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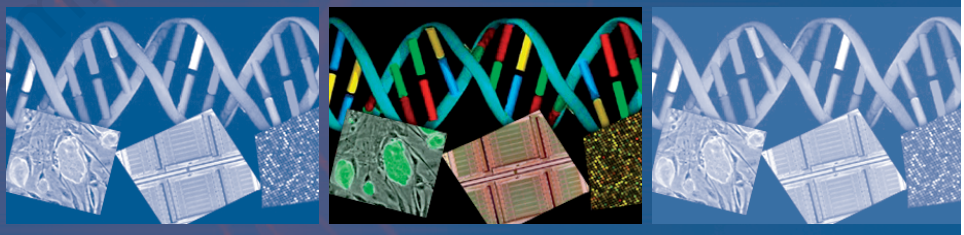



## Innovative approaches in cosmetic testing, in compliance with European regulations

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**Valeria Chiono** has a Master Degree cum laude in Chemical Engineering and a PhD in Chemical and Materials Engineering from the University of Pisa, Italy. She is currently Full Professor at the Department of Mechanical and Aerospace Engineering of Politecnico di Torino, Italy. Her research is aimed at the design of innovative bioengineering approaches to solve key problems in regenerative medicine and nanomedicine, and includes the development of bioactive materials and interfaces, tissue engineering, materials characterization, *in vitro* tissue models, drug delivery and non-viral gene therapy. One main research topic is cardiac tissue engineering. She has been the coordinator of several research projects, including STARIGEN FIRB2010 project, financed by the Italian Ministry of Education, University and Research, on the preparation of biomimetic scaffolds for cardiac regeneration. In 2017 she has been granted the ERC Consolidator project BIORECAR (contract number: 772168; http://www.biorecar.polito.it/) on advanced strategies for cardiac regeneration by direct reprogramming of fibroblasts into cardiomyocytes by an injectable hydrogel releasing miRNA-loaded nanoparticles. In BIORECAR *in vitro* models of fibrotic human cardiac tissue are also developed for preclinical investigation. Furthermore, she manages BIORECAR Laboratory at Politecnico di Torino. She is the main lecturer of the following courses at the Faculty of Biomedical Engineering at Politecnico di Torino: "Engineering for regenerative medicine", "Cell and tissue engineering" and "Laboratory of Tissues and Physiological Processes' Models". In 2021, she has been appointed Deputy-Director of Centro3R. She is author of 123 scientific publications (H-index: 33 Scopus, 38 Google Scholar) and 5 patents in the field of biomaterials, tissue engineering and nanomedicine.

# Innovative approaches in cosmetic testing, in compliance with European regulations

**June 22nd-23rd 2022**

**LABORATORIO DIDATTICO DIMES**
**University of Genova, Italy**


University of Genova



## SCIENTIFIC AND ORGANISING COMMITTEE

Anna Maria Bassi, Susanna Penco, Carla Villa, Stefania Vernazza, Sara Tirendi, Vanessa Almonti, Chiara Magliaro, Irene Carmagnola

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# Innovative approaches in cosmetic testing, in compliance with European regulations

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## ABSTRACT BOOK

### Introduction to the course "Innovative approaches in cosmetics in compliance with the European regulation"

Anna Maria Bassi,[1,2] Valeria Chiono,[2,3] Arti Ahluwalia[2,4]

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This collection includes the abstracts presented at the theoretical-practical course "Innovative approaches in cosmetics in compliance with the European regulation" (Genoa, 22-23 June 2022). The course aims to provide an update on alternative methods to animal experimentation, in the context of risk assessment in the European cosmetology field, based on the new regulations establishing the total ban on animal experimentation for cosmetic purposes (EC Regulation No. 1223/2009). Such regulation improves the safety of cosmetic products in the EU by strengthening safety requirements, simplifies procedures for companies and competent authorities in the sector, updates the rules to take into account the latest technological developments such as the use of nanomaterials, and keeps the ban on animal testing in place. From this point of view, it is essential to offer the scientific basis of these regulations and updated information to students of scientific degrees, to contribute to the training of technicians/cosmetic industry, with interest to eco-sustainability. The course promotes the interdisciplinary collaboration between biologists, chemists and ecologists. The course has been organized by Centro3R, Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research.

### Irritation and corrosion hazards under CLP regulation and waste framework directive

Tomaso Munari, Chiara Agrone

I.A. Industria Ambiente srl, Genoa, Italy

**Abstract:** Hazard classification of mixture according to CLP and of wastes according to WFD are not completely overlapping. Classification for irritation and corrosion is one of the cases in which the classification criteria are different.

**Introduction:** Hazard evaluation criteria for mixtures are defined in annex I of Regulation (EC) n. 1278/2008 (CLP). Hazard evaluation of a mixture can lead to the classification, *i.e.* attribution, of one or more hazard "H" phrases: H2XX for physical-chemical hazards, H3XX for health hazards and H4XX for environmental hazards. Wastes, while often approximated to mixtures, are outside the scope of CLP and classification is carried out according to Waste Framework Directive (WFD) 2008/98/EC. Classification process assign to the waste one or more hazard "HP" phrases: HP1-3 and HP15 for physical-chemical hazards, HP4-13 for health hazards (included HP9 - infective) and HP14 for environmental hazards. According to CLP there is a hierarchy of classification methods: first one is direct testing on a mixture; second one is the "bridging principle", in order to classify a mixture experimental data performed on a similar mixture could be used; third one is "calculation method" allowing to classify a mixture on the basis of component substances classification. According to WFD, direct testing on waste is preferred (using the same test methods foreseen by CLP); "bridging principle" are not acknowledged by WFD; "calculation methods" are also accepted by WFD and are widely used due to resource constraints. While, for some hazards, classification "by calculation" according to CLP and WFD broadly overlaps in others, such as irritation and corrosion, is quite different.

