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Molecular interactions and underlying mechanisms of dendritic cell activation in skin sensitization

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Molecular interactions and underlying mechanisms of dendritic cell activation in skin sensitization

Tim Lindberg

LUND UNIVERSITY

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Abstract

Allergic skin diseases caused by low molecular weight chemical compounds are a major health concern among the general population, especially in certain occupational groups. To limit exposure to such compounds, it is important that thorough risk assessments are performed before products reach the consumer market. Traditionally, such risk assessments have been based on animal tests, but due to several factors, including legislations and ethical concerns, the use of animal testing has become increasingly unjustifiable. In this context, one focus in predictive toxicology has been the development of alternative methods to replace animal experiments. Although several alternative methods have been validated for regulatory decision making, they lack certain endpoints for complete risk assessments, and molecular mechanisms underlying skin sensitization are still not entirely understood. The work described in this thesis aims at increasing our knowledge about the mechanisms in dendritic cell activation in response to skin sensitizers and to further develop a dendritic cell-based *in vitro* assay.

In **Paper I**, we investigated the microRNA regulation in response to stimulation of a DC model with structurally similar rubber sensitizers. The changes triggered by the rubber sensitizers in both mRNA and microRNA expression suggest a chemical-specific regulation, despite the structural similarity of the rubber sensitizers. In **Paper II**, the skin sensitizing properties of herbicidal formulations are investigated. Here, we predicted the herbicide glyphosate as a non-sensitizer, while the co-formulant polyethylated tallow amine and two glyphosate-based formulations were predicted as sensitizers. Additionally, we also investigated the proteomic alterations in response to these chemicals and formulations, and identified cellular responses associated with the differentially expressed proteins. In the last two papers, **Paper III** and **Paper IV**, we present additional applications for the *in vitro* assay setup used in this thesis. In **Paper III**, we identified a biomarker signature for the prediction of skin sensitizer potency, demonstrating a balanced accuracy of 78% targeting three potency classes, i.e., 1A (strong sensitizers), 1B (weak sensitizers) and no cat (non-sensitizers). In **Paper IV**, we applied a statistical method, the conformal prediction framework, to investigate the predictive boundaries of the *in vitro* assay, and concluded, based on 70 chemicals, that the assay can be applied to a large chemical space.

In conclusion, the work presented here can contribute to a better understanding of the mechanisms underlying the immunological response to skin sensitizing chemicals, which together with state-of-the-art predictive assays could be used for improved risk assessment of chemical compounds and to develop tools for prevention and treatment of allergic skin diseases.

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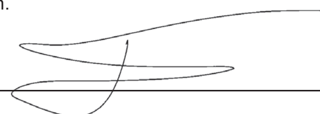

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Molecular interactions and underlying mechanisms of dendritic cell activation in skin sensitization

Tim Lindberg

Coverphoto by Juan Gärtner, illustrating a 3D rendering of a dendritic cell presenting antigen to T-lymphocytes.

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“You miss 100% of the shots you don’t take”

- Wayne Gretzky

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Author's contributions to the papers in this thesis

I. Involved in all parts of the experimental work and in the design of the study. Planned and performed data analysis and was the main writer of the manuscript.

II. Involved in all parts of the experimental work. Participated in design of study. Planned and performed data analysis and was the main writer of the manuscript.

III. Participated in design of the study and performed experimental work. Participated in data handing and statistical analysis. Involved in writing the manuscript. Read and approved the manuscript.

IV. Participated in design of the study and in data handling and statistical analysis. Read and approved the manuscript.

Original Papers

This thesis is based on the following Papers, which are referred to in the text by their Roman numerals (I – IV). Papers are appended at the end of the thesis.

I. Lindberg T., Forreryd A., Bergendorff O., Lindstedt M., Zeller K.S. *In vitro assessment of mechanistic events induced by structurally related chemical rubber sensitizers*. (Manuscript in revision).

II. Lindberg T., de Àvila R. I., Zeller K.S., Levander F., Eriksson D., Chawade A., Lindstedt M. *Skin sensitization testing and proteomic analysis of glyphosate, the co-formulant POEA and glyphosate-based formulations in a dendritic cell model*. (Manuscript).

III. Zeller K.S., Forreryd A., Lindberg T., Gradin R., Chawade A., Lindstedt M. (2017). *The GARD platform for potency assessment of skin sensitizing chemicals*. ALTEX, 34(4), 539-559.

IV. Forreryd A., Norinder U., Lindberg T., Lindstedt M. (2018). *Predicting skin sensitizers with confidence – Using Conformal Prediction to determine applicability domain of GARD.* Toxicology *In Vitro*, 48, 179-187.

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Additional Papers

i. Forreryd A., Zeller K.S., Lindberg T., Johansson H., Lindstedt M. (2016). *From genome-wide arrays to tailor-made biomarker readout – Progress towards routine analysis of skin sensitizing chemicals with GARD.* Toxicology *In Vitro*, 37, 178-188.

ii. de Àvila R. I., Veloso D. F. M. C., Teixeira G. C., Rodrigues T. L., Lindberg T., Lindstedt M., Fonseca S. G., Lima E. M., Valadares M. C. *Evaluation of in vitro testing strategies for hazard assessment of the skin sensitization potential of "real-life" mixtures: the case of henna-based hair coloring products containing p-phenylenediamine.* (Manuscript accepted for publication in Contact Dermatitis).

Abbreviations

AA	acylating agent
ACD	allergic contact dermatitis
AOP	adverse outcome pathway
APC	antigen-presenting cell
CD	cluster of differentiation
CLP	Classification, Labeling and Packaging
DA	defined approach
DC	dendritic Cell
DETBS	diethylthiocarbamylbenzothiazole sulfide
DMTBS	dimethylthiocarbamylbenzothiazole sulfide
DPCP	diphenylcyclopropenone
DPRA	direct peptide reactivity assay
EU	European Union
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
GARD	Genomic Allergen Rapid Detection
GPMT	guinea pig maximization test
h-CLAT	human Cell Line Activation Test
HRIPT	human repeated insult patch test
IATA	integrated approach to testing and assessment
IFN	interferon
IL	interleukin
KE	key event
LC	Langerhans cells
LLNA	local lymph node assay
MA	Michael acceptor

MHC	major histocompatibility complex
MIE	molecular initiating event
miRNA	microRNA
mRNA	messenger RNA
OECD	Organization for Economic Cooperation and Development
POEA	polyethylated tallow amine
PRR	pattern recognition receptors
QSAR	quantitative structure-activity relationships
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SVM	support vector machine
SVM DV	SVM decision value
TG	Test Guideline
Th	T-helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cell

1. Introduction

We are constantly exposed to chemicals present in various products such as hair dyes, household cleaning products and rubber gloves. Many of these chemicals can give rise to adverse immune responses in susceptible individuals and potentially cause severe reactions (Geier *et al.*, 2003; Handa *et al.*, 2012; Jongeneel *et al.*, 2018). Common inducers of these reactions are a particular group of such chemicals, termed sensitizers, which often are of low molecular weight. These chemicals can cause contact allergy, a disease with a prevalence of approximately 27% where the general population reacts to at least one allergen (Diepgen *et al.*, 2016). Allergic skin diseases are a major occupational health problem and in some countries considered the foremost disease in occupational work areas (Diepgen, 2003; Ring, 2017). If the exposure persists, the contact allergy can develop into a clinical manifestation of this condition called allergic contact dermatitis (ACD), which is a cell-mediated type IV hypersensitivity reaction (Rustemeyer *et al.*, 2012). Some of the cells involved in these reactions are dendritic cells (DC), keratinocytes and different subsets of T-cells, which all contribute to the observed symptoms of ACD, either by direct cellular interaction or release of inflammatory mediators (Kimber *et al.*, 2002). These symptoms include eczemas with profound instances of redness, itching and blisters, which could severely affect the quality of life. Furthermore, the symptoms and progression of ACD is often confused with, or indistinguishable from, related conditions such as atopic dermatitis, a hereditary disease and not acquired like ACD, or irritant contact dermatitis, which is an acute reaction with direct cellular injury due to contact with the chemical (Akhavan & Cohen, 2003).

The use and addition of chemicals in all sorts of products is essential to fulfil their function. It is, however, necessary to identify potentially dangerous chemicals to prevent adverse health effects caused by the products, and also to follow current legislations. Traditionally, different animal tests have been the main approach to determine if a chemical substance is hazardous or not. However, due to recent bans and legislative implementations, in addition to the ethical concerns associated with animal tests, there is a paradigm shift in

several parts of the world, including the European Union (EU), towards replacing or reducing animal tests with other non-animal approaches (Daniel *et al.*, 2018), thus, moving the field of toxicology closer to an animal-free testing standard. Several of the non-animal testing strategies are based upon measurements of a small set of biomarkers, while others utilize cutting-edge techniques coupled with different machine learning algorithms to predict the sensitizing hazards of chemicals. The common ground for the leading testing strategies is that they should cover a key event in the adverse outcome pathway (AOP) for skin sensitization (OECD, 2012). However, the immunological decision-making related to skin sensitization is still unclear, and more knowledge about how particular chemicals react in a biological system and what immunological pathways they activate can complement risk assessments for more precise decisions on the risks of a specific chemical.

The content of this thesis, which is based on four original research papers, aims to unravel underlying mechanisms in the immunological response towards skin sensitizers (**Paper I-III**). Additionally, **Paper III** and **Paper IV** concern the identification of a novel endpoint for an *in vitro* assay and investigation on its predictive boundaries, respectively. By using state-of-the-art techniques, targeting different omics areas such as transcriptomics or proteomics, we utilize the large amount of data gathered from our cellular stimulations to also elucidate mechanisms and genes regulated in response to sensitizing chemicals.

Paper I explored the regulation of the small non-coding RNA family of microRNAs (miRNA) in response to stimulation of a DC model with structurally similar rubber chemicals containing the same chemical reactivity domain. Expression profiling of miRNAs was conducted using the NanoString nCounter platform assaying 294 different miRNAs per sample. We identified few miRNAs that were commonly regulated in response to the investigated rubber chemicals. This observation was also mirrored in the transcriptomic analysis, with only 60% overlap of differentially regulated messenger RNA (mRNA) transcripts in response to stimulations with the rubber chemicals. Pathway analysis revealed the activation of unique pathways in response to each rubber chemical, as well as commonly activated pathways associated with known cellular events linked to skin sensitization. Together, these results indicate a chemical-specific immunological activation of DCs, possibly dependent on the unique structural properties of chemicals.

