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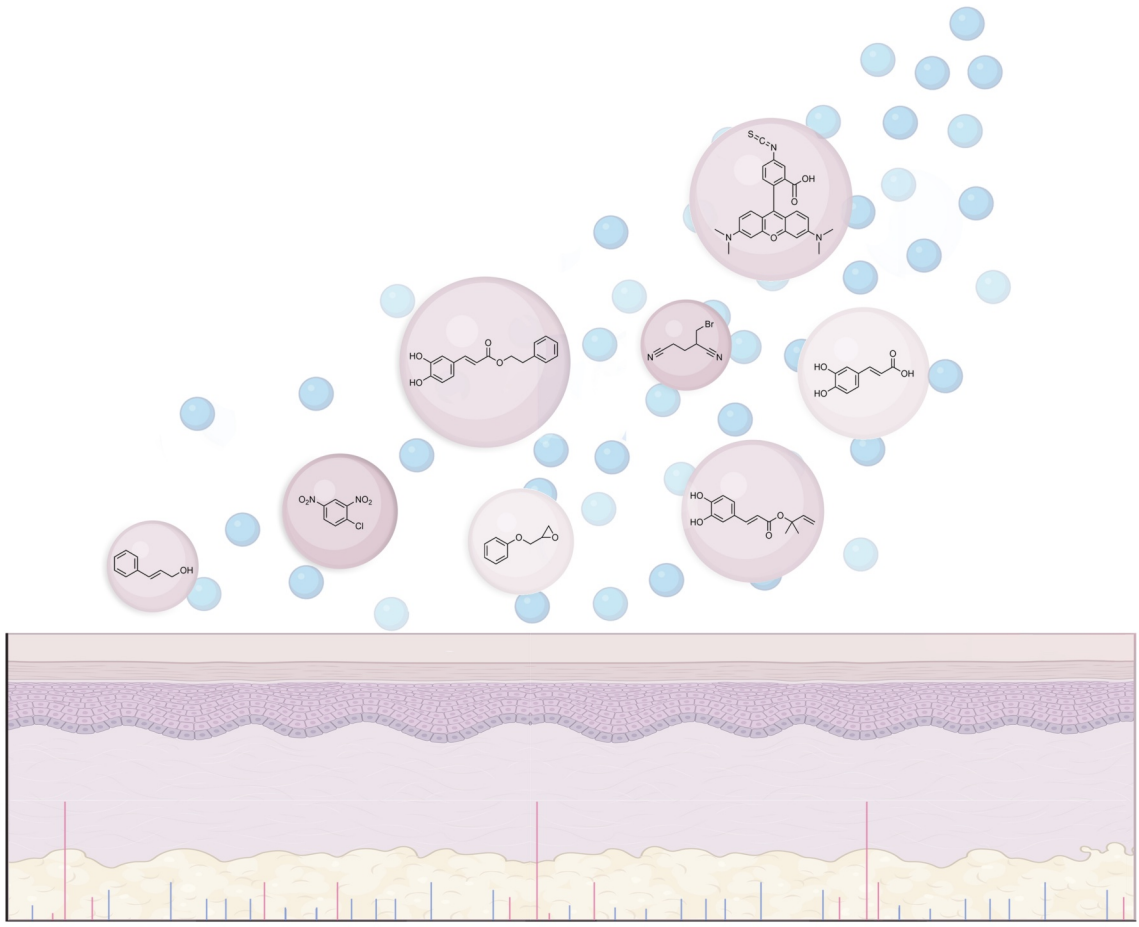
Mass Spectrometry Strategies for Characterization of Contact Allergens and their Protein Conjugates *in Vitro* and *in Vivo*

Lorena Ndreu



Doctoral Thesis in Analytical Chemistry at Stockholm University, Sweden 2023

Mass Spectrometry Strategies for Characterization of Contact Allergens and their Protein Conjugates *in Vitro* and *in Vivo*

Lorena Ndreu

Academic dissertation for the Degree of Doctor of Philosophy in Analytical Chemistry at Stockholm University to be publicly defended on Friday 26 May 2023 at 10.00 in Magnélisalen, Kemiska övningslaboratoriet, Svante Arrhenius väg 16B.

Abstract

Humans are daily exposed to chemicals from various sources, including cosmetics, jewelry, clothes, and hair dyes, which can result in the occurrence of contact allergy and subsequent allergic contact dermatitis (ACD), a type IV delayed hypersensitivity reaction. ACD is characterized by inflammation and eczema at the site of exposure, and no definitive cure for this condition has been identified to date, with only symptomatic treatment options involving corticosteroids being available.

The research presented in this thesis is centered around mass spectrometry (MS) strategies aimed at enhancing our comprehension of events that occur during the early stages of the development of contact allergy. Special emphasis is given to characterizing various contact allergens (haptens) and their interactions with endogenous proteins, as these interactions are considered crucial in the initiation of contact allergy. Moreover, the thesis endeavors to explore the activation of prehaptens and prohaptens, which are non-reactive compounds capable of transforming into haptens outside or inside the skin, respectively.

In **Paper I**, a bottom-up proteomics approach was employed to investigate the adductome of two major blood proteins, human serum albumin (HSA) and hemoglobin (Hb). The study aimed to identify the most reactive sites on these proteins upon exposure to different haptens with varying sensitization potencies. Highly susceptible sites on HSA and Hb were identified as the most likely targets for *in vivo* modification. This study is the first investigation of the Hb adductome in the context of contact allergy and may contribute to the development of improved diagnostic tools using blood samples. With Hb on focus, **Paper II** evaluated three different MS-based methods, including bottom-up proteomics, detachment of N-terminal adducts by FIRE, and limited proteolysis (LiP), to determine the most suitable approach for assessing exposure through this protein. The three methods showed different strengths and limitations depending on the nature of the hapten. In **Paper III**, the research conducted revealed the presence of a hapten-protein conjugate in blood samples mice treated with the synthetic hapten tetramethyl rhodamine isothiocyanate (TRITC) topically. The identified protein was the macrophage migration inhibitory factor (MIF), marking the first instance of such a conjugate being detected in blood samples after topical hapten application. The study also indicated that MIF could potentially be modified by other contact allergens, suggesting its potential as a biomarker for the condition. In **Paper IV**, contact allergy to propolis, a by-product of honey used in biocosmetics, was investigated. Air oxidation experiments with a model peptide and MS detection, revealed that quinones formed from the oxidation of major propolis components are responsible for adduct formation. The identified adducts are likely the cause of contact allergy to propolis, providing valuable insights into the underlying mechanisms of propolis contact allergy and potential implications for clinical diagnosis. In **Paper V**, the bioactivation of cinnamic alcohol, a common ingredient in many cosmetic products, was investigated using *in vitro* systems and a targeted MS approach. Two metabolites, namely pOH-cinnamic alcohol and pOH-cinnamic aldehyde, were identified as of particular interest and their sensitizing potency was evaluated, with the latter categorized as a moderate sensitizer.

In summary, this doctoral thesis employed MS techniques to characterize contact allergens and their protein conjugates, yielding valuable insights into the molecular mechanisms underlying contact allergy development. The findings have potential implications for improving diagnostic tools and strategies for preventing and treating contact allergy.

Keywords: *Mass spectrometry, Skin allergy, Protein Adducts, In vitro methods, Bioanalysis.*

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To my mom, who fought the monster…

List of papers

The present thesis is based on the following publications, which are denoted in the text by their Roman numerals.

I *Characterizing Adduct Formation of Electrophilic Skin Allergens with Human Serum Albumin and Hemoglobin*
Ndreu L., Erber N.L., Törnqvist M., Tretyakova N.Y., Karlsson I., Chemical Research in Toxicology, **2020**, 33, 2623−2636.