**Experimental Part:** Classification "by calculation" for irritation and corrosion of mixtures (CLP) and wastes (WFD) is compared.

**Results:** Comparison of "calculation criteria" for irritation and corrosion hazards for mixtures and wastes is shown in Tab. 1.

**Tab. 1. CLP and WFD irritation and corrosion.**

| Final classification of mixture or waste | | Relevant classification and concentration of substances contained in mixture or waste (substances are considered only if they are ≥ 1%) | | | |
|---|---|---|---|---|---|
| CLP | H314 cat. 1A, 1B, 1C Causes severe skin burns and eye damage | "extreme" pH of the mixture ≤ 2 or ≥ 11,5 or Acid with pH ≤ 2 ≥ 1% or Base with pH ≥ 11,5 ≥ 1% | Σ H314 cat. 1A, 1B, 1C ≥ 5% | | |
| | H315 cat. 2 Causes skin irritation | pH 2-11,5 | 1% ≤ Σ H314 cat. 1A, 1B, 1C < 5% | Σ H315 cat. 2 ≥ 10% | Σ 10*H314 cat. 1A, 1B, 1C + H315 cat. 2 ≥ 10% |
| | H318 cat. 1 Causes serious eye damage | "extreme" pH of the mixture ≤ 2 or ≥ 11,5 | Σ H314 cat. 1A, 1B, 1C + H318 cat. 1 ≥ 3% | | |
| | H319 cat. 2 Causes serious eye irritation | pH 2-11,5 | 1% ≤ Σ H314 cat. 1A, 1B, 1C + H318 cat. 1 < 3% | Σ H319 cat. 2 ≥ 10% | Σ 10*H314 cat. 1A, 1B, 1C + 10*H318 cat. 1 + H319 cat. 2 ≥ 10% |
| WFD | HP4 Irritant - skin irritation and eye damage | pH not relevant | Σ H314 cat. 1A ≥ 1% | Σ H318 cat. 1 ≥ 10 | Σ H315 cat. 2 + H319 cat. 2 ≥ 20% |
| | HP8 Corrosive (both for skin and eye) | pH not relevant | Σ H314 cat. 1A, 1B, 1C ≥ 5% | | |

**Discussion and Conclusions:** Irritation and corrosion classification "by calculation", according to CLP and WFD, are substantially diverging. It is not possible to simply infer hazard classification of a waste based on a CLP mixture classification.

**Acknowledgments:** Industria Ambiente SRL is an independent consulting company.

**Disclosures:** The authors declare they have no conflicts of interest to disclose.

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1. Regulation (EC) n. 1272/2008.
2. Directive 2008/98/EC.

## Conflitto tra REACH e regolamento cosmetici per quanto riguarda i test sugli animali

Costanza Rovida

CAAT-Europe, Konstanz University, Germany

**Abstract:** Nel database ECHA ci sono più di 3,200 dossiers REACH relativi a sostanze con un uso in ambito cosmetico. Di queste, 419 sono utilizzate unicamente come ingredienti cosmetici. Almeno 63 sono state testate su animali dopo la proibizione del regolamento cosmetici. Altre 20 hanno test in corso e molte altre potrebbero aggiungersi.
**Introduzione:** Il Regolamento REACH EC 1907/2006 (Registration, Evaluation, Authorisation and Restriction) chiede che chiunque immetta sul mercato UE sostanze chimiche in quantità superiori a 1 t/y queste siano registrate, inviando un dossier molto corposo a ECHA, l'agenzia europea delle sostanze chimiche. Mentre un dossier fino a 10 t/y può (e dovrebbe) essere preparato interamente senza fare nuovi test su animali, la cosa si fa via via più difficile e diventa praticamente impossibile per sostanze che superano le 100 t/y. D'altro canto, il Regolamento 1223/2009 sui prodotti cosmetici sancisce la totale proibizione di testare sugli animali i prodotti cosmetici finiti e anche tutti i loro ingredienti. Purtroppo, gli ingredienti cosmetici devono anche essere registrati secondo REACH e quindi soddisfare quello che questo regolamento prevede, compresa la richiesta di fare nuovi test sugli animali. Da qui nasce il conflitto tra i due regolamenti.
**Sperimentazione:** Per effettuare l'analisi[1], sono stati analizzati più di 400 dossier REACH relativi a sostanze con un uso esclusivo in ambito cosmetico.
**Risultati:** Da un punto di vista legale, un tribunale ha stabilito che il regolamento cosmetici proibisce nuovi test sugli ingredienti, ma solo se il test è fatto per valutare il rischio del prodotto utilizzato dai consumatori, mentre il REACH richiede dati per proteggere i lavoratori che maneggiano le sostanze prima che entrino a far parte del prodotto, quindi è lecito chiedere test sugli animali. Ciononostante, le aziende cosmetiche si trovano costrette a non poter più affermare che i prodotti cosmetici non contengono ingredienti testati sugli animali, con grande disappunto anche dei consumatori. Purtroppo, non si sta parlando di casi isolati, ma di numeri importanti sia per quel che riguarda il numero di ingredienti testati che di test eseguiti. Al momento non c'è una soluzione al conflitto, ma ci si sta muovendo per chieder alla Commissione Europea di prendere posizione ed eventualmente apportare degli aggiornamenti. La discussione è anche aperta a ciò che succede al di fuori dell'Unione Europea.
**Ringraziamenti:** Ringrazio Jean White di White Rabbit Beauty per il tremendo lavoro che ha fatto per arrivare alle conclusioni di questo lavoro.
**Dichiarazioni:** C. Rovida lavora come consulente in ambito REACH e ha legami con varie aziende coinvolte nella registrazione di prodotti cosmetici.