While **Paper I** focused on the gene regulatory mechanisms in DC activation and on a particular chemical reactivity, **Paper II** and **Paper III** focused on

more general skin sensitizing patterns by utilizing data gathered from proteomics and transcriptomics, respectively. **Paper II** aimed at investigating the immunotoxic aspects of glyphosate, a common agricultural herbicide, and two commercially available formulations containing this chemical. Here, we classified glyphosate as a non-sensitizer, while the two formulations were predicted as sensitizers. Furthermore, a surfactant commonly used in herbicidal formulations was also investigated and predicted as a sensitizer. Investigations of the pathways predicted to be activated in cells stimulated with the formulations and the surfactant revealed more pathways regulated by the formulations than by the surfactant. Additionally, the formulations and the surfactant commonly differentially regulated several protein groups linked to autophagy and cholesterol synthesis, suggesting a prominent role in the response to skin sensitizers for these processes. In **Paper III**, we investigated if the reactivity domains of chemicals influence the activation of DCs differently, by comparing predicted pathways induced by groups of chemicals that had different chemical reactivity domains. We observed a, to some extent, common response to chemical exposure, with prominent activation of cell cycle- and DNA damage-related pathways, but also chemical reactivity-specific activation of several pathways, many linked to immune-related events previously associated with skin sensitization. Also, with **Paper III**, a novel endpoint for an *in vitro* assay was investigated and expanded by introducing a biomarker signature for the prediction of skin sensitizer potency. In **Paper IV**, the boundaries of statistically valid predictions were explored, and the conformal prediction framework was implemented on *in vitro* assay predictions. This is a mathematical approach for determining the limitations of an assay to avoid false predictions on potentially harmful chemicals.

In summary, the combined discoveries in the papers included in this thesis contribute to a further understanding of the events involved in DC activation by skin sensitizing chemicals. By investigating the output from technologically advanced platforms, we have addressed several aspects of the response to skin sensitizing chemicals. In addition, a new approach for determining skin sensitizer potency was developed as well as the implementation of a mathematical method to establish the domains where an SVM-based assay gives reliable predictions.

2. Immunobiology of chemical hypersensitivity

The immune system is developed to protect us from foreign substances and pathogens that potentially can be harmful, and it consists of two parts: the innate and the adaptive immune system. While the innate immune system is responding to foreign substances with an immediate unspecific response, the adaptive immune system is shaped over time to encompass a specialized response against the antigen, which will enable faster and more persistent responses upon antigen reencounter. Even though this is a highly specialized system with several layers of checkpoints, characteristics of certain chemicals and allergenic proteins can give rise to adverse immune responses, such as hypersensitivity reactions or allergies (Paul, 2013).

This thesis addresses aspects of type IV hypersensitivity, also called delayed-type hypersensitivity. Compared to other hypersensitivity reactions, type IV does not involve antibodies but relies solely on cell-mediated responses, mainly from different subsets of T-cells (Uzzaman & Cho, 2012). A certain class of chemical substances, termed sensitizers, are common inducers of type IV hypersensitivity reactions. Sensitizers are often low molecular weight compounds, which are non-immunogenic themselves, that can penetrate the skin and react with endogenous proteins to form complexes capable of inducing an immune response in susceptible individuals.

The content of this chapter will cover the basis of the immunological reactions and responses towards chemicals that can elicit an adverse outcome such as ACD. There will also be a brief discussion around other relevant biological mechanisms and the presumptions that many of the predictive toxicological methods have been based upon (further discussed in **Chapter 3**).

2.1 Chemical properties linked to skin sensitization

Chemical compounds and mixtures are used on a daily basis, but the health risks associated with exposure to them is not always clear. First of all, not every chemical present in the environment around us is capable of eliciting an immune response, but many exist that are known to cause skin sensitization (De Groot, 2008). To begin with, a sensitizer needs to come in contact with cells in the dermal and epidermal layers of the skin as well as cells belonging to the immune system. This is done by breaching the outer layer of the skin, the stratum corneum, but lesions in the skin can facilitate easy penetration and uptake (Jakasa *et al.*, 2018). Further, chemical sensitizers are often defined as low molecular compounds with a molecular weight below 500 Da. However, recent data suggest that higher molecular weight compounds may also induce sensitization, independently of their ability to penetrate the stratum corneum (Fitzpatrick *et al.*, 2017a; Fitzpatrick *et al.*, 2017b; Roberts *et al.*, 2013).

Skin sensitizers are not capable of stimulating an immune response themselves, but rather need to react with endogenous proteins to form antigenic complexes, so-called hapten-protein complexes (Kimber & Dearman, 2003). In order to form this complex, skin sensitizers need to be inherently protein-reactive or activated to become protein-reactive, either metabolically in the skin (pro-haptens) or through autoxidation (pre-haptens) (Lepoittevin, 2006). These transformations turn the sensitizers into electrophilic compounds, in many cases the prerequisite for a chemical to act as a sensitizer, and they are thus capable of reacting with nucleophilic residues on the proteins. These residues are commonly found in amino acid side-chains such as cysteine and lysine (Divkovic *et al.*, 2005). The origin of the specific skin proteins involved in the formation of antigenic complexes is still not clear, but keratins in the skin are suggested as potential targets (Bauer *et al.*, 2011; Simonsson *et al.*, 2011). Although most chemical sensitizers need to have electrophilic characteristics to become antigenic molecules, some groups of contact allergens, mainly metal ions, have been recognized to interact directly with natural proteins in the groove of major histocompatibility complex (MHC) class II molecules in order to promote their allergenicity (Romagnoli *et al.*, 1991; Van Den Broeke *et al.*, 1999).

The mechanistic reactivity domains of skin sensitizers are functional reaction groups present in the structure of the chemical that is associated with its reactivity towards skin proteins. The most common reactivity domains are Michael acceptors (MA), acylating agents (AA), Schiff base formers (SBF), nucleophilic aromatic substitution (S_NAr) and uni- or bi-molecular nucleophilic substitution (S_N1/S_N2) (Aptula & Roberts, 2006). Additionally, several other chemical structures have been associated with the capacity to induce skin sensitization, for example aldehydes and lactams (Gerner *et al.*, 2004).

Another attribute to consider for skin sensitizers is their intrinsic sensitizing potency, i.e., the threshold dose where a sensitizer is able to either induce sensitization or elicit an ACD reaction (Kimber *et al.*, 2001). To this end, the aforementioned mechanistic reactivity domains have been suggested as important features in defining the skin sensitizing potential of chemicals (Chipinda *et al.*, 2011).

2.2 The immune response towards chemical sensitizers

A trait shared by all types of hypersensitivity reactions are the two phases, induction and elicitation, which describe the events necessary for an adverse outcome to occur. The induction phase (also called the sensitization phase), is a series of events that include the first encounter with the chemical compound leading to a specific adaptive immune response, orchestrated in part by cells from the innate immune system. Upon recurrent exposure to the specific chemical compound, the second phase is initiated. In this phase, called the elicitation phase or the effector phase, the specialized adaptive immunity is responding by recruiting compound-specific memory T-cells to the inflamed area, which in turn produces all kinds of inflammatory mediators and promotes infiltration of different effector cells, which together result in the symptoms of an allergic reaction (Kimber *et al.*, 2002; Silvestre *et al.*, 2018). A more detailed description of these two phases follows.

During the induction phase, the newly formed hapten-protein complex triggers an array of immune-related responses from several cells residing in the epithelial layers of the epidermis. Among the first cells encountered are the keratinocytes, acting as an initial line of defence, which also contribute to

the subsequent immune response during the elicitation phase of ACD (Albanesi, 2010; Kaplan *et al.*, 2012). Upon contact with the antigenic complex, keratinocytes secrete a large number of pro-inflammatory cytokines and chemokines necessary for the progression of the adaptive immune response (Corsini & Galli, 2000; Uchi *et al.*, 2000). The main mediators in terms of skin sensitization, secreted by keratinocytes, are tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-18, which are all important for maturation of the dermal antigen-presenting cells (APC) and their migration to a draining lymph node (Cumberbatch *et al.*, 2001). The release of pro-inflammatory mediators, and the activation of the innate immune system in response to stress induced by the antigenic complex, is required for a full-scale adaptive immune response to occur. Without these complementary signals, termed "danger signals" (Matzinger, 1998), the antigenic stimulation from the hapten-protein complex could induce tolerance instead (McFadden & Basketter, 2000). The danger signals are for example molecules released by cellular stress or cell death, so-called damage-associated molecular patterns, and are sensed by the immune cells through receptors called pattern recognition receptors (PRR). Furthermore, advances in the understanding of innate recognition of chemical haptens suggest an important role for several PRRs including the Toll-like receptors (TLR), for example the crucial role of TLR4 in development of contact allergy to nickel (Schmidt *et al.*, 2010), and the recognition of hapten-specific pathogen-associated molecular patterns through the inflammasome complex. These mechanisms subsequently activate immune cells to produce pro-inflammatory cytokines and engage the skin sensitizing compounds (Kaplan *et al.*, 2012).

In addition to keratinocytes, APCs in the epidermis and dermis are most important for orchestrating the subsequent immune response, serving as a link between the innate and adaptive immune system. Although Langerhans cells (LC) and dermal DCs are considered the main APCs in skin sensitization, the precise contributions from each subset remain unclear (Kimber *et al.*, 2009; Peiser *et al.*, 2012). Therefore, for the remainder of this thesis, if not specified otherwise, the APCs involved in the skin sensitization reaction will simply be summarized as DCs.

Upon recognition of the hapten-protein complex, the phenotype and function of the DCs are changed from antigen processing to immunostimulatory. This result in a maturation process that includes upregulation of co-stimulatory receptors, such as cluster of differentiation (CD) 80 and CD86 as well as MHC class I and II molecules for antigen presentation, in order to orchestrate

an adaptive immune response. Further, after initial antigen encounter the DCs migrate from the skin to local draining lymph nodes where they interact with naïve T-cells. The migration towards lymph nodes is enabled by upregulation of CCR7, and response towards chemotactic gradients of CCL19 and CCL21 present in high endothelial venules *en route* to, and on cells residing, in the local lymph node environment (Rustemeyer *et al.*, 2012).