The author was partly responsible for formulating the research hypothesis and planning of the study. The author conducted all the experimental work, data analysis and a major part of the writing.

II *Mass Spectrometry-Based Strategies for Assessing Human Exposure Using Hemoglobin Adductomics*
Rajczewski A.T. *, Ndreu L.*, Vryonidis E*., Hurben A.K., Jamshidi S., Griffin T.J., Törnqvist, M., Tretyakova N.Y., Karlsson I.
Manuscript

**These authors contributed equally.*
The author was responsible for a major part of the planning and experimental work and significant part of the writing.

III *Haptenation of Macrophage Migration Inhibitory Factor: A Potential Biomarker for Contact Hypersensitivity*
Ndreu L., Sasse S., Karlberg A.-T., Karlsson I., Frontiers in Toxicology, **2022**, 4:856614.

The author was partly responsible for formulating the research hypothesis and planning of the study. The author conducted all the experimental work, data analysis and a major part of the writing.

IV *Investigation into Propolis Components Responsible for Inducing Skin Allergy: Air Oxidation of Caffeic Acid and its Esters Contribute to Hapten Formation*
Ndreu L., Hurben A.K., Nyman G.S.A., Tretyakova N.Y., Karlsson I., Hagvall L.

Accepted for publication in Chemical Research in Toxicology

The author was responsible for a major part of the planning and experimental work and significant part of the writing.

V *Bioactivation of Cinnamic Alcohol in a Reconstructed Human Epidermis Model and Evaluation of Sensitizing Potency of the Identified Metabolites*
Ndreu L., Carlsson J., Ponting D. J., Niklasson I. B., McHugh L., Luthman K., Karlberg A.-T., Karlsson I.
Manuscript

The author was responsible for a major part of the planning and experimental work and significant part of the writing.

Papers not included in this thesis

VI. *Particle-based N-linked glycan analysis of selected proteins from biological samples using nonglycosylated binders*
Sroka-Bartnicka A., Karlsson I., Ndreu L., Quaranta A., Pijnappel M., Thorsén G., Journal of Pharmaceutical and Biomedical Analysis, **2017**, 132, 125-132.

VII. *Glycosylation patterns of selected proteins in individual serum and cerebrospinal fluid samples*
Karlsson I., Ndreu L., Quaranta A., Thorsén G., Journal of Pharmaceutical and Biomedical Analysis, **2017**, 145, 431-439.

VIII. *Glycosylation profiling of selected proteins in cerebrospinal fluid from Alzheimer's disease and healthy subjects*
Quaranta A., Karlsson I., Ndreu L., Marini F., Ingelsson M., Thorsén G., Analytical Methods, **2019**, 11, 3331-3340.

IX. *N-Glycosylation profiling of intact target proteins by high-resolution mass spectrometry (MS) and glycan analysis using ion mobility-MS/MS*
Quaranta A., Spasova M., Passarini E., Karlsson I., Ndreu L., Thorsén G., L. L. Ilag, Analyst, **2020**, 145, 1737-1748.

X. *Novel 4-Hydroxybenzyl Adducts in Human Hemoglobin: Structures and Mechanisms of Formation*
Rajczewski A.T.*, Ndreu L.*, Pujari S.S., Griffin T.J., Törnqvist M., Karlsson I., Tretyakova N.Y., Chemical Research in Toxicology, **2021**, 34, 7, 1769–1781.
**These authors contributed equally*

XI. *Nature-Derived Epoxy Resin Monomers with Reduced Sensitizing Capacity─Isosorbide-Based Bis-Epoxides*
Karlsson I., Ponting, D.J. Ortega M.A., Niklasson I.B., Ndreu L., Stéen E.J.L., Seifert T., Luthman K., Karlberg A., Chemical Research in Toxicology, **2023**, 36, 2, 281–290.

Populärvetenskaplig Sammanfattning

Genom daglig användning av olika hushålls- och hygienprodukter, kosmetika, kläder, smycken, hårfärgämnen, med mera, utsätter vi människor oss för en mängd olika kemiska ämnen, av vilka flera kan ha skadliga effekter på huden. Hudexponering för dessa kemikalier kan orsaka kontaktallergi (hudallergi), ett tillstånd som sedan ofta leder till inflammation och eksem, allergisk kontaktdermatit (ACD), vid minsta hudkontakt med ämnet. Kontaktallergi är ett livslångt tillstånd och dessvärre finns det ännu inget känt botemedel. För att inte riskera hudeksem eller andra symptom så måste en sensibiliserad person helt undvika hudkontakt med ämnet och för närvarande finns endast kortikosteroider tillgängliga för lindring av symptomen. För att på längre sikt kunna utveckla en effektivare behandling är det därför angeläget med mer forskning och en bättre förståelse av de molekylära mekanismer som ligger bakom kontaktallergi.

I min doktorsavhandling har jag använt masspektrometritekniker (MS) som verktyg för att kunna studera olika molekylära interaktioner under de tidigaste stadierna av kontaktallergi. Tyngdpunkten ligger på karakterisering av kontaktallergena ämnen (hapten) och deras interaktioner med proteiner i blod och hud, något som är helt avgörande för att kontaktallergi ska uppkomma. De immunogena hapten-proteinkomplexen som har identifierats belyser det invecklade samspelet mellan kemikalier och hudens immunsvar. Avhandlingen beskriver också hur vanliga föreningar som inte är allergena i sig själva, så kallade prehapten och prohapten, kan aktiveras och omvandlas till hapten antingen inuti eller utanför huden och på så sätt orsaka kontaktallergi.

Sammantaget har avhandlingsarbetet gett värdefulla insikter om de molekylära mekanismer som är involverade i utvecklingen av kontaktallergi. Dessa upptäckter kan öppna nya möjligheter till utveckling av effektivare diagnostiska verktyg och strategier för att förebygga och behandla vanliga typer av kontaktallergi.

Δημοφιλής Επιστημονική Περίληψη

Μέσω της καθημερινής χρήσης διαφόρων προϊόντων οικιακής χρήσης και υγιεινής, καλλυντικών, ρούχων, κοσμημάτων, βαφών μαλλιών κ.λπ., οι άνθρωποι εκτιθέμεθα σε μια ποικιλία χημικών ουσιών, αρκετές από τις οποίες μπορεί να έχουν βλαβερές επιπτώσεις στο δέρμα. Η έκθεση του δέρματος σε αυτές τις χημικές ουσίες μπορεί να προκαλέσει αλλεργία εξ επαφής (δερματική αλλεργία), μια κατάσταση που συχνά οδηγεί σε φλεγμονή και έκζεμα, αλλεργική δερματίτιδα εξ επαφής (ACD), στην παραμικρή επαφή του δέρματος με την ουσία. Η αλλεργία εξ επαφής είναι μια δια βίου πάθηση και δυστυχώς δεν υπάρχει ακόμη γνωστή θεραπεία. Προκειμένου να μην κινδυνεύσει το δερματικό έκζεμα ή άλλα συμπτώματα, ένα ευαισθητοποιημένο άτομο πρέπει να αποφεύγει εντελώς την επαφή του δέρματος με την ουσία και επί του παρόντος μόνο κορτικοστεροειδή είναι διαθέσιμα για την ανακούφιση των συμπτωμάτων. Για να μπορέσουμε να αναπτύξουμε μια πιο αποτελεσματική θεραπεία μακροπρόθεσμα, είναι απαραίτητη περισσότερη έρευνα και καλύτερη κατανόηση των μοριακών μηχανισμών που κρύβονται πίσω από την αλλεργία εξ επαφής.