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## *In vitro* skin irritation tests using reconstructed human epidermis model EpiDerm™

Jan Markus, Silvia Letasiova

MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia

**Abstract:** The EpiDerm™ 3D human tissue model is used across a diverse range of applications including safety and risk assessment, and biological efficacy. Simple protocols and the evaluation of early cellular endpoints allow research to acquire data in few days.
**Introduction:** EpiDerm, a Reconstructed Human Epidermis, is a ready-to-use, highly differentiated 3D tissue model consisting of normal, human-derived epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis (Fig. 1). NHEK are cultured on specially prepared cell culture inserts using serum free medium. This model exhibits *in vivo* – like morphological and growth characteristics which are uniform and highly reproducible.
**Experimental Part and Results:** Cultured at the air-liquid interface, EpiDerm™ allows for the evaluation of topically applied compounds, chemicals, cosmetic/personal care products, ingredients and final formulations. With multiple ECVAM validations and OECD accepted test guidelines, EpiDerm™ is a proven *in vitro* model system for chemical, pharmaceutical, cosmetics and skin care product testing. EpiDerm skin irritation test (SIT) is validated and accepted as OECD TG 439. EpiDerm Time-to-Toxicity assay (ET-50 assay) is used for screening, ranking and benchmarking of ingredients, for skin tolerance testing of final products (mildness testing) and for evaluation of minor changes in the formulations. This model can be used for different testing purposes, such as EpiDerm skin corrosion test (SCT), EpiDerm phototoxicity test, etc.

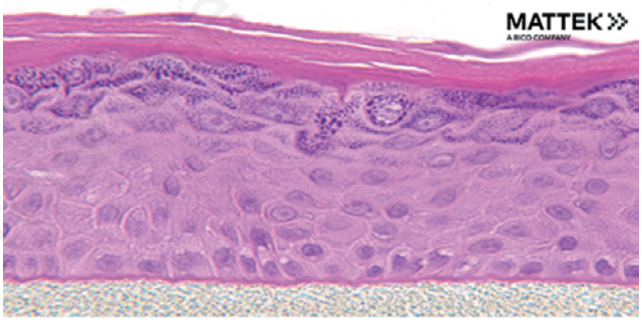


**Fig. 1. Histology cross-section of EpiDerm tissue model showing organized basal, spinous, granular layers and multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*.**

**Discussion and Conclusions:** The presentation will provide an overview of the *in vitro* skin irritation tests using reconstructed epidermis tissue model, EpiDerm™, for evaluation of safety and efficacy of cosmetics and skin care products.
**Disclosures:** The authors have nothing to disclose.

## Skin sensitization: validated alternatives and defined approaches

Laura Ceriotti

VitroScreen srl, Milan, Italy

**Abstract:** The evolution of the methods used for skin sensitization assessment is presented: from *in vivo* methods to *in vitro* and in chemico methods and define approaches (DAs) based on fixed combinations of *in vitro*, in chemico and *in silico* tools.
**Introduction:** Before a new cosmetic ingredient is placed on the European market, evaluation of its safety profile, including the assessment of skin sensitization hazards and potency is mandatory. According to the EC Regulation N. 1223/2009 on cosmetic products, substances exclusively intended for use in cosmetic products cannot

be tested *in vivo* with traditional animal test methods but only alternative non-animal approaches can be used.

**Experimental Part:** Since 2015, new in chemico and *in vitro* methods have been validated and included into official OECD test guidelines as alternative to animal testing (Tab. 1). These methods are designed to target individual Key Events (KE) in the Adverse Outcome Pathway (AOP) for skin sensitization (Fig. 1)(1): OECD TG 442C includes methods assessing covalent binding to proteins (KE1), OECD TG 442D keratinocytes activation (KE2), and OECD TG 442E dendritic cells activation (KE3). Each method has a specific protocol, applicability domain and limitations (*e.g.* solubility issues, non-applicability to complex mixtures, surfactants, pre- and pro-haptens). Furthermore, since each method address only one KE and being skin sentitization a complex process, none of them can be used as stand-alone method to conclude on skin sensitization potential of chemicals or to provide information on potency, but rather they can be used in the context of integrated approaches to testing and assessment (IATA)(2) or defined approaches (DAs)(3).

**Results:** Three DAs have been included in OECD Guideline n. 497 (Tab. 1) which is the first DA guideline intended as a full replacement for animal test methods. A DA consists of a fixed data interpretation procedure (DIP) (*e.g.* a mathematical model, a rule-based approach) applied to data (*e.g. in silico* predictions, in chemico, *in vitro* data) generated with a defined set of information sources to derive a prediction without the need for expert judgment. Conclusive prediction can be used on their own to make a similar conclusion as done by using a standard *in vivo* assay such as the Local Lymph Node Assay (LLNA). In case of inconclusive prediction, no stand-alone conclusions can be made based on the DA. However, the information generated from the individual information sources can still be used in a weight of evidence approach to conclude on skin sensitization or to indicate the need for additional information. DAs have been applied to assess the skin sensitization potential of cosmetic ingredients such as flavoring agents and fragrances.