At the site of the local draining lymph node, the matured DC presents the processed hapten-protein complex through MHC molecules to the T-cell receptor (TCR) on naïve T-cells. When the T-cells recognize the processed complex they are differentiated into heterogeneous hapten-specific effector subsets of $CD4^+$ T-helper (Th) 1 cells and $CD8^+$ cytotoxic T-cells (Cavani *et al.*, 2001; Martin, 2004). For a successful priming of T-cells, different co-stimulatory signals are necessary, for example interaction between T-cell specific CD28 and CD80/CD86 on DCs. In addition, the polarization of the T-cell subsets is driven by the cytokine profile during the priming of the T-cells, with IL-12 promoting type 1 T-cells, the main type associated with ACD, which produces interferon (IFN)-γ, IL-2 and TNF-α. After activation, the hapten-specific T-cell subsets differentiate into effector and memory cells and return to the circulation (Vocanson *et al.*, 2009).

Following sensitization of a susceptible individual to a specific chemical sensitizer, elicitation can occur upon subsequent exposure to the same hapten. This triggers an inflammatory response at the site of entry that activates the surrounding cells, including DCs and keratinocytes. At this stage, hapten-specific T-cells are recruited from the circulation attracted by the pro-inflammatory cytokine and chemokine cascades released from cells activated by the inflammatory environment. Upon interaction with hapten-specific DCs, the T-cells become activated and further amplify the inflammatory response by releasing more pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-4. Following the specific activation of T-cells, other inflammatory mediators are recruited to the area of inflammation in response to the increase in cytokine release. The characteristic clinical symptoms associated with ACD, including tissue damage and cutaneous lesions, are mainly a consequence of the infiltration of different leukocytes, among them the main effector cells, $CD8^+$ cytotoxic T-cells (Martin *et al.*, 2011; Vocanson *et al.*, 2009).

Importantly, several mechanisms to resolve the immune response towards skin sensitizers exist. IL-10, a cytokine involved in anti-inflammatory responses and deregulation of DC migration, is an important molecule in this

response (Cumberbatch *et al.*, 2003). IL-10 is produced by several types of skin resident cells, including mast cells and keratinocytes (Vocanson *et al.*, 2009). Additionally, a subset of T-cells called regulatory T (Treg) cells has been implicated as major players in the regulation and resolution of ACD. Their main regulatory function is exerted by the secretion of anti-inflammatory cytokines, mainly IL-10, and their subsequent suppressive function of effector T-cells (Silvestre *et al.*, 2018).

In summary, the events necessary for the development of contact allergy and subsequent manifestation of ACD can be divided into four steps, (1) hapten-protein complex formation, (2) stimulation and release of danger signals from cells residing at entry point, for example keratinocytes, (3) immunogenic activation and uptake of hapten-protein complex by DCs and (4) activation and proliferation of specific T-cell subsets. These central steps have been summarized into a so-called adverse outcome pathway (AOP) for skin sensitization, further discussed in **section 2.4**.

2.3 microRNAs in allergic skin disease and gene regulatory function

The transcription of mRNA and protein translation is essential in different responses induced by e.g. cellular stress or pathogen invasion. However, malfunctions in the regulation and maintenance of the transcription is a major factor in disease origin and progression (Lee & Young, 2013). The small non-coding RNA family of miRNAs is a group of biomolecules linked to several regulatory mechanisms. They exert their regulatory function by post-transcriptional complementary binding of the mRNA transcript, which induces silencing or disruption of the transcript (Bartel, 2004). Through this regulatory interaction, miRNAs play important roles in the pathogenesis of several diseases, including different cancers and the autoimmune skin disease psoriasis (Hawkes *et al.*, 2016; Rovira *et al.*, 2010). By being involved at all stages of a disease, miRNAs are ideal targets for therapeutic purposes as well as being prominent biomarkers for the identification of disease patterns (Wang *et al.*, 2016).

Although miRNAs have been identified as candidate biomarkers in many diseases, knowledge about their involvement in allergic skin diseases such as ACD is currently limited. However, a few studies have shown deregulation

of miRNAs in response to skin sensitizing chemicals. One study identified four miRNAs, miR-21, miR-142-3p, miR-142-5p and miR-223, to be upregulated in response to the chemical sensitizer diphenylcyclopropenone (DPCP) in chemically-challenged human skin biopsies (Vennegaard *et al.*, 2012). Furthermore, another study on healthy volunteers challenged with DPCP suggested a time-dependent expression of miRNAs in response to skin sensitizers (Gulati *et al.*, 2015). Unique miRNA profiles were identified at different time points post-chemical challenge, with a few miRNAs persisting until the last recorded time point of 120 days. Additionally, two separate studies have suggested an important role for miRNAs in the modulation of T-cell responses in allergic skin diseases. Firstly, miR-210 was suggested to have an inhibitory influence on the function of Treg cells upon chemical sensitization (Long *et al.*, 2016), and secondly, miR-150 was identified as mediating effector T-cell suppression in hapten-challenged mice (Bryniarski *et al.*, 2013). In addition, several miRNAs have been shown to be part of the differentiation and function of DCs (Smyth *et al.*, 2015), which are central players in the skin sensitization reaction. Altogether, these results suggest an important role for miRNAs at several stages in allergic skin diseases. Better understanding of the role of miRNAs in the mechanisms underlying the molecular and cellular events in skin sensitization could contribute to identify predictive biomarkers and aid in the prevention and treatment of ACD.

The lack of knowledge about the role for these regulatory molecules in skin sensitization was one of the major incentives for **Paper I**. Our group has earlier shown that transcriptional changes induced by chemical stimulation of a DC-based *in vitro* model predicted a de-regulation of several members of the let-7 miRNA family in response to skin sensitizing chemicals with different chemical reactivity domains (Albrekt *et al.*, 2014). In **Paper I**, following up the previous results on let-7 and chemical reactivity domains, we investigated the differential regulation of miRNAs in response to a particular group of sensitizers, namely rubber chemicals. Included in the chemical stimulations were two structurally similar chemicals, diethylthiocarbamylbenzothiazole sulfide (DETBS) and dimethylthiocarbamylbenzothiazole sulfide (DMTBS). They belong to the same reactivity domain, AA, and only differ in structure by DETBS having a diethyldithiocarbamate instead of the dimethyl analogue in DMTBS. Interestingly, the two chemicals activated different miRNA profiles displaying little overlap. Thus, the results in **Paper I** indicate a chemical-specific regulation of miRNAs in the DC model, which could be influenced by, possibly unknown, chemical interactions with proteins or the antigen

processing of DCs. These interactions could be the cause for a specific activation of the immune system in response to sensitizing chemicals. This would fit to observations made in clinical tests on patients, where exposure to DMTBS results in a stronger reaction compared to DETBS exposure (Bergendorff *et al.*, unpublished).

2.4 The adverse outcome pathway for skin sensitization

In predictive toxicology (further described in **Chapter 3**), the steps involved in the immune response towards skin sensitizers have been summarized in what is called an adverse outcome pathway (AOP). The AOP concept was initiated in 2010 (Ankley *et al.*, 2010), and is a framework for the overview of biological events, starting with one molecular initiating event (MIE) and always ending with one adverse outcome. In-between the MIE and the adverse outcome there could be several sequentially linked key events (KE) that are based on biologically relevant cellular responses or molecular interactions. AOPs are suggested to help in developing new mechanistic-based methods by streamlining the knowledge leading up to the adverse outcome (Wittwehr *et al.*, 2017). Further, the AOP idea aligns well with the notion from the distinguished report provided by the US National Academy of Science that the future of risk assessment and toxicity testing should preferably encompass *in vitro* systems based on human cells with measurements of cellular pathways changing in response to chemical exposure (NRC, 2007; Vinken, 2013). This view of incorporating mechanistic events into toxicity testing has been adopted by several of the regulatory agencies in the world (Edwards *et al.*, 2016; Leist *et al.*, 2014).

Although the AOP concept was designed to support the development of new mechanistic-based methods it is not without certain shortcomings. As an example, the proposed linearity from the MIE to adverse outcome might not be a true reflection of the reality, as feedback loops and modulators could affect a specific KE from several directions. Also, AOPs are independent of the intrinsic chemical properties of a compound, and do not describe the chemical´s mode of action for triggering an adverse outcome (Leist *et al.*, 2017). The compound only needs to have the ability to induce the MIE leading to the adverse outcome, which creates the possibility that some

information important for risk assessment could be lost along the way. However, other tools and concepts can be used together with AOPs to account for some of the limitations, and complement with quantifiable information (Leist *et al.*, 2017).

The concept of AOPs has been applied to describe several adverse outcomes (Vinken *et al.*, 2017), with the AOP for skin sensitization established by the Organization for Economic Cooperation and Development (OECD) in 2012 (OECD, 2012). The skin sensitization AOP comprises four KE, protein binding to endogenous proteins (KE1), activation of keratinocytes (KE2), activation of DCs (KE3), and proliferation of T-cells (KE4), all previously described in **section 2.2**. These KEs have been the targets during the last decade's development of new alternative assays for prediction of skin sensitization hazard (outlined in detail under **section 3.3**). As such, the AOP concept aids in supporting regulatory decision-making by describing a foundation where mechanistic data is stored and viewed in relation to associated events. Still, more knowledge about the specific molecular and cellular reactions that happen in connection with each KE is necessary to fully understand the consequences of exposure to a specific chemical, and to fuel the development of therapeutic tools or preventative measures to avoid adverse outcomes such as ACD.

3. Assessment of chemical sensitizers

The previous chapter discussed the sensitizing properties of chemicals and immunological events preceding an adverse outcome. Here, the usage of information from these events is translated into risk assessments of chemicals to prevent outbreaks of contact allergy. Due to the chronic nature of acquired contact allergy, exposure to sensitizers can cause life-long dermatitis. As complete avoidance of the sensitizing agent is the only way to prevent elicitation of ACD, it is imperative to implement proper risk assessments followed by appropriate risk management to characterize and reduce the exposure to compounds giving rise to contact allergy (Kimber *et al.*, 2002; Peiser *et al.*, 2012). Past examples have shown that strict regulations to minimize the exposure to a sensitizing agent has drastically decreased the occurrence of new ACD cases (Thyssen *et al.*, 2007). Historically, animal tests have been used to assess the allergenic properties of chemical compounds and despite limitations and ethical concerns associated with animal tests, they are still used (Daniel *et al.*, 2018). In this context, the last decade's development of non-animal alternative assays has moved the field of toxicology towards replacement of animal testing strategies.