Στη διδακτορική μου διατριβή, χρησιμοποίησα τεχνικές φασματομετρίας μάζας (MS) ως εργαλείο για να μπορέσω να μελετήσω διάφορες μοριακές αλληλεπιδράσεις κατά τα πρώτα στάδια της αλλεργίας εξ επαφής. Η έμφαση δίνεται στον χαρακτηρισμό των αλλεργιογόνων ουσιών εξ επαφής (haptens) και στις αλληλεπιδράσεις τους με τις πρωτεΐνες του αίματος και του δέρματος, κάτι που είναι απολύτως απαραίτητο για την εμφάνιση αλλεργίας εξ επαφής. Τα ανοσογόνα σύμπλοκα απτενίου-πρωτεΐνης που έχουν αναγνωριστεί υπογραμμίζουν την περίπλοκη αλληλεπίδραση μεταξύ χημικών ουσιών και της ανοσολογικής απόκρισης του δέρματος. Η διατριβή περιγράφει επίσης πώς κοινές ενώσεις που δεν είναι αλλεργιογόνες από μόνες τους, οι λεγόμενες prehapten και prohapten, μπορούν να ενεργοποιηθούν και να μετατραπούν σε απτένιο είτε μέσα είτε έξω από το δέρμα και έτσι να προκαλέσουν αλλεργία εξ επαφής.

Συνολικά, η εργασία της διατριβής έχει παράσχει πολύτιμες γνώσεις σχετικά με τους μοριακούς μηχανισμούς που εμπλέκονται στην ανάπτυξη της αλλεργίας εξ επαφής. Αυτές οι ανακαλύψεις μπορεί να ανοίξουν νέες ευκαιρίες για την ανάπτυξη πιο αποτελεσματικών διαγνωστικών εργαλείων και στρατηγικών για την πρόληψη και τη θεραπεία κοινών τύπων αλλεργιών εξ επαφής.

Përmbledhje Shkencore Popullore

Nëpërmjet përdorimit të përditshëm të produkteve të ndryshme shtëpiake dhe higjienike, kozmetikës, rrobave, bizhuterive, ngjyrave të flokëve, etj., ne njerëzit ekspozohemi ndaj një sërë substancash kimike, disa prej të cilave mund të kenë efekte të dëmshme në lëkurë. Ekspozimi i lëkurës ndaj këtyre kimikateve mund të shkaktojë alergji kontakti (alergjia e lëkurës), një gjendje që më pas çon në inflamacion dhe ekzemë, dermatit prej kontakti alergjik (ACD), në kontaktin më të vogël të lëkurës me substancën. Alergjia e kontaktit është një gjendje e përjetshme dhe fatkeqësisht ende nuk ka një kurë të njohur. Për të mos rrezikuar ekzemën e lëkurës ose simptoma të tjera, një person i sensibilizuar duhet të shmangë plotësisht kontaktin e lëkurës me substancën dhe aktualisht vetëm kortikosteroidet janë në dispozicion për të lehtësuar simptomat. Që të jemi në gjendje për të zhvilluar një trajtim më efektiv në një afat më të gjatë, më shumë kërkime dhe një kuptim më i mirë i mekanizmave molekularë që qëndrojnë në themel të alergjisë së kontaktit është thelbësor.

Në tezën time të doktoraturës, unë kam përdorur teknikat e spektrometrisë së masës (MS) si një mjet për të qenë në gjendje të studioj ndërveprime të ndryshme molekulare gjatë fazave më të hershme të alergjisë së kontaktit. Theksi vihet në karakterizimin e substancave alergjike të kontaktit (haptens) dhe ndërveprimet e tyre me proteinat në gjak dhe lëkurë, diçka që është absolutisht thelbësore për shfaqjen e alergjive nga kontakti. Komplekset imunogjene hapten-proteinë që janë identifikuar theksojnë ndërveprimin e ndërlikuar midis kimikateve dhe përgjigjes imune të lëkurës. Disertacioni përshkruan gjithashtu se si përbërësit e zakonshëm që nuk janë alergjikë në vetvete, të ashtuquajturat prehapten dhe prohapten, mund të aktivizohen dhe shndërrohen në hapten brenda ose jashtë lëkurës dhe kështu të shkaktojnë alergji kontakti.

Në përgjithësi, puna e tezës ka ofruar njohuri të vlefshme për mekanizmat molekularë të përfshirë në zhvillimin e alergjive të kontaktit. Këto zbulime mund të hapin mundësi të reja për zhvillimin e mjeteve dhe strategjive më efektive diagnostikuese për të parandaluar dhe trajtuar llojet e zakonshme të alergjive të kontaktit.

Table of Contents

Abbreviations

(d)DCs	(dermal) Dendritic Cells	LiP	Limited Proteolysis
2-MGN	2-methyleneglutaronitrile	LLNA	Local Lymph Node Assay
ACD	Allergic Contact Dermatitis	*m/z*	Mass-to-charge
APCs	Antigen Presenting Cells	MDBGN	2-(Bromomethyl)-glutaronitrile
CA	Caffeic Acid	MHC	Major Histocompatibility Complex
CAAE	1,1-dimethylallyl ester	MI	Methylisothiazolinone
CAPE	caffeic acid phenethyl ester	MIF	Macrophage Migration Inhibitory Factor
CD	Contact Dermatitis	MRM	Multiple Reaction Monitoring
Cr	Chromium	MS	Mass Spectrometry
DDA	Data Depended Acquisition	Ni	Nickel
DGEBA	Diglycidyl Ether of Bisphenol A	OECD	Organization for Economic Co-operation and Development
DNCB	2,4-Dinitrochlorobenzene	PGE	Phenyl Glycidyl Ether (1,2-epoxy-3-phenoxypropane)
(k)-DPRA	(kinetic) Direct Peptide Reactivity Assay	PRM	Parallel Reaction Monitoring
FDA	Food and Drug Administration	QSAR	Quantitative Structure Activities Relationships
FITC	Fluorescein Isothiocyanate	RHE	Reconstructed human epidermis
Hb	Hemoglobin	SI	Stimulation Index
HCD	Higher-Energy Collisional Dissociation	S_N2	Bimolecular Nucleophilic Substitution
h-CLAT	Human Cell Line Activation Test	S_NAr	Nucleophilic Aromatic Substitution
HLM	Human Liver Microsomes	TRITC	Tetramethyl Rhodamine Isothiocyanate
HSA	Human Serum Albumin	TRITC	Tetramethyl Rhodamine Isothiocyanate
LC	Liquid Chromatography	UV	Ultra Violet

Introduction to the thesis

Humans are continuously exposed to a wide variety of chemical compounds from diverse sources, both natural and man-made. There are two main types of exposure sources: endogenous, such as lipid peroxidation and oxidative stress, and exogenous sources, such as air pollution and occupational exposure. Many of the compounds to which individuals are exposed have the potential to interact with biomacromolecules and, as a result, pose a risk to human health.

Contact dermatitis which is irritation and eczema at the site of exposure, is negatively impacting the quality of life of affected individuals. The second most common form of contact dermatitis is Allergic Contact Dermatitis (ACD), which is the clinical manifestation of contact allergy. It is a type IV delayed hypersensitivity reaction that occurs at the site of exposure when a sensitized individual is exposed to the same chemical at levels that exceed their individual threshold. Only a minority of chemicals, known as haptens, can induce contact allergy and ACD (Karlberg et al., 2008). It is estimated that one in five people are allergic to at least one chemical in their environment (Nielsen et al., 2001), (Alinaghi et al., 2019) which makes it the most common adverse health effect caused by the interaction between our immune system and chemicals in our environment.

Unfortunately, the diagnosis and treatment of ACD have not seen much progress in recent decades. Diagnosis is performed in dermatology clinics, where a standard set of approximately 30 of the most frequent contact allergens, diluted in petrolatum or water, are applied to the patient's back. The development of an eczematous reaction at a test site indicates that the patient is allergic to the contact allergen applied there (Schnuch et al., 2008). Moreover, there is no cure for ACD, and only symptomatic treatment with corticosteroids is available. Since acquired contact allergy is chronic, avoiding the causing agent is the only way to prevent elicitation of the condition. However, this can be extremely difficult in cases of contact allergy to common allergens.

