Alternatives to animals

Dr Marc Teunis describes a successful European collaboration to prepare a combined approach for testing potential allergens using human skin cell cultures rather than current live animal models

Firstly, how can sensitisers and non-sensitisers be defined?

A sensitiser is a chemical that causes a substantial proportion of people or animals to develop an allergic reaction after repeated exposure. According to this definition, when a chemical is not capable of inducing an allergic reaction, it can automatically be classified as a non-sensitiser. In principle, however, people can become sensitised to almost every entity.

Can you explain the main aims of your project?

With respect to assessing the sensitising capacity and potency of chemicals, no formally validated and accepted *in vitro* method is currently available. The integrated EC project *Sens-it-iv*, which ended with a scientific conference in November 2011, aimed to develop promising methods to replace current animal models for chemical allergen testing.

Two of the assays from the *Sens-it-iv* toolbox were selected to be used in a two-tiered approach. Previously, a number of sensitising chemicals had been tested separately with these assays. The aim of our current project is to gather enough evidence to show the reliability of this approach; thus constituting a pre-validation study.

Why are no accepted *in vitro* methods for the identification of sensitising chemicals currently available?

Skin sensitisation is a complex multistep immunological process involving multiple cell types and biological mechanisms. The tests required for the *in vitro* identification of skin sensitisers are a matter of debate. Six key mechanisms have been proposed to cover the essential steps of sensitisation induction – haptenation, epidermal inflammation, dendritic cell (DC) activation, DC maturation, DC migration and T-cell priming – but such an approach would increase the expense of testing eight- to tenfold. The data seems to indicate that a haptenation test combined with a test addressing epidermal inflammation, and another determining dendritic cell (DC) activation, would provide more than 95 per cent predictive accuracy.

Animal-free test systems are increasingly accepted by regulatory authorities as bricks in integrated safety assessment strategies, provided that the methods are scientifically validated. But animal-free approaches are often squeezed between regulatory authorities demanding sufficient real-life data from industry demonstrating the strengths and limitations of the new methods, and industry hesitating to implement new approaches unless these methods are accepted by the authorities. Selected assays providing evidence about the capacity of chemicals to haptenise or to trigger epidermal inflammation and DC activation/maturation are currently being driven towards formal validation, but the process of acceptance is slow and can take up to 15 years.

Can you elucidate some *in vitro* methods that may identify the potential of chemicals to induce skin sensitisation?

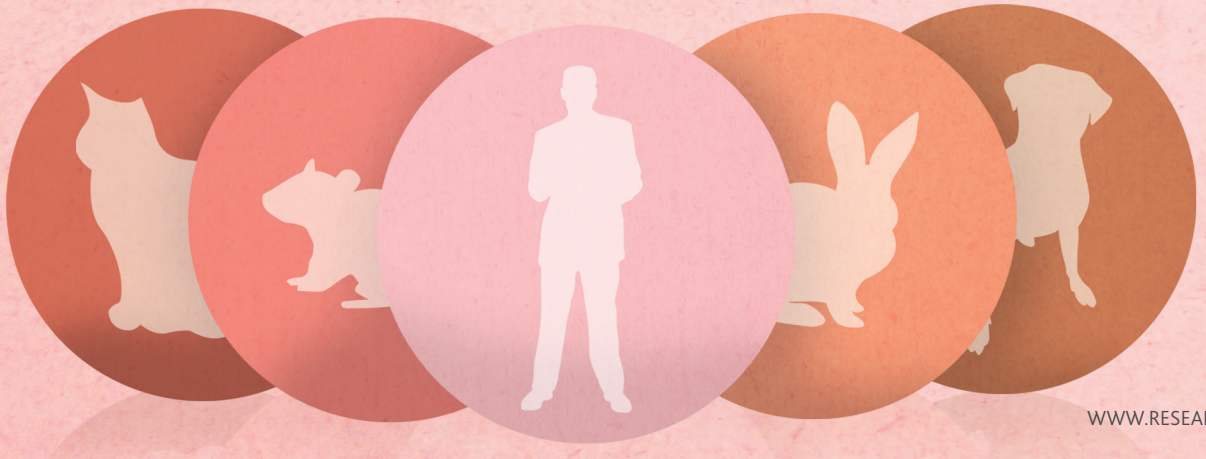
It is generally recognised that induction of contact dermatitis can be described by the six key mechanisms involved in sensitisation described above. With the exception of bioavailability, methods have been developed for each of these, ranging from computer-based assessment of the physicochemical and structural characteristics of a chemical over isolated cell systems to co-culture assays and three-dimensional (3D) reconstituted skin models. The readouts of these test systems are either single parameters or based on genomics and proteomics approaches allowing multiparametric analyses of pathways of toxicological concern.