The following chapter will give a brief overview of important legal frameworks to consider in skin sensitization testing. Additionally, the most common animal methods as well as current alternative assays, and their roles in determining skin sensitization commercially, will also be presented. Finally, the *in vitro* method used for the studies included in this thesis is described more in detail.

3.1 Legal frameworks and incentives for replacement of animal testing strategies

A new era of toxicity testing begun with the introduction of the three Rs in the 1950s (Russell *et al.*, 1959). Representing reduction, refinement and replacement of animal experiments, the three Rs can be utilized in various situations to introduce novel methods for toxicity testing. Applications such as reducing the number of animals required for the same results or refine the way animals are treated to induce less pain in the tests are means now well-established. In addition, demands from new legislations and regulatory implementations have spurred the development of alternative methods to replace animal studies. The REACH regulation, short for Registration, Evaluation, Authorization and Restriction of Chemicals, which was introduced in the EU in 2007, dictates that a substance with an output of more than one metric ton per year needs to be thoroughly tested and evaluated for safe use (EU, 2006). This would be demanding on the number of animals required to perform comprehensive toxicity tests of chemicals under the REACH regulation, with worst case estimates of 141 million animals needed for compliance with REACH (Hartung & Rovida, 2009). Further, Annex VII of the REACH legislation states that information from validated alternative methods should be used as a first step to determine the potential skin sensitizing properties of a chemical, and only if the information is insufficient to perform an adequate risk assessment should *in vivo* data be collected (EUR-Lex, 2017).

Thus, in the light of the last decade's progress in development of new *in vitro* alternative testing strategies, and also the implementation of regulatory frameworks on prohibiting the use of animals for testing of cosmetic ingredients (EU, 2009), the effort towards replacing animal methods has gained momentum. Test methods should be able to assess the skin sensitizing properties of a chemical, including skin sensitizing hazard and sensitizer potency, to achieve a proper risk assessment and provide safe guidance for the use of chemicals (Basketter, 2008). Performance of testing strategies are frequently evaluated using cooper statistics (Cooper *et al.*, 1979), that is the accuracy, sensitivity and specificity, which is obtained by comparing the predicted outcome to a reference of "correct" outcome. Furthermore, the Classification, Labelling and Packaging (CLP) regulation (EU, 2008), requires producers to appropriately classify, label and package the products before distribution. Revision of this regulation has added the notion to

subcategorize the sensitizer potency according to the categories 1A (strong sensitizers), 1B (weak sensitizers) or no cat (non-sensitizers) when sufficient data is available (EU, 2011).

3.2 Animal testing strategies for skin sensitization

Animal models for testing of skin sensitizing chemicals have been around for decades, with two of the most prominent being the Buehler method (Buehler, 1965) and the guinea pig maximization test (GPMT) (Magnusson & Kligman, 1969). The local lymph node assay (LLNA) was developed as a way to improve the previously established animal tests (Basketter *et al.*, 2002). Its introduction greatly reduced the number of animals needed per substance and also contributed to less distress and pain inflicted on test animals compared to the GPMT, a good illustration of reduction and refinement (Dean *et al.*, 2001). The LLNA readout is a measure of the proliferation of lymphocytes in draining lymph nodes after topical exposure to the ear of mice with the testing chemical. More precisely, each concentration of a test chemical is compared to mice treated with vehicle control and for a chemical to be classified as a sensitizer, one or more of those test concentrations must result in a threefold increase in lymphocyte proliferation (OECD, 2010). This value is called the stimulation index and can also give information regarding the skin sensitizing potency of the tested chemical. The effective concentration resulting in a threefold increase in lymphocyte proliferation, termed the EC3 value, indicates the potency of the assayed chemical. A lower EC3 value means a higher potency for the chemical, i.e., a lower amount of chemical is needed to induce a proliferative response and thus is deemed a stronger sensitizer (Basketter *et al.*, 2000).

In general, the LLNA is a test system incorporating the interaction between many cells in the skin sensitization reaction, as opposed to most *in vitro* methods, which usually target one KE, as described in the skin sensitization AOP. Although the results obtained from LLNA are currently seen as the gold standard for toxicity testing, it is not without limitations (Basketter *et al.*, 2009). Due to interspecies variability, one major concern has been the extrapolation of results from rodents to humans, but also the tendency of LLNA to produce false positives, i.e., predicting skin irritants as skin sensitizers (Anderson *et al.*, 2011). However, some studies have indicated a good correlation between human data and results obtained from the LLNA

(Api *et al.*, 2015; Schneider & Akkan, 2004). Still, considering LLNA is a method involving animal tests, there have been an increasing drive within many parts of the world, including Europe, to replace animal studies with *in vitro* alternatives when possible (Adler *et al.*, 2011).

3.3 Non-animal approaches for skin sensitization testing

Driven by ethical considerations and adaptation to recent legislations, new alternative methods to animal testing have emerged over the past decades. Comprehensive reviews on existing non-animal methods for skin sensitization prediction are available (Ezendam *et al.*, 2016; Reisinger *et al.*, 2015), and this section will provide an overview about a few selected assays. The common trait shared by all these alternative assays is that they target one of the KE in the skin sensitization AOP.

To begin with, the direct peptide reactivity assay (DPRA), which is an *in chemico* assay, targets the MIE of skin sensitization by measuring the protein reactivity of chemicals (KE1) (Gerberick *et al.*, 2004). Incubation of test chemicals with synthetic peptides of cysteine or lysine allows for hazard classification of chemicals by measuring the peptide depletion with high-performance liquid chromatography. Subsequent depletion values are then used in a prediction model to classify the chemical as either a sensitizer or non-sensitizer. Another assay targeting the initial stages of skin sensitization is the ARE-Nrf2 luciferase test method (KeratinoSens™) (Natsch, 2010). Here, keratinocyte activation (KE2) is used to predict sensitizing chemicals by utilizing cells derived from HaCaT human keratinocytes transfected with a plasmid containing a luciferase gene fused with an ARE element from the human AKR1C2 gene. The ARE element is activated through the Keap1-Nrf2-ARE pathway in response to skin sensitizing chemicals, and is detected through the luminescent signal corresponding to Nrf2-dependent activation. The Keap1-Nrf2-ARE pathway is a prominent cellular defence mechanism against xenobiotic damage and oxidative stress, and the transcription factor Nrf2 has been associated with inducing genes activated in response to skin sensitizers (Emter *et al.*, 2013). Another assay targeting KE2 is the LuSens assay (Ramirez *et al.*, 2014), also based on measurements of a luciferase gene, but it is under control by an ARE element from the rat NQO1 gene.

Furthermore, several *in vitro* assays have been developed that focus on KE3, DC activation. For instance, the human Cell Line Activation Test (h-CLAT) is an assay measuring the changes in cell surface expression of CD86 and CD54 in the human monocytic cell line THP-1 in response to skin sensitizing chemicals (Ashikaga *et al.*, 2006; Nukada *et al.*, 2011). Another assay associated with the DC activation step is the U-SENS™ assay (Piroird *et al.*, 2015), which evaluates changes of CD86 expression in the U937 cell line. As the field of predictive toxicology is continuously evolving and expanding, many more non-animal methods exist, but the ones presented here have been formally validated by OECD (discussed further in the next section, **3.4**).

Additionally, the assay used in this thesis, the Genomic Allergen Rapid Detection (GARD) assay (Johansson *et al.*, 2011), an *in vitro* assay asserted to KE3, utilizes transcriptomic alterations of 200 biomarkers in a derivative of the myeloid cell line MUTZ-3 to predict the skin sensitization hazard of chemicals. GARD is further developed and brought to market by SenzaGen AB, and registered under trademark GARD™. This product includes several endpoints and has been developed from the experimental setup of the *in vitro* assay used in this thesis. Two endpoints, GARD™skin and GARD™potency, are currently under review by OECD for formal regulatory validation. The experimental setup and other important aspects of the assay are presented further in **section 3.5**.

Another area of non-animal approaches developed for skin sensitization prediction is *in silico* methods. One such approach is the quantitative structure-activity relationships (QSAR), which utilize predictors based on different physicochemical properties of a certain chemical, as reviewed in (Patlewicz *et al.*, 2008). The chemical to be evaluated is compared to for example structural alerts to find similarities indicating whether it has sensitizing properties. However, as of now, *in silico* models are not yet at a stage where they alone can be used for prediction of either sensitizing hazard or potency (Teubner *et al.*, 2013; Verheyen *et al.*, 2017). Still, due to their cost-effectiveness and ease of use, QSARs have been suggested as complements to other alternative methods (Patlewicz *et al.*, 2014; Tollefsen *et al.*, 2014).

3.4 Routine testing and transition to animal-free testing strategies

For the transition from animal models to animal-free testing strategies to become the accepted way of performing toxicity testing, regulatory authorities must implement the decisions and assessments provided by the alternative methods. As a first step to accomplish this, alternative methods need to be formally validated and accepted for regulatory adoption. This is supervised by independent validation bodies such as the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) in the EU. Further, the OECD provides Test Guidelines (TG) for the validated methods to be used for regulatory purposes (Griesinger *et al.*, 2016). Currently, six alternative assays have been formally validated and given OECD TG, namely DPRA (OECD TG 442C), the ARE-Nrf2 luciferase test methods KeratinoSens™ and LuSens, both under OECD TG 442D and h-CLAT, U-SENS™ and IL-8 Luc assay, all described in OECD TG 442E (Casati *et al.*, 2018; OECD, 2018). However, the validated test methods are not able to predict all necessary endpoints for a comprehensive risk assessment. For example, all currently validated assays predict the skin sensitization hazard, i.e., if a chemical is sensitizing or not, and not the potency of the assayed chemical, which is crucial for a complete risk assessment. Although several assays have made an effort to predict sensitizer potency, like the U-SENS™ assay (Piroird *et al.*, 2015), none are yet validated for this purpose (ECHA, 2017). This information must then be provided by additional tests, i.e., animal experiments. This has led to difficulties for alternative test methods when it comes to industrial acceptance. One concern of the industry is that the use of animal tests still might be required by regulatory authorities, as not enough or appropriate information is given by the alternative methods and in consequence, is making them redundant (Clippinger *et al.*, 2016).