The limitations in both diagnosis and treatment of ACD are due to several factors, including a lack of understanding of the underlying mechanisms and the heterogeneity of haptens. Despite extensive research in recent years, there are still no specific biomarkers for the condition, and the biomarkers that have been identified can be described as general inflammation biomarkers (Koppes et al., 2017).

The purpose of this thesis was to utilize mass spectrometry (MS) based methodologies A) to investigate the initiating event in contact allergy, specifically the interaction between contact allergens and skin proteins after penetration, to identify hapten-protein conjugates with immunological significance, and B) to examine the activation of pre- and pro-haptens, compounds that do not have inherent reactivity but become haptens through transformations that occur outside or inside the skin, respectively.

Paper I describes a proteomic approach to characterize the conjugate formation between three contact allergens, with varying sensitization potencies, and the two major blood proteins, human serum albumin (HSA) and hemoglobin (Hb).

Paper II implements and compares three different MS-based strategies for determining chemical exposure through Hb and the methods usefulness for assessing exposure to contact allergens is discussed. (*Manuscript*)

Paper III describes the identification and quantification of a hapten-protein conjugate in blood and lymph nodes from mice that were topically treated with the fluorescent model contact allergen tetramethyl rhodamine isothiocyanate (TRITC), and examines its immunological relevance to contact hypersensitivity.

Paper IV describes the investigation into prevalent components of propolis, a by-product of honey production, responsible for inducing skin allergy. (*Accepted in Chemical Research in Toxicology*)

Paper V describes the bioactivation of cinnamic alcohol, one of the most widely encountered contact allergens, in the skin using both *in vitro* and *in vivo* systems combined with MS-based analysis. (*Manuscript*)

1. Background

1.1 Human skin

The human skin is considered to be the largest organ of the body, both in terms of surface area and weight. It has been estimated that in adult individuals, the surface area of the skin ranges from 1.6 to 1.85 square meters, and the weight may vary between 3 to 3.5 kg (Leider, 1949). The conventional view of the skin as an inactive barrier between the body and the external environment has been revised in the last decades, and it is now acknowledged that the skin performs a multitude of vital functions, including protection against exogenous pathogens, micro-organisms, physical trauma, and ultra violet (UV) radiation. Additionally, the skin plays a crucial role in maintaining body fluid regulation, temperature control, and immunologic surveillance (Bäsler et al., 2016).

1.1.1 Anatomy

The human skin is comprised of three distinctive layers, namely epidermis, dermis and hypodermis, **Figure 1**. The epidermis, the outermost layer of the skin, can be further divided into five sections: the stratum corneum, the stratum lucidum, stratum granulosum, stratum spinosum and the stratum germinativum, also known as the stratum basale. The primary building blocks of the epidermis are keratinocytes, which are responsible for its structural formation. These cells undergo a transformation over a period of approximately four weeks, from cuboidal dividing cells to flat, keratin-filled residues. In the stratum corneum, the keratinocytes are no longer viable and are referred to as corneocytes, primarily composed of keratin. Part of the epidermis are also a type of dendritic cells (DCs) known as Langerhans cells which act as professional antigen presenting cells (APCs), melanocytes, the role of which is melanin production to protect against UV-radiation, and Merkel-Ranvier cells that act as slowly adapting merchanoreceptors (Baroni et al., 2012). The dermis, responsible for the skin's elasticity, is composed of extracellular matrix components and is a network of collagen and elastin fibers. The dermis is also

home to blood and lymph node vessels, hair follicles, nerves and sweat glands. The hypodermis, the innermost layer of the skin, is mainly composed of fat and connective tissues (Richardson, 2003).

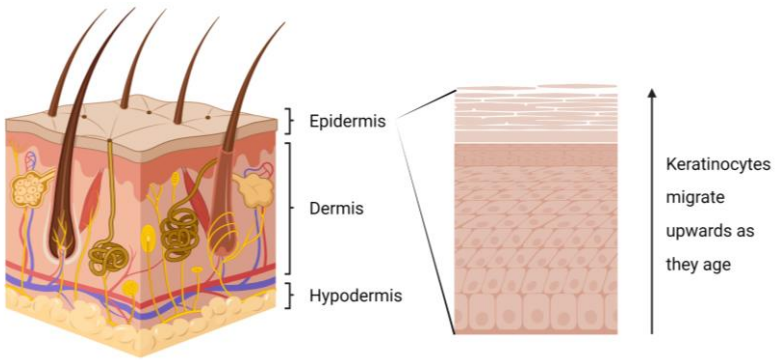


Figure 1: Skin anatomy, presenting the different skin layers, namely epidermis, dermis and hypodermis. [created with BioRender.com]

1.1.2 Pathologies of the skin and CD

As the first line of defense, the skin is designed to serve as a protective barrier, yet it often sustains damage. It has been estimated that there are over 3,000 known diseases of the skin (Bickers et al., 2006). Some of these skin diseases pose a significant threat to life, such as malignant melanoma. However, there are also skin conditions that are not life-threatening but can still have a significant impact on an individual's quality of life. Examples of such conditions are psoriasis, atopic dermatitis, ulcers and contact dermatitis. Contact dermatitis is one of the most common inflammatory dermatological conditions caused by exogenous substances that elicit an immune response leading to inflammation in the skin. The two most common types of contact dermatitis are irritant and allergic contact dermatitis (ACD). Irritant contact dermatitis is caused by direct skin cellular damage, due to exposure to chemicals such as soaps and detergents. During this condition, only the innate immune system is activated by the pro-inflammatory properties of the chemical triggering the response. On the other hand ACD is a type IV delayed-type hypersensitivity reaction that involves the activation of both the innate and acquired immunity (Nosbaum et al., 2009).

It is important to differentiate between ACD and contact allergy. An individual who has contact allergy may develop ACD upon exposure to the sensitizing contact allergen, but not necessarily (Scheinman et al., 2021).

1.2 Allergic contact dermatitis (ACD)

ACD is an acquired immune response triggered by the modification of skin proteins by contact allergens, also referred to as haptens, giving rise to an immunogenic epitope. In most cases, in a patient suffering from contact allergy, the symptoms of ACD typically manifest two to three days after exposure to the hapten and include itching, swelling, and redness at the site of exposure. These symptoms persist as long as the allergen remains in contact with the skin, even during the healing process.

1.2.1 Public health and economic impact

Contact allergy is a widespread public health issue, affecting over 20% of the Western population (Thyssen et al., 2007a), (Alinaghi et al., 2019). It is a cause of great concern for patients, as the symptoms it produces can be painful, cause permanent damage to the skin, and impact their quality of life negatively (Wallenhammar et al., 2004) (Saetterstrøm et al., 2014). Furthermore, contact allergy is a prevalent occupational disease in many countries, particularly among metal and construction workers, hairdressers, healthcare personnel, cleaners, and painters (Peiser et al., 2012) (Dickel et al., 2001). The estimated annual cost of occupational contact dermatitis in the United States alone is approximately $400 million (Thyssen et al., 2007a), (Diepgen and Coenraads, 1999). The risk factors related to the condition include age, sex, use of consumer products, genetic predisposition, and workplace exposure. Women are two times more likely to be affected than men, and the prevalence of the condition in young individuals aged 12-16 is estimated to be 15% (Peiser et al., 2012).

1.2.2 Immunological mechanism of ACD

The sensitization phase of ACD begins when the skin comes in contact with the hapten and results in the formation of an immunogenic epitope. During this phase, immune cells, such as T-lymphocytes, are activated and migrate to the site of exposure. This response leads to the formation of immunological memory, which

allows the individual to respond more rapidly to subsequent exposures to the same hapten.