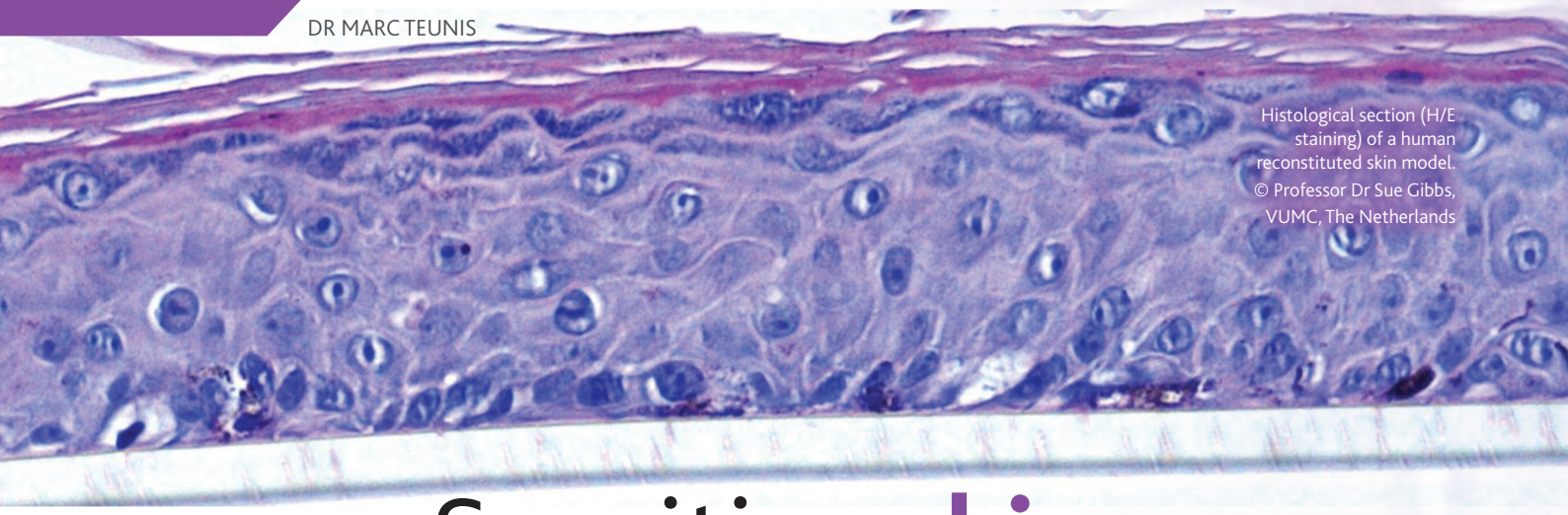
What are the advantages of testing chemicals in tiered strategies?

It is commonly accepted that no single assay can fully mimic all of the different *in vivo* aspects of sensitisation. One would need a set of specific, mechanistically driven assays to identify potential skin sensitisers with a non-animal approach. Another advantage of tiered testing is that, depending on the physicochemical properties of the chemical, it may not have to pass through the whole testing strategy. Sometimes a chemical will be labelled as a potential hazard after the first experiment, which saves time and money. Unequivocal results could lead to the testing of that particular chemical in animals in order to classify it. In order for a tiered approach to work, the applicability domain should be clearly defined in each tier.