**Disclosures:** The authors have nothing to disclose.

**Table. 1. Summary of the available in chemico/*in vitro* skin sensitisation test methods and defined approaches (4).**

| Latest update | AOP key event measured | Test method | Validation status, regulatory acceptance | OECD test guideline | Outcome according to the test method/guideline |
|---|---|---|---|---|---|
| 2021 | Key Event 1 (peptide /protein binding | DPRA | Validated and regulatory acceptance | OECD TG 442C | SS or NS with complementary information |
| 2021 | | ADRA | Validated and regulatory acceptance | OCD TG 442C | SS or NS with complementary information |
| 2021 | | kDPRA | Validated and regulatory acceptance | OECD TG 442C | Cat 1A or Cat 1B/NS |
| 2018 | Key Event 2 (Keratinocyte response) | Keratinosens™ | Validated and regulatory acceptance | OECD TG 442D | SS or NS with complementary information |
| | | LuSens | Validated/under regulatory review | OECD TG 442D | SS or NS with complementary information |
| 2018 | Key Event 3 (Monocytic / dendritic cell response) | h-CLAT | Validated and regulatory acceptance | OECD TG 442E | SS or NS with complementary information |
| 2018 | | U-SENS™ | Validated and regulatory acceptance | OECD TG 442E | SS or NS with complementary information |
| 2018 | | IL-8 Luc | Validated and regulatory acceptance | OECD TG 442E | SS or NS with complementary information |
| 2021 | Defined approach | 2 out of 3 | Validated and regulatory acceptance | OECD TG 497 | SS or NS |
| 2021 | | ITS v1 or v2 | Validated and regulatory acceptance | OECD TG 497 | SS (Cat 1A or 1B) or NS |

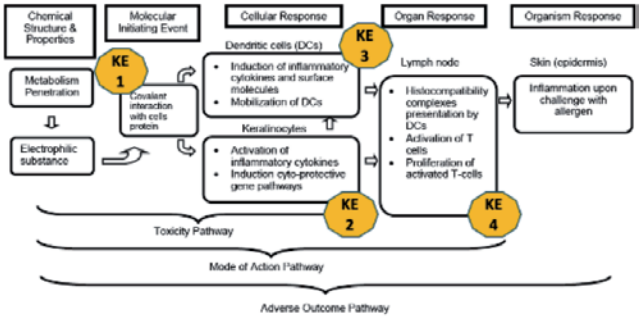


**Fig. 1. Adverse Outcome Pathway (AOP) for skin sensitization (1).**

**Discussion and Conclusions:** As DAs are fixed data interpretation procedure they can be harmonized and provide a useful tool for hazard classification. However they have the same limitations of the individual in chemico/*in vitro*/*in silico* information sources they included. Thus, other methods are under validation or regulatory acceptance (*e.g.* SENS-IS and GARDSkin) to address the regulatory needs for surfactants, difficult-to-test samples and complex mixtures, which are currently outside the applicability domain of validated test methods and DAs.

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## The GARD assay for skin sensitization hazard identification and risk assessment

Andy Forreryd

SenzaGen AB, Lund, Sweden

**Abstract:** A variety of chemicals of interest to the cosmetic sector remains challenging to accurately assess in the OECD validated New Approach Methods (NAMs) for skin sensitization. Novel state-of-the-art scientific methods, such as the GARDskin assay, has shown promise to fill some of the remaining data gaps and contribute to an expanded applicability domain of NAM-based strategies.

**Introduction:** A variety of New Approach Methods (NAMs) has recently been adopted into the OECD TGs for assessment of skin sensitization potential and provides acceptable hazard classifications to replace animal assays for a variety of chemical classes. However, it has been recognized that certain chemicals of interest to the cosmetic sector, including indirect acting haptens and hydrophobic substances, may be challenging to accurately assess in conventional OECD assays. In addition, there is still an apparent lack of NAMs that can effectively and quantifiably characterize the skin sensitizing potency which is of importance to establish a threshold concentration of sensitizers in cosmetic formulations. The GARDskin assay is

a next-generation *in vitro* assay based on genomics and machine learning. The assay is currently considered for adaption into OECD TG. This study present data aiming to explore the applicability of GARDskin for testing of challenging substances, and to present a novel strategy based on dose-response measurements in the GARDskin assay which can be utilized for continous potency predictions and contribute to the establishment of safe concentrations of sensitizers when used in consumer products.

**Experimental Part:** The objective of the current study was to compile and present experimental data for a variety of substances which has traditionally been considered as challenging to test in conventional OECD assays. Necessary adaptions to the validated protocol for GARDskin included the selection of less polar solvents to facilitate testing of hydrophobic materials. Furthermore, a protocol based on dose-response measurements in GARDskin have been developed. A case study involving the testing of the chemical resorcinol will be presented to demonstrate how this approach can be utilized for potency predictions.

**Results:** Results from the testing of a selection of challenging substances, including hydrophobic materials, UVCBs and indirect acting haptens demonstrates accuracies in the range between 80-95%[1]. For quantitative potency predictions, the case study (Fig. 1) demonstrates how the experimentally derived output in GARDskin Dose-Response can be used as input into a linear regression model to derive a continous prediction of skin sensitizing potency in the terms of an LLNA EC3 value[2].