The elicitation phase of ACD occurs when the individual is re-exposed to the hapten and symptoms of ACD develop. This phase is characterized by the activation of immune cells, release of mediators and the recruitment of immune cells to the site of exposure, resulting in inflammation and the typical symptoms of ACD, such as itching, redness, and swelling.

In summary, the development of ACD involves both innate and acquired immune responses and the interplay between haptens, immune cells, and mediators during the sensitization and elicitation phases, **Figure 2**.

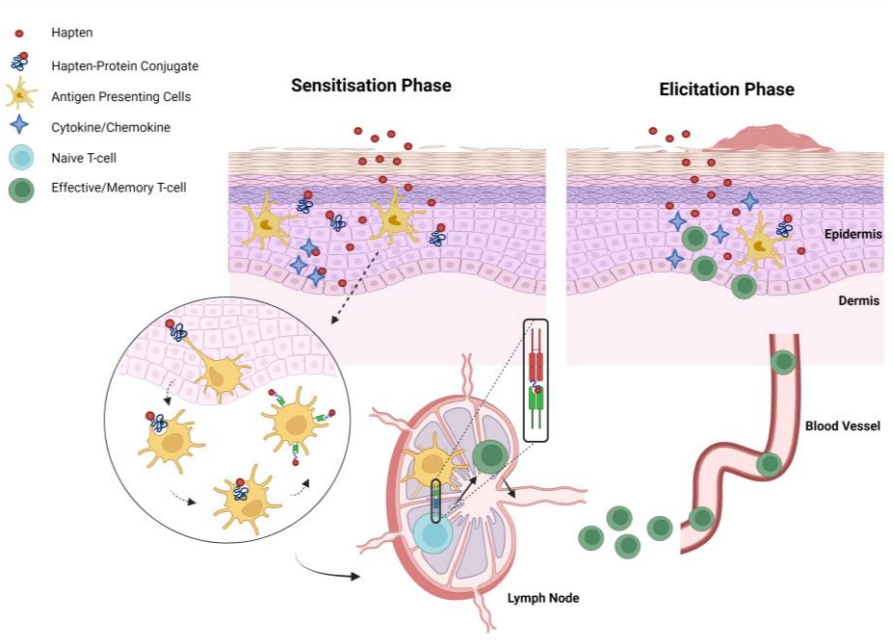


Figure 2: General mechanism of ACD. During the sensitization phase, the hapten penetrate into the epidermis and the hapten-protein conjugate is formed. The antigen formed is recognized by APCs which take up and process the antigen into epitopes while migrating in the nearest local lymph node. In the local lymph nodes, APCs present part of the antigen to naïve T-cells, causing them to proliferate, and differentiate into effector and memory T-cells. the formed T-cells circulate via the blood and the lymphatic system, to the entire body, including the site of exposure. Upon re-exposure to the same hapten, the elicitation phase occurs, leading to the visible symptoms of ACD. [created with BioRender.com]

Penetration of xenobiotics into the epidermis

Although the primary function of the skin is to serve as a protective barrier, it is not completely impermeable. A wide range of compounds, such as environmental pollutants, pharmaceuticals, and many industrial chemicals, have the capacity to infiltrate the skin. The skin's permeability to a particular compound is influenced by several factors, including its molecular weight and LogP (the logarithm of the compound's partition coefficient between polar and non-polar solvents) (Mackay et al., 2006). Small electrophilic compounds (Mw < 500 Da) are more likely to penetrate the skin than larger molecules. Additionally, compounds with a LogP value around 2 are generally considered to have a higher potential for skin penetration (Tsakovska et al., 2017). Sufficient hydrophobicity of the compound facilitates its moving into the lipid envelope of the stratum corneum and into the epidermis. (Karlberg et al., 2008) (Abraham et al., 2011). However, the specific threshold values for molecular weight and LogP of compounds that penetrate the skin may vary depending on the compound, the integrity of the skin and the conditions of application.

Haptenation of Proteins

Haptens are too small to trigger the immune system as such, however, they come in contact with and modify self-proteins in the skin, forming neo-antigens that are recognized by the immune system as “altered self” (Kaplan et al., 2012).

The hypothesis that a chemical's potential to act as a skin sensitizer is related to its capacity to covalently modify a carrier protein, was first proposed by Landsteiner and Jacobs in 1936. (Landsteiner and Jacobs, 1936) The conjugate that is formed is recognized by the immune system as "altered self", which triggers an immune response. The amino acids with nucleophilic side chains, such as lysine, cysteine, and histidine, are capable of facilitating binding. However, the nucleophilicity of these sites is influenced by their ionization state , which is dependent on the pH of the microenvironment and influenced by the surrounding amino acids and the epithelium, with the unprotonated form generally being the most nucleophilic (Divkovic, et al. 2005). Exposure factors that affect the hapten-protein conjugate formation, are the dose, frequency (Paramasivan et al., 2010), duration of exposure (van Och et al., 2003), the vehicle used during application (Rozieres, et al. 2010) as well as genetic factors, such as genetic deficiency of detoxifications enzymes (Friedmann et al., 2015).

Haptens can react with different amino acid residues through various mechanisms. Some of the most significant reactions include nucleophilic substitution (S_N2 mechanism), nucleophilic aromatic substitution (S_NAr), and Michael addition reactions, **Figure 3a-c**.

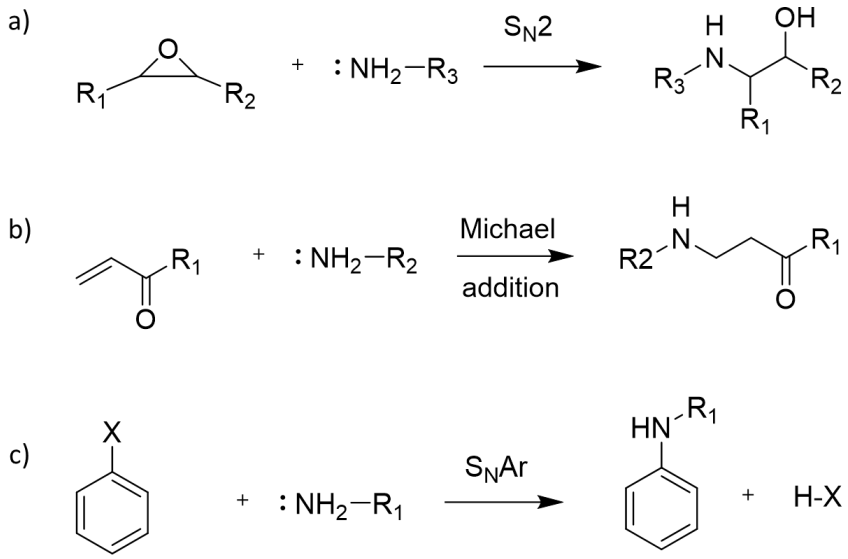


Figure 3: a) An example of nucleophilic substitution (S_N2-type mechanism) is the reaction between epoxides and nucleophiles, such as amines, to form adducts. With epoxides, substitution can occur at either carbon. b) During a nucleophilic aromatic substitution, a nucleophile, represented here by an amine, displaces a leaving group (X) in an aromatic ring, to form adducts. c) α,β-Unsaturated carbonyl compounds, react with nucleophiles, represented here by an amine, via Michael addition.

Radical mechanisms can also occur between compounds prone to autoxidation and proteins. The radical mechanism involved in autoxidation starts with the initiation step, where a radical species is formed from the original compound. In the next step this radical undergoes propagation steps, generating new radicals that can react with other molecules, leading to a chain reaction. The process eventually slows down as radicals react with each other in termination steps, resulting in the formation of non-radical products (Karlberg et al., 2008). Metals belong to a separate category and mainly react with skin proteins through the formation of non-covalent coordination-chelation complexes (Schmidt and Goebeler, 2015).