Can you outline the importance of partnerships to your endeavours?

Confidence and trust in sharing views, comments and difficult issues are of the utmost importance in order to succeed. The project was initiated in 2009 and the participants remained involved over the complete duration – the participants collaborated very well together to make this project successful. Organising a formal end meeting has helped to evaluate the progress made and to make plans for future enterprises. The project also recieved substantial financial contributions from the partners, on top of the contributions from ZonMw and *Sens-it-iv*.





Histological section (H/E staining) of a human reconstituted skin model. © Professor Dr Sue Gibbs, VUMC, The Netherlands

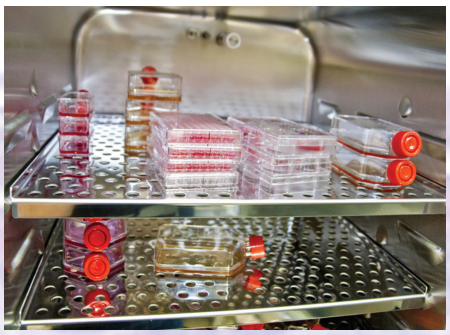
Sensitive skin

An innovative project funded by the **Netherlands Organisation for Health Research and Development** and ***Sens-it-iv*** – Prevalidation of a two-tiered approach for evaluating dermatological allergic reactions to chemical compounds – has determined a viable strategy for reducing the use of animals in experimentation

THE INCIDENCE OF allergies is increasing and so becoming an important health issue worldwide. An allergic reaction following the exposure of a tissue or organ – most often the skin or the lungs – to a sensitising agent or allergen is generated by the human immune system. Repeated exposure to chemical allergens as a result of environmental or occupational factors can exacerbate the immune response and engender severe dermatological or respiratory conditions.

The European Registration, Evaluation, Authorisation and Restriction of Chemicals regulation came into force in June 2007. This requires the testing and retesting of all substances produced or marketed in the EU if their quantity exceeds 1 tonne per year. Consequently, more chemical compounds have required testing for human safety in the last five years and the number of experiments conducted within the EU has increased. Testing of chemical compounds intended for application in cosmetics, agriculture and household products to assess the likelihood of their causing an allergic reaction in humans – either directly through contact with the skin, or indirectly through inhalation – is currently largely carried out using live animal subjects. For example, the Organisation for Economic Cooperation and Development (OECD) guidelines for skin sensitisation testing recommend testing on mice and guinea pigs, which account for more than 5 per cent of animals used in commercial tests. The current OECD guidelines require the use of 30 guinea pigs for the guinea pig maximisation test (GPMT) and Buehler test, and 25 mice for the local lymph node assay (LLNA). The latter is deemed to be a highly reliable predictor of sensitisation potential and is the preferred test in terms of animal welfare.

An incubator containing cell culture vessels: cells of mammalian origin are typically placed in a special incubator with temperature and CO_2 regulation: 37 °C, 5 per cent CO_2.



ALTERNATIVE STRATEGIES

A significant trigger towards animal-free testing is that the Seventh Amendment to the European Cosmetics Directive, which will be enforced from March this year, bans animal testing of ingredients in European and imported cosmetics. Viable alternative means of testing for hazards to human health from chemicals are currently actively being sought by the European Centre For the Validation of Alternative Methods (ECVAM) based in Ispra, Italy. The Centre's mission is to coordinate and compile independently evaluated non-animal tests so that reliance on animal test procedures is reduced.

Within the EU Sixth Framework Programme (FP6), a project called *Sens-it-iv* was designed to establish a non-animal testing strategy that could be used to replace current animal-based assays. *Sens-it-iv* centred on human biological markers for identifying allergic reactions and skin sensitisation, or dermatitis, and for classifying sensitisers according to their potency.