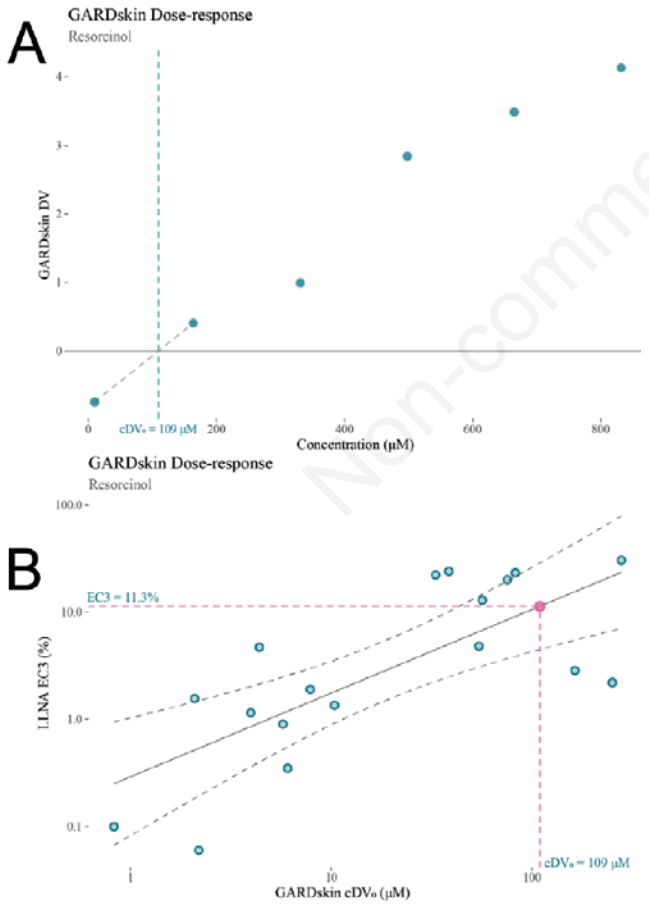


**Fig. 1. (A) The concentration in GARDskin dose-response (cDV0) required to generate a positive classification in the assay (DV>0)(B) is used as input in a linear regression model for prediction of LLNA EC3 values on a continous scale.**

**Discussion and Conclusions:** Available data supports the inclusion of a variety of challenging substances into the AD of GARDskin. The GARDskin dose-response approach illustrates a novel and promising protocol to predict skin sensitization potency on continuous scale. Together, data may contribute to a reduced need for animal studies.

**Disclosures:** AF is employed by SenzaGen, the developers of the GARD assay.

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## From AOPs (Adverse Outcome Pathways) to the Next Generation Risk Assessment (NGRA) for the final goal of full animal replacement in toxicology

Costanza Rovida

CAAT-Europe, Konstanz University, Germany

**Discussione:** Al momento il risk assessment delle sostanze si basa sul calcolo del DNEL (Derived No Effect Level) che si ottiene partendo dal NOEL (No observed effect level) che costituisce la dose di no effetto misurata con un esperimento a dosi ripetute sugli animali. Ma allora, come si fa a sostituire i test sugli animali? La procedura più semplice e più utilizzata è quella del Read Across, cioè partendo dai dati tossicologici di una o più sostanze simili, chiamate source substances, si stabilisce la similarità con la sostanza da analizzare, la target substance[1] e si trasferiscono le informazioni esistenti. In questo passaggio, si adatta anche il valore di NOEL, a volte correggendolo considerando la molarità delle sostanze, visto che invece il NOEL è espresso in mg/Kg. Questo va bene nel breve periodo, ma rimane un adattamento dell'approccio tradizionale. Se si vuole pensare a un sistema più innovativo e che rifletta maggiormente quello che è il vero rischio per gli esseri umani esposti alle varie sostanze chimiche, è necessario rivoluzionare completamente questo paradigma. Prima di tutto i test *in vivo* vanno sostituiti con test *in vitro* che rappresentino gli adverse outcome pathways (AOP), cioè i meccanismi che partendo dal contatto con una sostanza estranea possono portare all'insorgenza di un meccanismo avverso. Deve essere chiaro che gli AOP non sono una metodica, bensì un modo per organizzare le informazioni che è possibile raccogliere attraverso test studiati per rappresentare determinati meccanismi che possono avvenire nell'organismo umano. I dati organizzati all'interno degli AOP, vanno combinati con informazioni di tipo tossicocinetico per prevedere la relazione tra esposizione e dose di effetto, considerando i parametri per assorbimento, che può avvenire attraverso la pelle, per via inalatoria o attarverso l'apparato gastrico, distribuzione, cioè come si muove la sostanza assorbita all'interno dell'organismo, metabolismo ed escrezione (ADME, Absorption, Distribution, Metabolism, Excretion). Queste valutazioni vanno fatte attraverso modelli computazionali basati però su dati sperimentali e sulle proprietà chimico fisiche della sostanza. Questo è il concetto su cui si fonda il cosiddetto Next Generation Risk Assessment, che studia la valutazione del rischio all'uso di sostanze chimiche partendo dall'esposizione reale sull'uomo e combinandola con informazioni ottenute attraverso metodi *in vitro*, *in chemico* e/o *in silico*. L'utilizzo di NGRA è già realtà in ambito cosmetico, ma è necessario approfondirne le possibilità e allargarne l'ambito di applicazione. E' importante aggiungere che la Commissione Europea ha finanziato il progetto

RiskHunt3R (www.risk-hunt3R.eu) che è proprio focalizzato su questo argomento.
**Ringraziamenti:** Ringrazio Jean White di White Rabbit Beauty per lo straordinario lavoro che ha fatto per arrivare alle conclusioni di questo lavoro.
**Dichiarazioni:** C. Rovida lavora come consulente in ambito REACH e ha legami con varie aziende coinvolte nella registrazione di prodotti cosmetici.