As the current belief is that no stand-alone assay will be able to provide all the information necessary for a complete risk assessment (Rovida *et al.*, 2015), innovative strategies have been suggested to integrate science-based information from different sources to make the best decision possible, despite the fact that test methods lack certain aspects of chemical risk assessment (MacKay *et al.*, 2013). Such an approach used in toxicity testing is called integrated approach to testing and assessment (IATA) where results from different methodological approaches are weighed against each other. An

IATA should strive for incorporating all available data to perform risk and safety assessments, which is in concordance with the concept of evidence-based toxicology (Hoffmann & Hartung, 2006). Examples of subcategories included in an IATA could be information derived from the AOP framework, which could form a mechanistic basis for the decision, or information provided from the use of several alternative assays in defined approaches (DA) (Casati *et al.*, 2018). DAs, or integrated testing strategies, are rule-based decisions originating from predictions provided by the alternative assays (OECD, 2017). In their simplest form, DAs only give information about the skin sensitization hazard through majority voting in a "2 out of 3" combination of alternative assays (Bauch *et al.*, 2012). The scientific validity of such an approach has been questioned as the actual added value from combining several methods could be overestimated compared to a top-performing stand-alone assay (Johansson & Gradin, 2017; Roberts & Patlewicz, 2018). A comprehensive review on existing DAs is provided in (Kleinstreuer *et al.*, 2018), where all the evaluated DAs are performing equally good or better than the gold standard LLNA in terms of both skin sensitization hazard and potency. However, no DA has yet gained regulatory acceptance, thus, the use of animal tests still remains (Daniel *et al.*, 2018)

In conclusion, to correctly predict skin sensitizers, both animal-based and alternative methods need to have their strengths and limitations defined and carefully weighed against each other. To account for some of these limitations, the current state of predictive toxicology moves towards integration of several methods to compensate for the shortcomings of each individual assay. However, the possibility of a stand-alone testing strategy is not entirely impossible (Roberts, 2018).

3.5 GARD – Genomic Allergen Rapid Detection

The research presented in this thesis is focussed on the use and discovery of other endpoints for the experimental setup of the GARD™ assay, which is a multiparametric assay targeting KE3, DC activation, in the skin sensitization AOP. Initially developed to predict skin sensitizing hazard, the assay measures the transcriptional changes in a biomarker signature, comprising 200 genes following chemical exposure. These 200 genes were identified using complete genome transcriptomic analysis of MUTZ-3-derived cells stimulated with a panel of well-characterized sensitizing and non-sensitizing chemicals (Johansson *et al.*, 2011), and a detailed description of the initial protocol has been published (Johansson *et al.*, 2013).

In brief, the concentration of the sensitizer is titrated for cytotoxic chemicals targeting a relative cell viability of 90%. If no toxicity or solubility issues are observed, the chemical is used at 500 µM. Cell stimulations are then performed in biological triplicates with each test chemical and after 24 h stimulated cells are harvested and RNA is purified. Following the RNA purification, transcriptome analysis of the cells is performed using Affymetrix Human Gene 1.0 ST Arrays. During the initial development, the 200 genes comprising the predictions signature were filtered out from redundant and uninformative genes using p-value filtering, backward elimination and machine learning based on a Support Vector Machine (SVM) (Cortes & Vapnik, 1995). The SVM prediction model is thereon used for predicting the sensitizing capacity of test chemicals previously unseen to the model. It generates an SVM decision value (SVM DV) for each unknown test chemical, which indicates if the chemical is classified as a sensitizer (positive SVM DV) or a non-sensitizer (negative SVM DV).

By utilizing an *in vitro*-model of human myeloid cells, the assay reflects an integral part in the response of APCs to skin sensitizing chemicals. DCs are central players in this response, and involved in initiating adaptive immune responses, and therefore, obvious targets for mechanistic studies about the complex events leading to an adverse outcome like ACD. The human DC cell model used for the cellular stimulations in the assay is a derivative of the acute myelomonocytic leukaemia cell line MUTZ-3 (Hu *et al.*, 1996). These cells serve as $CD34^+$ progenitors to DCs, which through cytokine stimulation can differentiate into populations of $CD14^+$ precursors of LCs and dermal DCs (Santegoets *et al.*, 2006). Further characterization has demonstrated the ability of MUTZ-3 cells to endocytose antigens and present them through

functional MHC class I and II molecules as well as CD1d (Masterson *et al.*, 2002). In addition, MUTZ-3 cells have been shown to be able to activate naïve T-cells antigen-independently, and have a similar transcriptional expression profile to that of *in vivo* DCs (Larsson *et al.*, 2006).

As an important step in the development from a biomarker discovery platform into a state-of-the-art testing platform for commercial use, the microarray format was identified as a throughput-limiting step. Therefore, the gene expression measurements were transferred from whole genome transcriptomics to the NanoString nCounter platform to allow customized analysis of the 200 genes in the prediction signature (Forreryd *et al.*, 2016), now employed by SenzaGen in the commercialization of the GARD™ assay. In a recent evaluation of the accumulated predictive performance on skin sensitizing hazard by the GARD™skin assay, an accuracy of 86% across a dataset comprising 127 chemicals in total was reported (Johansson *et al.*, 2017).

While the initial assay was developed to predict skin sensitization hazard, the assay format, as an alternative method to animal testing, has further been shown to be applicable to other endpoints such as chemical respiratory sensitization (Forreryd *et al.*, 2015) and sensitizer potency (**Paper III**). Further, a framework to assure statistically valid predictions (**Paper IV**) has been investigated on the assay format, which will be further described in **section 4.2.1** together with the initial identification of a biomarker signature linked to sensitizing potency of chemicals (**Paper III**). Although the work performed in this thesis has contributed to the expansion of the assay, the main scope has been to go beyond the assay development and investigate underlying mechanisms in the DC activation step of skin sensitization (**Paper I-III**).

In summary, by utilizing the high predictive power that the GARD™ assay format generates, as an *in vitro* alternative to animal models, I believe it will be an important component for future non-animal-based risk assessments. Furthermore, an advantage of using the initial microarray assay setup, utilized in this thesis, is the opportunity to collect large datasets in order to gather mechanistic information about DC activation in skin sensitization.

4. Predictive toxicology beyond binary skin sensitization testing

The field of predictive toxicology has been moving forward at an incredible speed during the last decade, fuelled by the development of novel omics-technologies, *in vitro* methodologies and *in silico* tools for data analysis. Ever since the introduction of the three Rs, which first lead to improvements of animal models, we now have technologically advanced alternative methods for prediction of chemical sensitizers. Although some of these assays have been formally validated and given an OECD TG, no assay is currently accepted as a stand-alone method to be used in risk assessments of sensitizing chemicals. Most assays inform about skin sensitizing hazard and should other endpoints be required, complementary information needs to be provided by other sources. IATAs comprising different DAs are one suggested solution for incorporating information from several endpoints (Casati *et al.*, 2018). However, the additional information should be carefully selected to provide actual value to the final decision. As the ultimate goal is to prevent adverse effects in humans such as ACD, the predictive tests need to reflect the human immune responses, and not rely solely on the outcomes observed in animals.

This chapter aims to highlight some of the limitations in current predictive toxicology and considerations that need to be addressed to move towards an animal-free testing standard. Also, our contributions to address some of the challenges in predictive toxicology are presented.

4.1 Challenges in predictive toxicology

Skin sensitizer risk assessment is inevitably moving towards the replacement of animal testing with alternative non-animal methods. Although some alternative methods have been validated and accepted for regulatory implementation, many challenges remain. First of all, the methods need to be correct in their assessment of skin sensitizers and relevant for the true situation, i.e., human skin sensitization. Secondly, for a complete risk assessment, the alternative methods should be able to inform about several aspects of risks involved in the exposure to chemicals, including hazard and potency of the chemical. This section will cover the basics for two challenges that I believe are necessary to consider more carefully, accurately predicting the true human outcomes and the current assessment of single substances instead of chemical mixtures.

4.1.1 Predictions of the human outcome

Unlike most alternative assays, which only target a specific part of the skin sensitization reaction by focusing on one KE in the AOP, the LLNA is a biological system with interactions between many cell types. In addition, the LLNA assay provides skin sensitization hazard information as well as potency information, e.g. by sub-categorization of the EC3 values into CLP categories (Ezendam *et al.*, 2016). However, extrapolation of results from animal models to the human outcome is not without issues. In the case of LLNA, one limitation is the difference in the skin immunology between mice and humans, such as the different phenotypic and functional roles of human and murine DC populations (Shortman & Liu, 2002; van de Ven *et al.*, 2011). Adding to this, the murine TLR4 lacks two histidine residues, which for example makes mice unable to develop contact hypersensitivity to the common human sensitizer nickel (Schmidt *et al.*, 2010). This highlights the need to understand cellular and molecular interactions in the response to chemicals (further discussed in **section 4.2.2**) and to use models relevant for the human situation. In this context, the problem arises what the reference for the alternative methods should be, i.e., what is “the true response” to a skin sensitizer?

In the development of alternative methods, the predictive performance is evaluated based on the outcomes from other assays, or “true” instances. To this end, the use of LLNA as reference is widespread and the major

advantage is the easy accessibility of comprehensive datasets, like the one provided by Natsch *et al.* (Natsch *et al.*, 2013). However, the validity of comparing to LLNA data can be questioned, as LLNA performance values have been shown to not entirely translate to human hazard, with an accuracy ranging from 63-73% (Haneke *et al.*, 2001). Thus, it would be more relevant to use human references as standard for assessment of the predictive power of alternative methods. Subsequent risk and safety evaluation of chemicals would then be more related to the human outcome. However, human data is scarce and not easily interpretable, but efforts have been made to compile datasets (Api *et al.*, 2017; Basketter *et al.*, 2014). In these datasets, chemicals are categorized in six classes according to their human potency, where sensitizers are categorised from class 1 (extreme sensitizers) to class 5 (very weak sensitizers) followed by class 6 representing the true non-sensitizers. The compiled data were based solely on human experimental observations, derived from no observed effect level of human repeated insult patch tests (HRIPT) (Marzulli & Maibach, 1974) or the human maximization tests (Kligman, 1966), and refined in some cases by data from diagnostic patch tests. However, as noted by the authors, the aspect of expert judgement in asserting the classes could impose a bias depending on the quality of the available evidence. Although the patch testing in determining occupational contact dermatitis is standardized, the grading relies on the expertise of a skilled physician (Sasseville, 2008). The ethical and scientific validity of using HRIPT must also be considered (Basketter, 2009).