A further project was then staged under the auspices of the Netherlands Organisation for Health Research and Development (ZonMw) to evaluate the use and portability of two human cell-based assays that had been developed within *Sens-it-iv* and held promise as a substitute for the LLNA test in a combined two-tiered approach: the NCTC2544 assay to determine skin sensitisation capacity of a chemical and the epidermal equivalent potency assay which, as the name suggests, assesses the level of its potency.

This follow-on project involved a consortium of representatives from institutes across Europe. The project leader, Dr Marc Teunis from the Department of Innovative Testing, University of Applied Sciences in Utrecht, The Netherlands explains that the undertaking constituted a pre-validation exercise prior to attempting to gain ECVAM consideration for formal validation of the assays and methodology involved: "Performing a pre-validation can eliminate key hurdles to later success, which reduces disappointment and delays in getting the method accepted and implemented".

Epidermal skin equivalents are exposed to sensitisers and non-sensitisers, thereafter viability of the skin model is determined using a specific enzymatic colour reaction. The amount of staining is a direct measure for viability and for sensitising potential (panel **A**). The data can be used to calculate an EC50 concentration for a certain compound (panel **B**).

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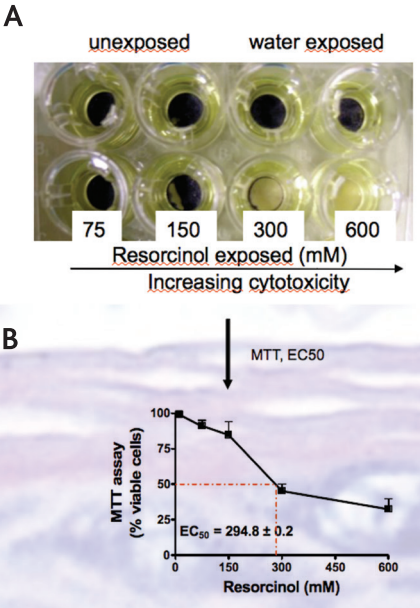

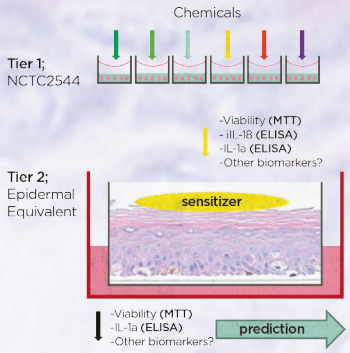

The two-tiered approach for determining skin sensitising capacity and potency of a chemical. Skin cells are exposed to ranging concentrations of chemicals after which IL-18 – a signal molecule of the skin-immune system – is determined, together with viability. Skin sensitising chemicals induce a significant increase in the levels of IL-18, compared to non-sensitisers. The second tier is a reconstituted epidermal skin model in which exposure of sensitisers leads to cell death and cytokine release. The amount of cell death is a measure for the potency of the compound.

PRE-VALIDATION APPROACH

A pre-validation study is designed not only to evaluate a proposed method but also to improve it. Teunis' project comprised the testing of a set of predefined but undisclosed chemicals by independent laboratories according to guidelines laid down by the European Reference Laboratory of ECVAM. Originally, three laboratories were to be involved, but an additional three volunteered to participate at their own cost.

Such a study is usually managed in two stages. The first stage is technology transfer to a number of laboratories. A small set of identified and disclosed chemicals is selected to train staff in the technology and to review the protocol, which is then adopted as the draft standard operating procedure (SOP). The second stage involves pre-validation, where a larger set of independently selected chemicals are submitted to double blind testing. The results of these tests are then analysed by an independent statistician. The project adopted this framework along with an additional preliminary step of investigating how easily the technology might be transferred to laboratories unfamiliar with the assay and method but experienced in the techniques required, and a second check against the training set of chemicals before the SOP was reviewed and finalised for the pre-validation stage.