Activation of compounds: pro-and pre-haptens

Complete haptens are electrophilic compounds that have inherent reactivity and can be conjugated to skin proteins. Apart from this class of compounds, those without reactive moieties can be activated either before permeating the skin (such as through autoxidation), referred to as prehaptens, or through bioactivation in the skin (such as through metabolism facilitated by skin enzymes), known as prohaptens (Karlberg, et al. 2013), **Figure 4**.

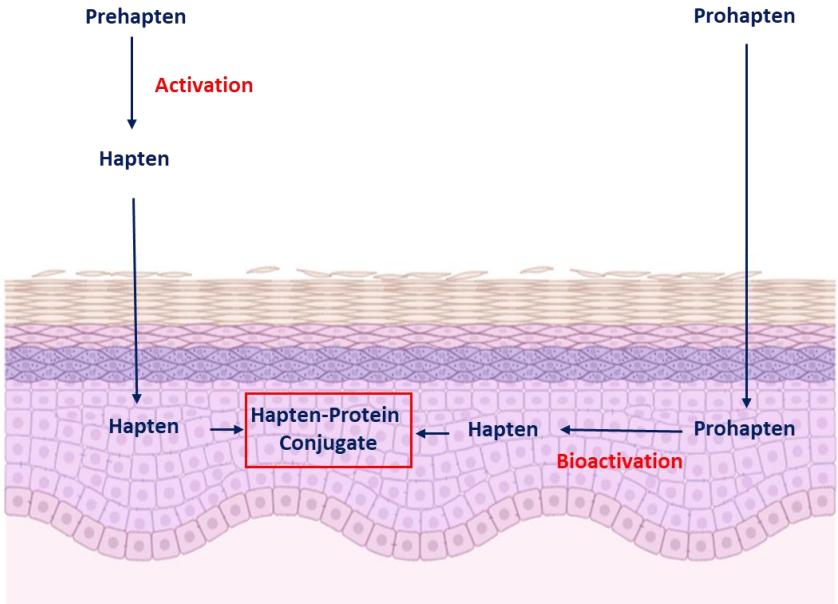


Figure 4: Schematic overview of the activation of prehaptens outside the epidermis and of prohaptens inside the epidermis. [created with BioRender.com]

The skin is a major metabolic organ that is capable of undergoing both phase I and phase II metabolism, thereby enabling biotransformation of xenobiotics that penetrate the epidermis (SMITH, 2001). Both keratinocytes and Langerhans cells present in the epidermis have been shown to be metabolically active (Swanson, 2004), (Modi et al., 2012). While this process aims to detoxify the body from foreign substances, it can sometimes result in the activation of otherwise non-harmful compounds.

Phase I metabolism involves the addition of functional groups, mostly polar, to the xenobiotics in order to aid the excretion from the body. This is achieved

through processes such as oxidation, reduction, and hydrolysis. Phase I enzymes include cytochrome P450, alcohol and aldehyde dehydrogenases, monoamine oxidases, flavin-containing monooxygenases, and hydrolytic enzymes. Phase II enzymes present in the skin include glutathione-S-transferase, uridine 5'-diphosphoglucuronosyltransferase, N-acyl transferase, and sulfotransferase (SMITH, 2001).

In addition to activation within the skin, some inactive compounds can be converted into protein-reactive species through contact with air, such as through the process of autoxidation. These compounds are known as prehaptens, and typical examples include limonene, linalool, cinnamic alcohol, and geraniol (Karlberg et al., 2013).

Activation of innate responses

Strong haptens, both of electrophilic nature and metals, have the potential to activate innate immune responses, in a similar fashion to irritants, by direct or indirect activation of pattern recognition receptors. Accumulation of these haptens within the cell induces cell death which is accompanied by the release of damage-associated molecular patterns and production of reactive oxygen species (Esser et al., 2012) that leads through a chain of events to the activation of Toll-like receptors and finally the production of different inflammatory cytokines (Klekotka et al., 2010), (Yasukawa et al., 2014). Innate immune responses can also be triggered by the misfolding and unfolding of proteins inside cells due to strong allergens, resulting in the activation of the unfolded protein response pathway (Luís et al., 2014).

Activation of adaptive responses: Antigen processing and DC migration

The acquired immune system develops when antigens are presented to T-cells by APCs, such as DCs, B-cells, and macrophages. Langerhans cells and dermal dendritic cells (dDCs) are two types of DCs that are involved in the immune response in the skin. Langerhans cells are located in the epidermis, while dDCs are found in the dermis. These cells recognize hapten-protein conjugates as antigens, take them up, and migrate to local lymph nodes via the lymphatic vessels. During migration, the antigen is internalized, processed into peptide epitopes, and displayed on the cell surface through major histocompatibility complex (MHC) receptors (Vocanson et al., 2009).

Antigen presentation and T-cell activation

After migrating to the nearby lymph node, the APCs presenting the immunogenic peptide initiate the activation, proliferation, and differentiation of hapten-specific naive T-cells. This differentiation results in the creation of two subsets, memory T-cells, which serve as a repository for the specific antigenic response, and effector T-cells, which mediate the immune response to the antigen. These T-cells then leave the lymph nodes and circulate through the blood and lymphatic system (Martin, 2004), (Rustemeyer et al., 2006).

The elicitation of phase

Elicitation is the second stage of ACD and takes place when an individual who has been sensitized to a hapten is exposed to the same hapten again. During this stage, a rapid and amplified response is triggered by the antigen-specific memory T-cells formed during the sensitization phase (Rustemeyer et al., 2006). However, elicitation can also occur after a single application, since elicitation occurs within 10-15 days and a sensitizer can remain in the skin for approximately two weeks. This type of ACD is known as primary ACD (Saint-Mezard et al., 2003).

1.3 Diagnosis

The gold standard for diagnosing contact allergy is the patch test, **Figure 5**. In this test, small areas of the patient's skin, typically on the back, are exposed to the suspect substances using chambers affixed to the skin with tape. The substances are usually applied using a vehicle such as petrolatum or water, although alcohol is sometimes used. The concentrations used depend on the allergen and should induce an allergic response but cause minimal skin irritation or adverse health effects. Dosing is an essential aspect of the patch test as overdosing can result in false-positive results or sensitization, while underdosing can lead to false-negative results (Bruze et al., 2007). The test lasts for 48 hours, and the results are read following an internationally accepted semi-quantitative scale, performed at three different times after patch removal at day two (D2), day three or four (D3 or D4), and day seven (D7). Research has shown that if the D7 reading is not performed, 15% of positive reactions can be missed (Johansen et al., 2015) (Amerongen et al., 2019).

The selection of appropriate substances to be tested on the patient's back is a crucial step in the patch testing process, as the number of substances that can be tested is limited. Different baseline series containing 30 to 100 substances are available, and patients are tested with these series, which can be modified by adding specialized test series or individual allergens relevant to the patient's exposures, including skin care products and occupation (Friis et al., 2013).

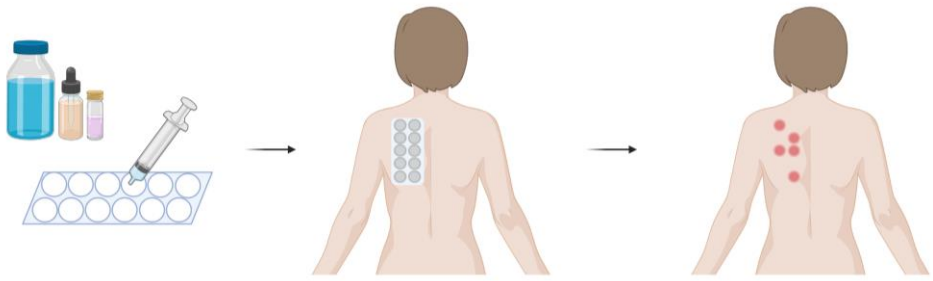


Figure 5: Schematic representation of the patch testing procedure. A set of the most common contact allergens are applied on the back of the patient and the development of an eczematous reaction is monitored during different time points. [created with BioRender.com]

1.4 Prevalent contact allergens

On a daily basis, individuals are exposed to a substantial number of chemicals derived from various sources, including cosmetics, jewelry, household items, dyes, and others. The chemicals responsible for causing contact allergy encompass a broad range and vary in terms of their potency. To date, 5,000 compounds have been identified as potential allergens (de Groot and Frosch, 2001).