Considering the importance of reference data and predicting chemicals with sensitizing capabilities in humans, it is interesting to note that some of the misclassifications according to CLP in **Paper III** seemed to correlate better to their human potency class than to the reference CLP category. A similar pattern was also observed with mercaptobenzothiazole in **Paper I**, which indicated a CLP category of 1B, more in line with its human potency class 3, instead of the established classification of 1A. This is based on the suggestion that human potency categories 1 and 2 would correspond to CLP 1A, categories 3 and 4 to CLP 1B, and 5 and 6 to the no category classification (Basketter *et al.*, 2014). Furthermore, one solution could be to harmonize assessments of alternative methods when comparing different assays (Kleinstreuer *et al.*, 2018), where a standardized dataset (Hoffmann *et al.*, 2018) to evaluate all the methods based on the same assumptions and criteria could be used. However, caution needs to be taken, as many of the individual assays are evaluated on LLNA data, which could have a negative influence

on the prediction of human sensitization categories (Kleinstreuer *et al.*, 2018).

In conclusion, I believe that an open dialogue between assay developers and dermatology clinics would be a first step to provide the high-quality data needed for relevant prediction of human responses to sensitizer exposure. In my opinion, this could bring the confidence to regulatory authorities to make alternative assays the first choice for toxicity testing of skin sensitizers.

4.1.2 Testing of mixtures instead of pure substances

The current testing of pure substances instead of final products or mixtures, which most individuals are exposed and sensitized to, could have consequences and should be properly investigated (EC, 2012). Limitations in testing individual components of a mixture or only focus on the active ingredient can cause adverse effects, as seen in cases with for example excipients in drug products (Reker *et al.*, 2019) or co-formulations in glyphosate-based herbicides (Young *et al.*, 2015). In addition, effects, such as synergism or additive interactions between different components, could also contribute to mixture toxicity (Bonefeld *et al.*, 2017), which further complicates toxicity predictions of mixtures. Thus, it is important to perform risk assessments of compounds and mixtures that people actually are exposed to, instead of assessing pure chemicals (Karlberg *et al.*, 2008). Although several attempts have been made to facilitate the risk assessment of mixtures, more efforts are needed to have a transparent and adequate evaluation of chemical mixtures (Kienzler *et al.*, 2016).

A frequently used group of mixtures are glyphosate-based herbicidal formulations, commercially available worldwide (Benbrook, 2016). The most common active ingredient in these herbicidal formulations, glyphosate, is reported to have a negligible toxic effect on humans (Myers *et al.*, 2016; Steinrucken & Amrhein, 1980) and thus, minimal risk assessments of glyphosate-based herbicides have been performed. However, disagreements about the human toxicity caused by these herbicides exist (Portier *et al.*, 2016) and highlight the need for more thorough investigations on this topic. Notably, it has been shown that human toxicity, such as endocrine disruption, can be caused by contaminants and co-formulations in the herbicidal mixture, rather than the active ingredient glyphosate (Bradberry *et al.*, 2004; Young *et al.*, 2015). However, co-formulations are often referred to as inert or kept confidential by the manufacturers. As glyphosate-based herbicides are used

all over the world, systematic safety evaluations and risk assessments of the individual components and functional formulations is vital to prevent adverse effects in humans.

In **Paper II**, we address the skin sensitizing capacity of mixtures by investigating glyphosate-based herbicidal formulations. We evaluated the skin sensitization hazard of pure glyphosate, the surfactant polyethylated tallow amine (POEA) and two commercially available glyphosate-formulations. Firstly, pure glyphosate was predicted by the GARD™skin assay as non-sensitizing while POEA and the two formulations were predicted as sensitizers. Secondly, we also investigated changes in the proteome induced in the cellular model in response to the investigated chemicals and mixtures. Using mass spectrometry, the generated peptide fragments were assembled to protein groups, which in turn were associated with a corresponding gene. Subsequent pathway analysis revealed that the two formulations are predicted to activate more pathways than pure POEA, among them immune response- and oxidative stress-related pathways, which is well in line with previous knowledge about the cellular response to skin sensitizing chemicals. Furthermore, we identified several proteins commonly differentially regulated in response to the formulations and POEA to be linked with autophagy and cholesterol biosynthesis, which could be cellular processes important in the DC activation towards skin sensitizing chemicals. Altogether, these results stress the importance of conducting risk assessments of all ingredients in a mixture as well as the mixture itself.

In summary, the current state of predictive toxicology faces several challenges, and to address some of these challenges, more knowledge about the underlying mechanisms in skin sensitization is needed.

4.2 From method development to mechanistic insight

When new chemicals are evaluated, the predictive models must be able to also determine the hazard associated with chemicals possibly unlike those they were trained on, or not applicable to the chemical space of the assay. Thus, chemicals possessing unique chemical properties or specific reactivity towards certain skin proteins might fall outside the applicability domain of a particular predictive assay. Additionally, for a complete risk assessment, a prediction on the sensitizer potency is also necessary. Depending on the initial development of the predictive model, and to what extent it is able to incorporate other information, problems arise if potentially harmful chemicals are predicted as safe. To prevent this, more research efforts should focus on the specific properties of the assayed chemical and what type of immunological response is expected based on the traits of the chemical, which also could be used to define model boundaries.

4.2.1 Novel applications for the experimental setup of the GARD assay

The *in vitro* experimental design exploited in this thesis, used during the GARD™ assay development and predictive biomarker discovery, provides an excellent opportunity to study cellular and molecular responses towards skin sensitizers. The microarray platform provides information of transcriptional changes induced by individual chemicals, and these comprehensive datasets provide a source of mechanistic data, such as pathways engaged and biomarkers triggered by xenobiotics.

In **Paper III** we used complete genome transcriptomics to gain insight about the cellular mechanisms and to construct a biomarker signature for potency predictions. While the main aim was to identify novel endpoints for the experimental setup of the GARD™ assay to include potency predictions, we also investigated the pathways predicted to be activated in stimulations with skin sensitizing chemicals. Previous observations using the assay setup have showed that more signalling pathways (many of them linked to cell cycle regulation and control) were induced when the model was challenged with chemicals of stronger sensitizing potency. A similar trend was observed for the number of molecules in each pathway, where more molecules were

engaged within each pathway depending on the increasing potency of the assayed chemical (Albrekt *et al.*, 2014).

Based on these results, the changes in the cellular transcriptome were hypothesized to be able to discriminate between different potency classes. As opposed to the initial prediction signature (Johansson *et al.*, 2011), which distinguished between two classes, the legal requirements for potency classes comprises three categories (1A, 1B and no cat). Thus, we aimed to identify a predictive biomarker signature capable of determining the sensitizer potency according to the CLP regulation. We utilized the machine learning algorithm random forest (Breiman, 2001), which has been shown to be applicable to multiclass problems (Díaz-Uriarte & Alvarez de Andrés, 2006). By combining historical data from previous experimental campaigns with newly generated data, a balanced dataset comprised of chemicals from all three CLP categories was created for building of the prediction model. Care was taken to include chemicals representing the main chemical reactivity domains (mentioned in **section 2.1**). After random forest modelling, a biomarker signature consisting of 52 genes was identified, having the best discriminatory power to separate the three CLP categories. Similar to the established prediction signature for hazard identification, the potency prediction signature also includes genes previously linked to skin sensitization or immune responses, such as NQO1 (Ade *et al.*, 2009) and PLK1 (Hu *et al.*, 2013).

The predictive performance of the 52 genes was evaluated on a test dataset comprising 6 chemicals from each CLP category, previously unseen by the model. The final classification yielded a balanced accuracy of 78%, showing good predictive power of CLP categories 1A (5/6 correctly predicted) and no cat (6/6 correctly predicted). However, the weak sensitizers belonging to CLP category 1B were more challenging to predict (3/6 correctly predicted), but as previously mentioned in **section 4.1.1**, these predictions seemed to better correlate with their human potency. Reasons for the misclassification of chemicals belonging to CLP category 1B could be the broad span of chemical characteristics and potencies comprised in this category or the problems associated with what classification reference should be used, i.e., human potency or LLNA data. Interestingly, the 52 genes of the prediction signature were demonstrated to also contain information capable of grading the training and test samples according to their human potency.

In addition to providing a predictive biomarker signature for potency determination, we also explored the cellular response in stimulations with

sensitizing chemicals. As mentioned earlier, the chemical reactivity domain is a major contributing factor in the skin sensitization potential of a chemical (Chipinda *et al.*, 2011). Chemicals in CLP category 1B possessed many of the reactivity domains while category 1A was dominated by chemicals with MA domains. The no cat class was predominantly represented by unreactive chemicals expressing no apparent protein binding, but a few chemicals displayed protein reactivity domains belonging to the SBF and S_N groups. Further, we investigated pathways predicted to be activated in response to chemicals grouped according to their reactivity domains. Overall, a common activation of pathways related to cell cycle and DNA damage was seen for all reactivity domains. However, reactivity-specific patterns were also seen, as unique pathways were identified in response to each reactivity domain. This suggests a specific cellular activation linked to the distinct properties of chemicals. Interestingly, MA was predicted to induce most pathways, possibly due to many strong sensitizers (CLP category 1A) in this reactivity domain, thus inflicting greater damage and subsequently more cellular defence responses.