The NCTC2544 and epidermal equivalent potency assays are based on keratinocyte cell cultures from human skin. The first tier test – the NCTC2544 assay – determines cell viability and production of intracellular concentrations of the cytokine IL-18 after exposure to chemicals at mildly toxic concentrations. The second tier potency test (reconstituted epidermal equivalent cultures) assesses cell viability and production of cytokines after exposure of the skin models to chemicals. All six laboratories participated in the first tier testing and four in the second. Training chemicals included three sensitisers and one non-sensitiser for the first tier test and two sensitisers of different potencies for the second. The chemicals then used in the double blind tests numbered 29 for the first tier and 13 for the second.

OUTCOMES AND OBSERVATIONS

Double blind testing was conducted over a period of nine months. Analysis of the large quantities of data obtained from these tests then took a further four months: "The results from the pre-validation study were very promising for the second tier potency assay," highlights Teunis. "Its predictive capacity was well within the required value and the potency class of the chemicals corresponded very nicely with *in vivo* data obtained in LLNA experiments." The results from the first tier were, however, not as good as expected which Teunis attributes to the low number and diversity of training chemicals, procedural oversights and the omission of formal training for laboratory staff.

Key lessons learned from the project were that planning should be highly structured and allow sufficient time for preliminary activities, which should include a double blind in-house chemical trial at the developing laboratory before the method is ported to other laboratories, and clear mapping of factors that might introduce variability in results. In addition, sufficient financial resources should be allocated to the independent selection and distribution of chemical samples for double blind trials, of the order of 5-10 per cent of a project's budget. Lastly, allowance should be made for the management of the huge quantities of test data and results accumulated in such a pre-validation study.

In addition to well-publicised ethical questions, testing on animals whose genetic makeup, physiology and biochemistry are so different from those of humans raises doubt about the efficacy of such an approach. While developing alternative tests for allergens is a complex and lengthy process, the use of *in vitro* approaches based on human cell cultures has the potential to substantially improve on the current paradigm in addition to reducing the burden placed on animals.

Key publications

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PRE-VALIDATION OF A TWO-TIERED APPROACH TO DETERMINING THE SKIN SENSITIZING POTENCY OF CHEMICALS AND CAPACITY

OBJECTIVES

To (pre)validate a method to evaluate the potential of a chemical to induce allergic dermatitis. The potential of a chemical to induce allergic reactions of the skin is an important health issue.

KEY COLLABORATORS

Dr Raymond Pieters, vice-Project Leader, University of Applied Sciences, Utrecht, The Netherlands • **Dr Erwin Roggen**, Prevalidation Manager, Novozymes, Denmark • **Professor Dr Sue Gibbs**, Principal Investigator, VU Medical Center, The Netherlands • **Dr Emanuela Corsini**, Principal Investigator, University of Milan, Italy • **Dr Charlotte Bernard Madson**, Principal Investigator, Danish Technical University, Denmark • **Dr Janine Ezendam**, Principal Investigator, National Institute for Public Health and the Environment, The Netherlands • **Dr Robert Landsiedel**, Principal Investigator, BASF, Germany

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CONTACT

Dr Marc Teunis
Senior Scientist

Research Centre for Technology and Innovation
University of Applied Sciences
FC Dondersstraat 65
3572JE Utrecht
The Netherlands

T +31 88 481 88 60
E marc.teunis@hu.nl

DR MARC TEUNIS performed a PhD project on Psychoneuroimmunology at the Wilhelmina Children's Hospital, Utrecht after having graduated in Biology at the Faculty of Biology, Utrecht University. He started teaching Immunology, Neurosciences, Animal Sciences and Pharmacology at the University of Applied Sciences in 2003.

In 2006, Teunis founded the research group on Innovative Testing with Dr Cyrille Krul, and coordinated the group until 2010 when he was appointed Senior Scientist. His current lines of research are on reproductive and immunological toxicology. Teunis' teaching focuses on toxicology and alternative approaches to animal use.