1.4.1 Fragrances

Contact allergy to fragrances is the most prevalent form in the general population, with a prevalence ranging from 1.0-4.2% (Thyssen et al., 2007b). Despite the fact that fragrances are complex mixtures comprising over 2,000 compounds, only

a limited number of these are known to cause contact allergy. A mix of eight compounds, known as Fragrance Mix I, is part of the standard diagnostic series used to identify contact allergies (Thyssen et al., 2008). A second mix of six compounds, Fragrance Mix II, has recently been added to this diagnostic series (Krautheim et al., 2010). The increased frequency of fragrance allergy is attributed to the formation of allergens through the process of autoxidation of fragrance compounds. For example, the autoxidation of limonene from citrus and linalool from lavender can lead to the formation of hydroperoxides, which are known to cause ACD. In contrast, the parent compounds rarely cause sensitization themselves (Bennike et al., 2019), (Sköld et al., 2004).

1.4.2 Metals

Metals, such as nickel (Ni), cobalt and chromium are also prevalent contact allergens. In fact, Ni is the most common source of contact allergy in most industrialized countries (Ahlström et al., 2019). It is estimated that approximately 8-19% of European adults are allergic to Ni (Diepgen et al., 2016). This high prevalence of nickel allergy is not due to the allergen being the most potent, but rather due to frequent and prolonged exposure to the allergen through sources such as the metal parts of jewelry and watches. However, the incidence of Ni allergy has decreased in many European countries following the implementation of the European Union (EU) Nickel Directive in 1994, which came into full effect in 2001 (Thyssen et al., 2009). Unfortunately, this regulation does not apply in the United States, where the release of Ni is still unregulated. In a similar manner, the prevalence of contact allergy to chromium present in cement and leather products that have been tanned, has been reduced due to the introduction of relevant legislation in 2005 (Bregnbak et al., 2015). However, this legislation does not extend to leather products, resulting in an increase in the incidence of contact allergy from this type of products.

1.4.3 Others

One of the commonly reported allergens is *p*-phenylenediamine, which is present in oxidative dyes and is a widespread occupational allergen. However, recent studies have shown a decline in the incidence of ACD caused by *p*-phenylenediamine, due to its substitution with alternative derivatives (Kirchlechner et al., 2016). Preservatives have also been identified as a common cause of contact allergies. For instance, methylisothiazolinone (MI) was responsible for an outbreak of ACD

in 2010. This situation was later rectified by the implementation of appropriate legislation that limited the concentration of MI in rinse-off products to 15 parts per million (Uter et al., 2020). Another preservative, 2-bromo-2-(bromomethyl)-glutaronitrile (MDBGN), which was introduced in the 1980s and was widely used in industrial and cosmetic products, has been banned in EU in 2016 due to an increase in contact allergy cases. However, exposure to MDBGN can still occur through occupational exposure or the use of topical medications (Giménez-Arnau et al., 2017), (Johansen et al., 2005). Contact allergy to epoxy resin systems components, such as diglycidyl ether of bisphenol A (DGEBA) is also very common (Kligman, 1966). These are classes of thermosetting products that are used as adhesives, coatings (Niklasson et al., 2009), and are among the most frequent causes of occupational allergic contact dermatitis (Pesonen et al., 2015).

1.5 Assessment of skin sensitization potency

ACD is a major concern for society and industries, particularly with the introduction of new compounds on the market. Cosmetics and toiletry products, which are in direct and prolonged contact with the skin, must be free of chemicals that can potentially cause ACD or contain amounts below an established threshold to ensure consumer safety. Historically, animal testing has been the standard method for evaluating the sensitization potential of novel compounds. However, since 2003, cosmetics and toiletries tested on animals have not been allowed for sale in EU, as per the 7th amendment to the European Union's Cosmetic Directive (76/768/EEC). Efforts have been made to develop accurate and reliable non-animal-based methods, however, to date, none of the methods used can be considered a stand-alone method.

1.5.1 Local lymph node assay

The Local Lymph Node Assay (LLNA) is a widely recognized *in vivo* test used to evaluate the potency of chemicals to cause skin sensitization. It replaced the Guinea Pig Maximization Test, which was the initial test used for potency assessment (Magnusson and Kligman, 1969). The LLNA is considered to be the "gold standard" test in the field and has been officially adopted by the Organization for Economic Cooperation and Development (OECD) and the U.S. Food and Drug

Administration (FDA). (OECD, Guideline for the testing of Chemicals 429. Skin sensitization: Local Lymph Node Assay) (OECD, 2010).

The LLNA is based on the principle that skin sensitization can trigger an immune response, resulting in the activation and proliferation of immune cells in the draining lymph nodes. Young adult female mice of CBA/Ca or CBA/J strain, typically between 8 to 12 weeks of age and weighing between 18 to 25 grams, are selected for the test. The test substance is applied topically to the ears of the mice in three consecutive days (day 1, 2 and 3). On day 6, the animals are injected with radioactive thymidine through the tail vein and euthanized after 5h. The size of the draining lymph nodes is assessed through the uptake of the isotope, **Figure 6**. The increase in the number of cells in the lymph nodes is used as an indicator of skin sensitization potential, and the data collected from the assay is analyzed to determine the Stimulation Index (SI). The SI is calculated as the ratio of the radioactivity in the lymph nodes of the treated mice to the radioactivity in the lymph nodes of the control mice. Compounds causing an SI value greater than 3 are considered positive for skin sensitization. Compounds EC3 values, which are the estimated concentrations to cause an SI of 3, are reported and used for classification regarding sensitization potency (Gerberick et al., 2007).

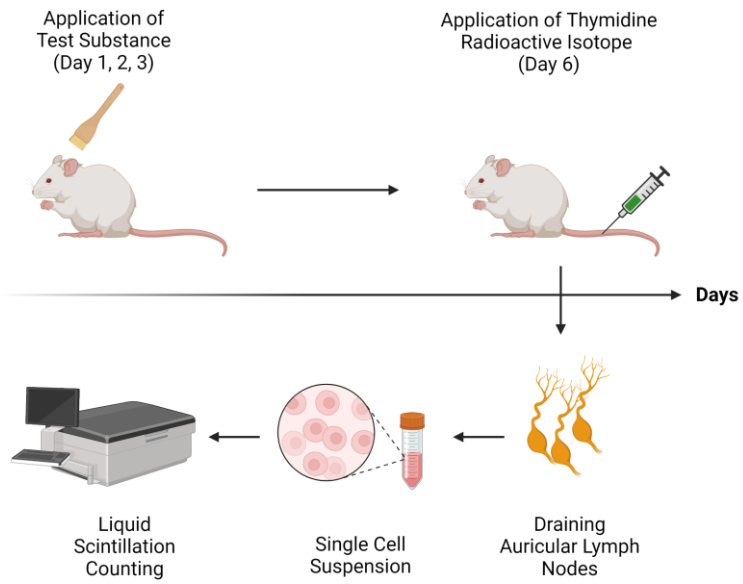


Figure 6: Schematic representation of the local lymph node assay. [created with BioRender.com]

1.5.2 *In vitro* methods

To date, a comparable *in vitro* alternative to the LLNA has yet to be developed. Despite ongoing efforts to create a method that can accurately predict the sensitization potency of compounds and mitigate instances of ACD in the general population, the complex molecular and cellular processes involved in ACD may always necessitate the use of a combination of assays.