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## *In vitro* experimental system relevance and factors influencing the outcome of the study

Emma Di Consiglio

Istituto Superiore di Sanità, Rome, Italy

**Abstract:** The development of New Approach Methodologies (NAMs), based on the use of human cellular models, empowered by use of computational methods (*e.g.* QIVIVE), already used in the research field, could be improved and imported in the regulatory area of toxicity testing. In this context, the integration of *in vitro* and *in silico* methods into specific testing strategies as well as considerations about the fit for purpose and kinetics of the used experimental systems can increase the predictivity of health effects in particular in repeated-use scenario.
**Introduction:** Cosmetic ingredients evaluation by using NAM-based strategies is an essential step to ensure their safety profile (1). The NGRA process, recently described, is allowing the paradigm shift from the evaluation of apical endpoints in animal testing, to the safety assessment based on the bioactivity measured in a set of human relevant *in vitro* assays. To this aim, several NAMs, including those reported in peer reviewed studies, could be valuable in a Weight of Evidence approach, provided that each method is scientifically valid, properly developed and described (2). In order to increase their applicability, reducing uncertainty, the experimental systems should be assessed for the intrinsic characteristics (*e.g.* metabolic and transport capability) of the cellular models, the suitability of the applied QIVIVE, including the kinetic parameters used to develop it. In this lecture, by way of case-studies, practical issues and possible solutions will be discussed.
**Results and Discussion:** Some obstacles in accepting data from *in vitro* toxicity tests could be overcome by taking into account some prerequisites which can improve the outcome of the study: i. application of a step-wise procedure for estimating cellular bioavailability to determine the Bio Effective Concentration (BEF): if *in vitro* conditions (*e.g.* protein-free culture medium) do not reflect the *in vivo* situation, different kinetic parameters might be obtained (e.g organ/blood partition coefficient) (3); ii. following the kinetic behavior of a substance in repeated use-scenario can inform about crucial processes occurring in the cellular system (*i.e.* bioaccumulation, metabolism); iii. an appropriate QIVIVE should be based on a well-defined dose metric: the use of nominal concentration vs BEC can affect the assessment of a substance toxic potential (3); iv. *in vitro* models should be associated to human relevant key events, in human relevant cellular models (*e.g.* exploring the presence of the main metabolic enzymes and /or transporters can give a mechanistic insight of toxic insults caused by the substance) (4); v. a broad quality assessment of more complex cellular models, including a standardization process, is needed to demonstrate their relevance and promote the application both by users and regulators (5).
**Acknowledgments:** Special thanks to all the colleagues, who took part to the cited papers and made this dissertation possible.
**Disclosures:** The author has nothing to disclose.

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## Advanced wound dressings and models for their preclinical validation

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**Abstract:** Despite the wide availability of commercial wound dressings (WDs), more effective products are urgently required to improve the treatment of chronic skin wounds. Simultaneously, the design of *in vitro* preclinical evaluation tools has become a new need to assess the safety and the effectiveness of advanced WDs under the 3Rs.
**Introduction:** Chronic skin wounds affect more than 40 million patients globally representing a severe burden. To satisfy the huge demand, different types of WDs have been introduced on the market. The advancements in material design and processing, biotechnology, imaging and electronic fields have successfully contributed to the development of formulations actively participating to tissue healing. Recently, the effectiveness of advanced WDs has been further improved working on patient's personalization. However, their clinical translation is suffering a setback due to the limited availability of preclinical evaluation tools. The development of skin wound models (SWMs) can speed up the validation process and thus, the quick market entry of advanced WDs. This work describes the design of personalized wound patches (PWPs) and SWMs combining the engineering of ad hoc synthesized biomaterials and green processing techniques.
**Experimental Part:** PWPs and SWMs were fabricated through the 3D extrusion of customized biomaterial inks based on multi-stimuli-responsive polyurethanes (PU)[1] and cellularized methacryloyl gelatin (GelMA), respectively. Ibuprofen (IBU) was selected to study the pH-controlled release kinetics from PWPs[2]. Cell survival upon SWM fabrication was preliminarily evaluated through NIH-3T3 murine fibroblast encapsulation.
**Results:** PU synthesis and functionalization with alkaline pH-responsive moieties were first chemically assessed showing the synthesis of a high molecular weight polymer with $5.3\times10^{18} \pm 0.6\times10^{18}$ – $COOH/g_{polymer}$. Then, hydrogel internal pH changes when in contact with alkaline fluids proved its potential responsiveness to infected wound exudate. The pH-controlled drug release was proved through the significantly higher IBU release from PU hydrogels compared to the control. Lastly, differently-shaped structures were successfully extruded, demonstrating hydrogel capability to be processed according to a patient-personalized CAD model. GelMA synthesis was first physico-chemically proved; then, NIH-3T3-loaded GelMA

hydrogels were extruded into square-meshed structures showing good shape fidelity to the CAD model. No cell sedimentation phenomena and shear stress-induced damages were observed after extrusion. Lastly, strong proliferation within GelMA scaffolds was observed up to 7 days.

**Discussion and Conclusions:** The combination of biomaterial engineering and mild fabrication techniques represents a promising approach towards the development of PWPs and SWMs as preclinical evaluation tools.

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## The assessment of eye irritation potential of chemicals, cosmetics, and household products using reconstructed human cornea-like model EpiOcular™

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**Abstract:** Determination of serious eye damage/irritation originally involved the use of laboratory animals (OECD TG 405). In 2015, a new test guideline (OECD TG 492) was accepted which enables the use of an *in vitro* procedure based on reconstructed human cornea-like epithelium (RhCE), EpiOcular™ Eye Irritation Test (EIT), to distinguish between chemicals (substances and mixtures) not requiring classification and those that must be labeled for eye irritation or serious eye damage.