In **Paper IV** we focus on an important aspect in predictive toxicology, the confidence in generated predictions, i.e., are the predictions suitable for the applicability domain for the particular test method? The applicability domain of a model encompasses the predictive boundaries of where it is able to provide reliable predictions. As information about the applicability domain for *in vitro* assays is not yet a regulatory requirement, this is often ignored by test developers (Roberts & Patlewicz, 2018). *In vitro* assays are instead assumed to be applicable to the entire chemical space. Therefore, some models could provide false statements outside of the chemical space for which they are developed. In cases where a statement is made on the applicability domain, it often refers to technical limitations such as solubility issues with the test substance due to aqueous test systems or the lack of metabolic activity for the cell model (Bauch *et al.*, 2012).

To establish the predictive boundaries of the hazard identification for the original design of the GARD™ assay, the conformal prediction framework (Vovk *et al.*, 2005) was evaluated and implemented on assay predictions in **Paper IV**. This framework is built on assigning classes to each sample based on the similarity of that test sample to the samples in the initial training dataset, which in turn is based on a user-defined confidence level. In our case, this similarity came from the distance to the hyperplane in the SVM prediction model, which was defined as a conformity score. These

conformity scores for the test sample were then compared to conformity scores of the training samples to acquire a conformal prediction p-value, which indicates the similarity to the training samples. Subsequently, this p-value was used to assign samples into one of four labels depending on a comparison to the user-defined significance level. Thus, samples could be assigned as sensitizer, non-sensitizer, both classes (meaning the model could not assign either of the classes to the sample) or empty domain (indicating that the model could not provide a reliable prediction to the sample as it was too different compared to the samples in the training data). To evaluate this approach, a test set of 70 chemicals was analysed. The overall balanced accuracy was determined to 88% and no chemical was found to be outside the applicability domain of the model, i.e., no chemicals were assigned the empty domain category.

4.2.2 Mechanism-based risk assessment

One objective with this thesis was to study the immunological mechanisms involved in the response of DCs to chemical exposure. Previous studies have implicated several signalling pathways in DC activation in skin sensitization, including phosphorylation of p38 MAPK, changes in the cell surface thiols, the Nrf2/ARE pathway and regulation by the transcription factor NF-kB (Neves *et al.*, 2011). Additionally, the importance of TLRs in skin sensitization have been highlighted due to their involvement in providing danger signals required for a proper adaptive immune response initiated by DCs. Thus, these receptors, for example TLR4, have been proposed as potential drug targets for treatment of ACD (Martin *et al.*, 2011). Furthermore, it has been discussed if all sensitizers engage common traits, like the activation of TLRs and inflammasome-associated pathways, which could be targeted in therapeutic applications (Kaplan *et al.*, 2012). Mechanistic knowledge about specific pathways and molecular interactions could give valuable insights in determining different endpoints associated with chemical exposure and could contribute to assessing the chemicals. This resembles what has been done in classifications of breast cancer metastasis and ovarian cancer survival time (Kim *et al.*, 2012), as well as the prognostic use of 38 subnetworks for prediction of disease progression in chronic lymphocytic leukaemia (Chuang *et al.*, 2012). Many mediators are responsible for the initiation and development of allergic diseases such as ACD, ranging from innate effector cells to a multitude of cytokines, chemokines and T-cell subtypes. As several of these interactions and

activations are still not completely understood, more investigations are needed to expand the mechanistic understanding (Shane *et al.*, 2019).

We aimed to expand the knowledge about DC activation in response to skin sensitizers by investigating the miRNA regulation in **Paper I**. As mentioned previously, the role of miRNAs in skin sensitization is not entirely clear, although part of diagnostic and clinical applications in other inflammatory skin diseases (Lovendorf & Skov, 2015). However, none of the miRNAs identified in **Paper I**, except miR-1973 (Van Loveren *et al.*, 2014), had previously been associated with skin sensitization, and the low number of commonly expressed miRNAs in response to the rubber chemicals was surprising considering their structural similarity. Even though the rubber chemicals contain the same reactivity domain, they still seem to regulate different targets. This observation was also reflected in the limited number of commonly differentially regulated mRNA transcripts in response to the rubber chemicals. However, when translating the transcriptomic variations into pathways predicted to be activated in response to the stimulations with the rubber chemicals, we observed an overlap between the immune-related pathways predicted to be regulated by both rubber chemicals.

The complexity of the interactions involved in the skin sensitization reaction should be considered when performing risk assessments of sensitizing chemicals. Even though the reactivity domain seems to be an important aspect of skin sensitizing agents, the regulated transcripts and associated pathways activated could respond to small structural differences in chemicals, possibly due to unknown modifications in the reactivity towards skin proteins or traits associated with their ability to bind certain skin proteins. To this end, a recent investigation into the haptenation of keratinocyte and skin proteins showed a small degree of total proteins haptenated by the assayed chemicals, and there was a trend correlating a lower number of modified proteins with weaker skin sensitizer potency. In addition, the protein tertiary structure was identified to have a role in the likelihood of haptenation (Parkinson *et al.*, 2018). However, it has been argued that structural elements outside the protein reactivity has little to do with the skin sensitizing properties of a chemical (Natsch, 2010; Roberts & Aptula, 2008). In light of these discussions, I believe that specific immunological activation towards certain skin sensitizing chemicals is likely varying depending on several chemical characteristics, not excluding small structural differences that could influence protein binding or initiation of subsequent immune responses.

To summarize, the field of toxicology should focus more on the evaluation of mechanism-derived data to better understand the molecular and cellular interactions in response to skin sensitizers. As different chemicals have been observed to activate unique immunological responses (Dhingra *et al.*, 2014; Peiser *et al.*, 2012), to some extent also observed by us (**Paper I-III**), this could be summarized as “that ACD cannot be considered a single entity” (Dhingra *et al.*, 2014). In many cases, I believe the over-simplification of a complex biological system, by only interpreting few biomarkers or focusing on a particular part of the induced cellular responses, is responsible for misclassification of chemicals. This again is linked to a lack of knowledge about the human immunological response to that particular chemical. In the light of this, the development of novel predictive assays might need to focus on characterization of chemical subgroupings instead of applying models to the entire chemical space (Hoffmann *et al.*, 2018). Although this would be a huge challenge and may not be feasible due to the large quantity of chemical sensitizers present in our environment. However, more studies to map specific immunological patterns induced by unique properties of the skin sensitizers could improve the prediction of skin sensitizers and contribute to identifying therapeutic targets for treatment and prevention of ACD.

5. Concluding remarks

Allergic reactions caused by repeated exposure to small chemical compounds, commonly known as chemical haptens or sensitizers, is a major health concern. As this kind of compounds is encountered every day, thorough risk assessments need to be performed to prevent harmful chemicals from reaching the consumer market. Several legislative regulations control the use and distribution of chemicals and require information about characteristics of the chemicals, such as their capacity and potency to cause skin sensitization. These assessments have traditionally been performed using animal models, but recent advances in the development of alternative methods have shifted the field of predictive toxicology towards replacing the animal models with non-animal alternatives.

However, before non-animal testing strategies can replace animal tests, several challenges remain to be addressed. Firstly, the currently validated alternative assays are limited to predicting the skin sensitizing hazard. Other endpoints such as skin sensitizer potency, which is imperative for a complete chemical risk assessment, are currently provided by other means, i.e., testing on animals. To complement existing alternative methods with the information that they are lacking, the development of innovative approaches to incorporate information from different sources has been investigated, but no such method has yet gained regulatory acceptance. Secondly, there is still a lack of knowledge about the underlying mechanisms in response to skin sensitizing chemicals. While the need for accurate validated methods to replace animal tests is of highest importance, knowledge about the underlying immunology in allergic reactions to chemical sensitizers is necessary to better understand the complex network of cellular and molecular interactions induced by sensitizing chemicals. Such knowledge allows for the evaluation and improvement of existing strategies and the development of new approaches for the prediction, prevention and treatment of adverse effects induced by sensitizing chemicals in humans.

The objectives of this thesis, which is based on four original research papers, has been to provide more knowledge about the mechanisms in DC activation in response to sensitizing chemicals (**Paper I-III**) as well as to further develop a DC-based *in vitro* assay, an earlier adaptation of the GARD™ assay, to face unmet requirements in current predictive toxicology (**Paper III** and **IV**).

In **Paper I**, we investigated the gene regulation in DCs, focusing on the small regulatory RNA family of miRNAs and their regulation in response to structurally similar rubber chemicals. The role of miRNAs in skin sensitization is not well understood, but they are described as prognostic biomarkers in several other diseases. Here, we demonstrated mainly a chemical-specific regulation of miRNAs, but also a few commonly regulated miRNAs in response to the investigated structurally similar rubber chemicals. Furthermore, we performed an analysis of the transcriptional regulation to the same rubber chemicals. Significant changes in the transcriptome, in cell stimulations with the rubber chemicals, were predicted to activate several pathways linked to cellular events in skin sensitization such as antioxidant response-related pathways. This information paves the way for further mechanistic investigation in order to broaden the knowledge about DC activation in response to skin sensitizing chemicals. Furthermore, the unique cellular response to exposure of the rubber chemicals shows that the cell model used in this study is capable of differentiating between highly structurally similar chemicals. However, further studies with more sensitizers are needed in order to define if miRNAs could generally be used as biomarkers in skin sensitization, or if miRNA and subsequent gene expression is mostly dependent on unique properties of the respective sensitizing chemical. More knowledge about miRNA regulation could of course contribute to develop tools to prevent and treat ACD.

In **Paper II**, we attended to an important aspect of predictive toxicology, the assessment of chemical mixtures instead of pure substances. The combination of several sensitizers could have unexpected consequences. Therefore, it is important to evaluate the risks associated with exposure to the complete mixture or final products. Here, we focused on two herbicidal formulations containing glyphosate as main ingredient, and the commonly used co-formulant POEA. Both herbicidal formulations and POEA were predicted as sensitizers by the GARD™skin assay. Glyphosate in contrast was predicted as a non-sensitizer, and when mixed with POEA, no additional effects were observed. Interestingly, the two herbicidal formulations containing other

surfactants than POEA had a higher SVM DV than POEA alone, indicating additive or synergistic effects induced by the ingredients present in the formulations in addition to glyphosate, likely driven by potent skin sensitizing capacity of their co-formulants. Additionally, we used a proteomic-based approach to evaluate the protein expression in response to the investigated herbicidal formulations and POEA. Proteins were associated with their corresponding genes and investigated on their biological relevance in terms of skin sensitization. We identified several predicted pathways based on the regulated proteins, which were linked to known cellular events in skin sensitization, confirming the skin sensitizing properties of the formulations and POEA. Furthermore, several differentially regulated proteins, found in response to both the formulations and POEA, were linked to autophagy- and cholesterol synthesis-related processes. These cellular functions could be interesting targets for investigating the DC response towards skin sensitizers. However, further studies are needed to understand the importance of these processes in the skin sensitization reaction. In conclusion, the results in **Paper II** show the importance of evaluating complete chemical mixtures with regard to their impact on human health. It should thus be considered equally important to risk assess the final products as it is to determine the skin sensitizing capacity of individual chemicals. In addition, the use of technologically advanced methods could contribute to find novel immunological processes relevant in the context of skin sensitization.

