce IL-18 neo-synthesis, above threshold levels indicating that the chemical must reach the viable epidermis, otherwise the induction of IL-18 would not be possible. Our results indicate that exposure to metal salts, in contrast to other chemical sensitizers, only results in a small or no increase in IL-18 production in RhE. This finding was not related to a lack of chemical penetration since cytotoxicity (decrease in metabolic activity; MTT assay) was observed after topical RhE exposure to 8 of the 15 metal salts. Since most metal salts did result in some IL-18 production, the pathways leading to IL-18 neo-synthesis and posttranslational cleavage was not entirely obsolete. Also, even though we detected very low IL-18 release, it has recently been shown that nickel, cobalt and palladium can bind directly to TLR4 resulting in dimerization and without prior haptenization can trigger the secretion of inflammatory cytokines like CXCL8 from dendritic cells. For gold the response can be attributed to TLR3 binding (Rachmawati et al., 2015, 2013; Raghavan et al., 2012). Our finding that potassium dichromate (VI) results in much more IL-18 production than chromium (III) chloride is in line with literature which reports hexavalent chromium induced ROS production and NF- κ B release from keratinocytes (Wang et al., 2010) and hexavalent chromium acting as a pro-hapten and penetrating the skin where it is then reduced enzymatically to trivalent chromium (Burrows, 1984). It is therefore possible that our results can be explained by the inability of trivalent chromium to penetrate the stratum corneum and activate TLRs. However, clearly the molecular pathways resulting in the generalized low IL-18 production by metal salts and the significance of this with respect to sensitization needs further investigation.

Using traditional human diagnostic skin patch testing techniques, the number of patients showing false negative reactions to metals is suspected as being very high. To improve patch test diagnostics, the correct label and classification of different metal salts needs to be defined so that optimal test panels can be selected. The outcome of the traditional skin patch test is important in deciding the treatment regime and whether or not the implant requires removal. Since skin patch testing is accompanied with the risk of sensitizing the individual, physiologically relevant human in vitro methods are preferred for identifying relevant metal salts for use in improved diagnostics. Here we show that the RhE IL-18 assay can be used to determine metal salt penetration and (irritant) potency and therefore identify metal salts suitable for patch testing. Since we could not label salts which may give a positive patch test due to insufficient IL-18 release, a combined test with e.g. a dendritic cell maturation assay (with CXCL8 secretion as readout) may be considered (Rachmawati et al., 2015; Toebak et al., 2006). Previously we have shown that sodium tetrachloropalladate is a superior diagnostic salt for use in patch testing compared to palladium chloride (Muris et al., 2012). Using RhE we can now explain this finding since we show that sodium tetrachloropalladate could penetrate the stratum corneum, as indicated by an EC50 being obtained, whereas palladium chloride cannot. We also show that gold (I) chloride readily penetrates the stratum corneum whereas sodium aurothiosulphate (I) does not in line with the absence of LLNA and human data for the later, and neither salts were able to increase IL-18 production. However, it has been described that gold (I) needs to be oxidized to gold (III) to become antigenic and therefore a gold (III) salt may be preferred for patch testing in the future (Schuhmann et al., 1990; Goebel et al., 1995). Notably, none of the 4 tested titanium salts resulted in a decrease in metabolic activity of RhE indicating that these salts do not penetrate the stratum corneum and therefore are not optimal salts for diagnostic patch testing of suspected titanium allergy.

In summary, we have expanded on the panel of chemicals which have been tested with the RhE IL-18 assay. We clearly show that metal salts fall outside of the applicability domain of the assay due to insufficient amounts of IL-18 being released and low cytotoxicity. The

reason for this is currently unknown, however, it is important to remember that only chemical-inducing type IV hypersensitivity reactions (i.e. allergic contact dermatitis) will be positive in the RhE IL-18 assay. Therefore, it should not be surprising if metals inducing primarily humoral immune responses will be negative. With regards to chemical potency testing, metal salts, with the exception of potassium dichromate (VI) and mercuric (II) chloride all scored as moderate/weak sensitizers. Since for a number of these metals this prediction was lower than expected, it may reflect the poor ability of the metal salts to penetrate the stratum corneum. Notably, all metal salts, for which no in vivo data was available, scored as weak sensitizers in the RhE IL-18 assay.

Conflict of interest

None.

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