**Introduction:** EpiOcular™ corneal model consists of normal, human-derived epidermal keratinocytes which have been cultured in serum free medium to form a stratified, squamous epithelium, similar to the cornea *in vivo*. This model is mitotically and metabolically active and releases many of the pro-inflammatory agents which are important in ocular irritation and inflammation.

**Experimental Part and Results:** EpiOcular EIT has been validated as part of OECD TG 492 and is used to discriminate between ocular irritant/corrosive materials (GHS Categories 1 and 2 combined) and those that require no labeling (GHS No Category). Chemicals identified as requiring classification for eye irritation/serious eye damage must be further tested to distinguish between eye irritants and those causing serious eye damage. EpiOcular Time-to-Toxicity (ET-50) protocols (Neat protocol, Dilution protocol, Sub-Draize Mildness protocol) are another types of eye irritation tests, they can be used for assessment of irritation potency, tolerance and mildness of cosmetics, cosmetic formulations, etc. In the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project, a new testing strategy for EpiOcular ET-50 protocols to achieve optimal prediction for all three categories (Category 1 (Cat 1), Category 2 (Cat 2) and No Category (No Cat)) was developed. It combines the most predictive time-points of neat and dilution protocols and tests liquids and solids separately (1).

**Discussion and Conclusions:** The presentation will provide an overview of the *in vitro* eye irritation tests using cornea-like tissue model, EpiOcular™, for evaluation of hazard and risk assessment of chemicals, cosmetics, personal care products, household products and the testing strategy proposed in CON4EI and verified in ALT4EI (ALTernatives for Eye Irritation) projects to achieve optimal prediction for all three categories – prediction models for liquids and solids seems to be a very promising tool in an integrated testing strategy (ITS) that can discriminate chemicals to No Cat, Cat 2 and Cat 1.

**Disclosures:** The authors have nothing to disclose.

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## A new highly representative *in vitro* model of human skin

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**Abstract:** The predictivity of a skin model can be increased using a human biopsy. This refinement requires the use of an advanced *in vitro* platform to preserve the viability and functionality of the tissue. In this work, we introduce the preliminary results about this field of research, achieved by the University of Verona, in collaboration with IVTech.

**Introduction:** The human skin is characterized by a complex multi-layered structure. Its main function is its protective role against microbial attack, UV irradiation, as well as exogenous substances, such as chemicals and immunological barrier. Considering that skin is one of the most studied tissues for test of drugs/cosmetic compounds, several approaches are available to *in vitro* recreate a viable and functional model. The wide variety of *in vitro* models spans from conventional mono-layered cell cultures to more complex 3D co-cultures. The first type is widely accepted/diffused but it is not enough predictive of the reality [1, 2]. An example of a complex 3D co-culture is represented by the commercially available reconstructed tissues [3]. These models are composed by human cells, cultured in a 3D structure which is topologically closed to the real skin. However, 3D micro-structures, such as sebaceous glands or hair bulbs, are not always reproduced. Moreover, they are developed and sold in transwell/inserts, characterized by a static environment: the dynamicity of the native environment is not simulated. Considering the state of the art, is it possible to increase the predictivity of skin model?

**Experimental Part:** The predictivity of a skin model can be increased using a human biopsy. This approach fills the lack of certain 3D micro-structures in some of the commercial tissue. However, the maintenance of a viable tissue, as well as its functionality are still a challenge. In this context, Tommaso Sbrana, CEO of IVTech [4], introduces the results of a collaboration between IVTech srl and the research group, directed by Prof. Laura Calderan (University of Verona) [5]. He describes the set-up of an advanced *in vitro* model, involving the use of a human skin biopsy and a fluidic platform (*i.e.* LiveBox and LiveFlow produced by IVTech), where the tissue is irrorated by the flow of medium, allowing for good results in maintenance of viability and functionality for a long time experiment.

**Results:** We think that the combination of a 3D biopsy and a dynamic environment has a key role in the increase the predictivity of an *in vitro* model if compare with human reality. This is demonstrated in this work, focusing the attention on 3 different cases of study: *in vitro* administration of hyaluronic acid on a healthy tissue, and the characterization of 2 disease models, such as inflamed skin and skin affected by contact dermatitis.

**Discussion and Conclusions:** These results are the baseline to demonstrate the efficacy of the model, which can be used to test as a service the effects of drugs on a highly representative model of the human skin.

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## Grape-derived extracts as potential active pharmaceutical and cosmetic ingredients

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Oxidative stress plays a key factor in the neurodegeneration process related to pathologies such as Parkinson's disease. To counteract the uncontrolled increase of reactive oxygen species (ROS) and oxidative stress-dependent cell death, several preclinical and clinical tests exploit natural-derived organic antioxidants, such as polyphenols. Despite some promising results, free antioxidants show scarce brain accumulation and may exhaust their scavenging activity before reaching the brain. In this work, we developed an antioxidant therapeutic nanoplatform consisting of nanosized functionalized liposomes loaded with selected polyphenol-rich vegetal extracts with high blood-brain barrier crossing capabilities. The antioxidant extracts were obtained from grape seeds and skins as a by-product of wine production in the Cinque Terre territories (La Spezia, Liguria, Italy), following a sustainable circular approach with reduced environmental impact. The antioxidant nanoplatform was successfully tested in a relevant *in vitro* model of Parkinson's disease (Fig. 1), where it completely rescued the ROS levels, prevented the aggregation of α-synuclein fibrils, and restored cell viability, paving the way for preclinical translation of the approach [1]. Further possible applications in the nutraceutical and cosmetics sectors will be finally discussed.