The second objective of this thesis was to expand the experimental setup of the GARD™ assay to investigate other relevant endpoints beyond the binary predictions of skin sensitizing hazard that it was originally developed for. As a multiparametric *in vitro* alternative to animal tests, the initial GARD™ assay utilizes information from several genes to determine the skin sensitizing hazard of chemicals. However, this prediction algorithm was not designed to predict skin sensitizer potency, a prerequisite for a complete chemical risk assessment. Therefore, we investigated the applicability of the GARD™ assay experimental setup to also predict skin sensitizer potency in **Paper III**. We identified a predictive biomarker signature consisting of 52 genes, which was designed to predict the sensitizer potency according to the three CLP categories 1A (strong sensitizers), 1B (weak sensitizers) and no cat (non-sensitizers). Both the training dataset and the test set were carefully selected to include chemicals with different chemical reactivity, as this previously has been argued to be one of the major chemical characteristics that influence the sensitizer potency. We also demonstrated that the 52 genes contain information that could group the training and test set according to

their human potency. The development of a predictive biomarker signature that targets the six human potency classes would be highly relevant, but would require an even larger dataset to allow for both training and evaluation of the algorithm. In addition, we demonstrated that transcriptional changes and subsequent pathway activation were influenced by the chemical reactivity of the sensitizers, although common cellular events, such as cell cycle- and DNA damage-related pathways, were identified as well. Again, we show that unique properties of chemicals can activate specific pathways and cellular responses, which is well in line with the results of **Paper I**, albeit here detected with a larger set of samples and several reactivity domains. In **Paper IV**, we investigated the application of a framework to assure statistically valid predictions, an often overlooked feature in assay development. Here, we applied the conformal prediction framework on *in vitro* assay predictions and evaluated the uncertainty of the generated predictions. The approach was tested on a dataset of 70 chemicals, and no chemical was assigned to the empty domain. This proves that the experimental setup and assay predictions are applicable to a large chemical space. This type of additional information, i.e., the predictive boundaries of a given assay, could bring confidence to regulatory authorities and industrial stakeholders to accept *in vitro* assays on a broader scale and to entirely move away from the use of animal testing strategies.

In conclusion, I believe that using a dynamic approach to testing of skin sensitizers is the ideal way to go for routine screening of sensitizing compounds. Incorporating knowledge about the specific immunological response together with state-of-the-art non-animal predictive methods, be it a top-performing stand-alone assay or suitable combinations of different methods, should ultimately lead to the best possible decisions on chemical risk assessments. Only then can we achieve an animal-free testing standard in risk assessment of chemicals. Furthermore, knowledge about the activation of the immune system in response to specific skin sensitizers could be used to find novel predictive biomarkers or targeted applications for treatment and prevention of ACD.

Populärvetenskaplig sammanfattning

Dagligen använder vi, och utsätts för, många olika kemikalier. Detta har gett upphov till att allt fler människor får allergiska reaktioner framkallade av ämnen som ingår i produkterna vi använder. Dessa allergiska reaktioner är vanligt förekommande hos yrkesgrupper inom olika industriella områden men återfinns också bland befolkningen i allmänhet. Orsaken bakom dessa reaktioner är att vissa ämnen har förmågan att aktivera kroppens försvarsmekanism, trots att de i grunden är ofarliga. Bland annat kan en viss typ av kemikalier, så kallade sensibiliserande kemikalier, ge upphov till kontakteksem med påföljande negativa hälsoeffekter. Sensibiliserande kemikalier aktiverar celler som ingår i immunsystemet, kroppens försvar mot inkräktande mikroorganismer och farliga ämnen, som då reagerar och ger upphov till de kliniska symptom som är vanliga vid kontakteksem, såsom klåda och svullnader. Kontakteksem uppkommer vanligtvis genom att kemikalien vid upprepade tillfällen kommer i kontakt med huden och på så sätt aktiverar immunförsvaret.

För att motverka användningen av sensibiliserande kemikalier i produkter har stora ansträngningar gjorts för att utveckla metoder som kan förutspå de allergiframkallande egenskaperna hos kemikalier. Historiskt har dessa metoder involverat djurtester men på senare år har det tillkommit lagar och förordningar som kräver att dessa tester ska utföras utan djurförsök. Detta har varit den drivande faktorn bakom de senaste årens utveckling av alternativa metoder, som ska ersätta de djurmodeller som tidigare har använts. Trots att många av de alternativa metoderna har visat god förmåga att förutspå kemikalier med sensibiliserande förmåga, är det ingen metod som för sig själv har kapaciteten att behandla alla nödvändiga detaljer som krävs för att riskklassificera en kemikalie. De flesta metoderna har utvecklats så att de kan ge information om en kemikalie har sensibiliserande förmåga eller inte. Det är dock också viktigt att kunna fastställa kemikaliens potens, det vill säga om kemikalien är starkt eller svagt allergiframkallande. Därtill finns det mycket kvar att förstå om de bakomliggande mekanismer som sker vid exponering av sensibiliserande kemikalier och de olika signalvägar som immunförsvaret använder sig av.

Mot bakgrund av ovanstående har målet med denna avhandling, som är baserad på fyra vetenskapliga artiklar, varit att bidra till en fördjupad kunskap om de bakomliggande reaktioner som är involverade i utvecklingen av kontakteksem förorsakade av sensibiliserande kemikalier. Dessutom har

avhandlingen berört vidareutvecklingen av en djurfri testmetod, genom att utöka dess användningsområde samt kunna bestämma hur tillförlitliga metodens kemikaliegrupperingar är. En central del i denna avhandling har varit användandet av immunsystemets ”känselspröt”, de så kallade dendritcellerna, där vi har tittat på biologiska signalvägar som aktiveras i respons mot kemikalier som ger upphov till kontakteksem.

I den första publikationen, **artikel I**, berörs genuttrycket och genregleringen av dendritcellernas respons mot en specifik grupp av sensibiliserande kemikalier, nämligen gummikemikalier. Kontrollen av genuttrycket styrs bland annat av en grupp små RNA-molekyler, så kallade microRNA. Dessa har som uppgift att styra uttrycket av gener och på så sätt är de involverade i många typer av cellulära reaktioner. Vi fann att de strukturellt lika gummikemikalierna gav upphov till olika uttrycksprofiler av microRNA. Dessutom kunde samma tendens också observeras när vi jämförde vilka gener som var mest uttryckta i stimuleringar med gummikemikalierna. Detta pekar på att det är strukturspecifika egenskaper hos kemikalierna som styr aktiveringen av immunförsvaret.

Artikel II undersökte aspekten av kemikalieblandningar och fokuserade på växtbekämpningsmedel som innehåller glyfosat. Många av de klassificeringar som görs på kemikalier är baserade på rena kemikalier och inte på produkter eller blandningar som personer kommer i kontakt med. Detta kan medföra att synergistiska eller additiva effekter från olika komponenter kan missas och därigenom ge en felaktig bedömning av risken. Dessutom är användandet av glyfosat-baserade medel ett stort miljöproblem och troligtvis farligt vid kontakt med huden (det råder dock ingen samstämmighet om vilket som är det farliga ämnet i blandingen). Resultaten från **artikel II** visade på att rent glyfosat inte verkar ha några allergiframkallande egenskaper men att ett ytaktivt ämne som används i vissa blandningar gav ett klart sensibiliserande svar. Genom att använda en metod för att kartlägga alla uttryckta proteiner i kemikaliestimulerade celler upptäckte vi flera signalvägar som kunde kopplas till den immunologiska responsen mot sensibiliserande kemikalier.

De två avslutande publikationerna, **artikel III** och **artikel IV**, fokuserade på vidareutvecklandet av en djurfri testmetod där **artikel III** bidrog till att få fram en biomarkörsignatur för att förutspå kemikaliers potens att orsaka en allergisk reaktion. Detta är viktigt för att kunna göra en korrekt bedömning av riskerna som användningen av en specifik kemikalie medför. Vi identifierade en biomarkörsignatur, bestående av 52 gener, som kunde klassificera

kemikalier i tre olika grupper, enligt rådande regleringar. När biomarkörerna utmanades med en okänd uppsättning av kemikalier kunde träffsäkerheten fastställas till 78%. Dessutom undersöktes signalvägsaktivering inducerat av kemikalier grupperade enligt deras kemiska reaktivitet. Denna undersökning visade att både antalet gener och de specifika signalvägarna var till viss del beroende av kemikaliens reaktivitet. Vidare implementerades i **artikel IV** ett matematiskt ramverk för att statistiskt säkerhetsställa korrektheten i klassificeringarna från den prediktiva modellen. Detta medför att det går att avgöra om en specifik kemikalie är för olik kemikalierna som modellen har tränats på. Om denna olikhet är för stor går det inte att med säkerhet fastställa den korrekta klassen för den aktuella kemikalien.

För att genomföra ingående riskbedömning av sensibiliserande kemikalier behövs både exakta testmetoder, som ger all information som krävs, och kunskap om de bakomliggande mekanismer som sker vid exponeringen av kemikalierna. I situationer då en testmetod inte kan bedöma risken av en kemikalie kan det bero på att kemikalien är utanför användningsområdet för metoden eller att metoden inte kan tillämpas på de specifika immunologiska signalvägar som aktiveras. De artiklar som avhandlingen är baserad på försöker utöka kunskapen för att kunna ge säkrare metoder och bättre förstå de mekanismer som sker vid exponering av kemikalier för att till slut minska uppkomsten av och kunna ge bättre behandling mot kontakteksem orsakade av kemikalier.

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