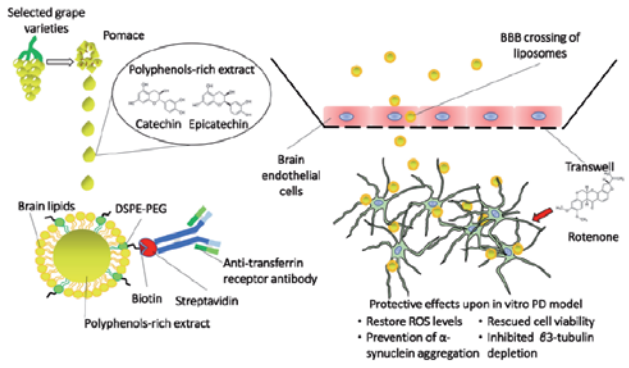


**Fig. 1. Schematization of the experimental design.**

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**Disclosures:** Competing interest statement: G.C. is a co-founder of Kidaria Bioscience SRL, a company aiming at the production, characterization, and commercialization of cosmetics based on natural products-derived ingredients.

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## Ecofriendly microwave extractions from agrifood of potential cosmetic ingredients

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**Abstract:** Microwave mediated green extractions were applied to agro-food waste to obtain new bioactive cosmetic ingredients.

**Introduction:** Nowadays the highest priorities for the cosmetic industry are green products and processes, in terms of safety and respect of the environment. Following the Green Chemistry targets [1] the use of substrates from renewable sources, the reduction of organic waste and solvents and the development of sustainable and mild procedures represent a main objective. In an extractive context, studies on the exploitation and recycle of solid organic waste are gaining increasing interest. The agro-food division produces a large amount of organic residues, often highly polluting and quite expensive to be disposed. Their conversion from worn out plant matrices with high environmental impact to recycled sources with significant added values represents a great challenge in the eco-sustainable industrial compart, due to the general demand of environment respect, circular economy, high quality and safe products and processes [2]. In the framework of the innovative extractive methodologies, microwave-assisted extractions (MAE) are considered green and flexible procedures suitable to extract different substrates using alternative energetic sources. They present several advantages over conventional extraction techniques such as reduction of extraction times, solvent and energy consumption, coupled to a better extraction efficiency as well as high extract quality. In order to assess several kinds of extraction methods with only one microwave applicator, in the present work a specifically designed oven has been developed and applied. Different MAE techniques, such as solvent-free microwave extraction (SFME) [3] and microwave hydro-diffusion and gravity (MHG) [4], were exploited in order to obtain, from agrifood waste, essential oils and the inner water phases of the plants ("essential waters"®) useful as green ingredients in cosmetic products.

**Experimental Part:** The extraction of several worn out plant matrices, such as grape marc (from the wine making process), rose petals, (from rose syrup production), crocus exhaust flowers (from saffron production and pomegranate peels (from juice production) was carried out. The multipurpose microwave applicator was a multimode prototype with two optical fibres (NEOPTIX Reflex) for temperature measurement and control.

**Results:** SFME allowed to isolate (5 min) the rose essential oil

(0,40% vs 0.1% steam distillation). MHG allowed to recover (10 min) the "essential waters"® containing several water-soluble bioactive principles.

**Discussion and Conclusions:** Extraction was characterized by speediness, higher extraction efficiency and increased quality. The procedures applied afford to transform discarded agrofood exhaust matrices into recycled sources with an added value for the cosmetic market.

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## *In silico* approaches for cosmetics

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**Abstract:** The aim of this work is to review the *in silico* models currently exploited in different research areas of cosmetic testing.

**Introduction:** The 7th amendment to EU D. 1976/768 banned animal testing for cosmetics with a final firm deadline of 2013. This boosted the implementation of alternative or non-animal methods, in particular *in vitro* experimental or *in silico* models. In particular for *in silico* models, several approaches based on machine intelligence-enabling extrapolation, prediction and risk assessment has been developed in the last decades. However, one of the biggest challenges for the integration of non-animal methods, into mainstream basic and applied research is the demonstration of their translational value. In the light of this, the course will review the computational-based techniques providing meaningful and animal-free predictions about chemicals used in cosmetics.

***In silico* models employed in cosmetics:** After definition and classification of computational modelling, the lesson will focus on the main toxicological areas of interest for cosmetics: toxicokinetics, repeated dose toxicity, carcinogenicity and skin sensitization [1]. Then, the lesson will provide an overview on traditional *in silico*/mathematical models exploited in cosmetics research. Specifically, models which describe the concentration versus time profiles of substances in organ and tissue compartments through differential equations (such as physiologically based pharmacokinetics – PBPK) and statistical methods based on comparison, classification and pattern recognition (often used for analysing molecules with potential risks according to their structure, as in QSAR – quantitative structure-activity relationship) will be presented [1-2]. In this context, the importance of databases for identifying similar molecules that may share similar properties, thus predicting their toxicological effect and for providing a means to select chemical for testing, as a part of an *in vitro* testing strategy, will be highlighted.

Finally, some examples of simple models employed to estimate dermal exposure will be presented [3].

**Discussion and Conclusions:** *In silico* models represent a promising non animal alternative for cosmetic testing, since they give useful predictive information for experimental design, even across scales, and allow the investigation of systemic effects that cannot be obtained by means of non-invasive investigations in humans or *in vitro*.

**Disclosures:** The corresponding author declares that there is no conflict of interest with the authors.

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