There are four critical events in the development of ACD, which pose significant challenges in the development of *in vitro* methods that are independent of animal testing. These events include: (1) molecular initiation, or the covalent binding of contact allergens to skin proteins; (2) the inflammatory responses and gene expression linked to specific signaling pathways that occur within keratinocytes, such as the antioxidant/electrophile response element-dependent pathways; (3) the activation of DCs; and (4) T-cell proliferation. The Direct Peptide Reactivity Assay (DPRA) is widely accepted as the most effective method for addressing the first key event, molecular initiation. The second event, inflammatory responses, is being addressed through the use of the OECD-recommended methods KeratinoSens and LuSens. The human cell line activation test (h-CLAT) assay is used to address the third event, activation of DCs. Despite ongoing efforts, the fourth event, T-cell activation, is still being addressed through the use of the LLNA method, which also address all the previous events.

Quantitative structure activities relationships (QSAR)

Physicochemical parameter of compounds have been exploited to access their sensitizing potential, through what is known as the ‘Quantitative Structure Activities Relationships’ (QSAR), first introduced in 1930s (Landsteiner, 1936). Such properties include partition coefficient, as well as chemical and thermodynamic constants. QSAR predictions are always accurate for compounds that react via the same mechanism, but are more challenging for compounds, such as aldehydes that can react via several mechanisms (Franot et al., 1994).

Direct peptide reactivity assay (DPRA)

The Direct Peptide Reactivity Assay (DPRA), developed at the company Procter & Gamble, is a method for evaluating the skin sensitization potential of a chemical, offering an alternative to animal testing that is quicker and more cost-

effective. DPRA was previously considered the standard method for assessing sensitization potential, in accordance with the 2015 OECD test guideline 442C. DPRA measures the reactivity of a chemical towards synthetic peptides that mimic proteins found in the skin, containing cysteine and lysine moieties. The assay evaluates changes in fluorescence intensity or absorbance following a 24-hour incubation with a single concentration of a test substance, which reflects the depletion of peptide as a result of the amount of hapten-peptide conjugate formed (OECD, 2021).

The kinetic DPRA (k-DPRA) is a variation of DPRA that measures the rate of reaction between a tested substance and a synthetic cysteine-containing peptide. The k-DPRA incorporates various concentrations and timepoint measurements, to provide additional information about the reaction kinetics and offer a more comprehensive evaluation of skin sensitization potential. The assay involves measuring changes in fluorescence over time, after incubation with fluorescent monobromobimane that rapidly reacts with unmodified cysteine residues, allowing for the calculation of reaction rate constants and other kinetic parameters (Wareing, 2020).

DPRA and k-DPRA have several advantages over traditional animal testing methods, including the ability to test a large number of substances in a short period of time, the absence of ethical concerns associated with animal testing, and a lower cost compared to animal testing methods. However, both assays are extremely simplified systems and do not provide structural information about the hapten-peptide conjugates formed in each case. This drawback is particularly problematic when testing compounds without inherent reactivity that require activation to become haptens. Additionally, DPRA assays cannot predict at all the sensitization potential of compounds that require bioactivation.

KeratinoSens™ and LuSens

KeratinoSens™ and LuSens are *in vitro* alternatives to animal testing for skin sensitization assessment, developed at Givaudan, a leading global company in the fragrance and flavor industry. They address key event two of the development of ACD, which involves inflammatory responses and gene expressions linked to specific signaling pathways that take place inside keratinocytes. These assays quantify the production of cytokines in response to chemical exposure. Both KeratinoSens™ and LuSens are based on the same principle and use immortalized human keratinocyte cells to measure the activation of the Keap1-Nrf2-antioxidant/electrophile response element pathway. This pathway is a crucial regulator of

cytoprotective responses to electrophiles and oxidative stress, and it is known to be involved in cellular processes during skin sensitization. In the assays, the test substance is incubated with the keratinocyte cells that have been engineered to express the luciferase gene under the control of the antioxidant/electrophile response element. The activation of the antioxidant/electrophile response element in connection to the test substance results in the expression of luciferase, which is the endpoint measured after 48 hours of incubation with the test substance (OECD, 2022a).

Both methods have been validated and recognized as an alternative method for skin sensitization testing by regulatory agencies such as the OECD. These assays offer the same advantages as DPRA and kDPRA over traditional animal testing methods. However, while they can predict skin sensitization potential of certain chemicals, they may not be accurate for all chemical classes, such as metals, which may cause skin sensitization through mechanisms that are not related to the Keap1-Nrf2-ARE pathway. Additionally, they cannot fully replicate the complex biological and physiological processes in the sensitization process.

Human cell line activation test (h-CLAT)

The Human Cell Line Activation Test (h-CLAT) is a reliable *in vitro* assay that is utilized for evaluating the skin sensitization potential of various chemicals, jointly developed by Shiseido and Kao Corporation in Japan. The test addresses key event three of the sensitization process, which involves the activation of DCS. The h-CLAT is based on the principle that exposure to skin sensitizers activates immune cells, leading to the release of cytokines and other signaling molecules. The measurement of these signaling molecules serves as an indicator of the substance's ability to cause skin sensitization. The h-CLAT is a quantitative method that measures changes in the expression of cell surface markers (CD86 and CD54) on a human monocytic leukemia cell line after a 24-hour incubation with the test chemical. The changes are detected by flow cytometry following staining of the cells with fluorochrome-tagged antibodies. The relative fluorescence intensity of the surface markers compared to the solvent/vehicle control is calculated and used to differentiate between sensitizers and non-sensitizers (OECD, 2022b). However, h-CLAT may not be able to predict the skin sensitization potential of all chemicals with high accuracy, especially those that require metabolic activation *in vivo*. In the same line as the previous *in vitro* methods described, h-CLAT assay uses a

single cell type and does not account for the complexity of the skin and the interaction of different cell types and biological mechanisms involved in the sensitization process.

Genomic allergen rapid detection (GARD)

Genomic Allergen Rapid Detection (GARD) is a genomics and machine-learning based *in vitro* skin sensitization assay evaluating the potential of chemicals and other substances to cause allergic reactions, developed by the company SenzaGen based in Lund, Sweden. GARD is included in OECD TG 442E for *in vitro* skin sensitization and addresses the third molecular key event of the sensitization process (OECD, 2022b). The GARD assay uses a combination of cell-based and gene expression technologies to evaluate the ability of a chemical or substance to activate the immune system and cause an allergic response. The GARD technology platform comprises of two components, namely GARDskin and GARDpotency. The method utilizes the nCounter system to measure the expression level of a set of genes in the human myeloid cell line MUTZ-3 exposed to chemicals. The GARDskin component involves the assessment of the expression of 200 genes, known as the GARD prediction signature, using a prediction model based on a support vector machine trained with 38 chemicals. This model classifies chemicals as skin sensitizers or non-sensitizers. GARDpotency serves as the second tier of the testing strategy after GARDskin. It sub-categorizes sensitizers into GHS/CLP categories 1A (strong/extreme sensitizers) or 1B (weak/moderate sensitizers) based on the expression of 52 genes, known as the GARD potency prediction signature.

Although GARD assays have shown promising results, the analysis of GARD data requires specialized expertise and software tools, which can be challenging to interpret accurately, especially when dealing with complex gene expression patterns involving multiple genes or pathways.

Reconstructed human skin models

Organotypic skin models are gaining popularity for allergenicity testing in both research and industrial settings. These models are designed to replicate the complex structure and function of human skin and are cultured at the air-liquid interface, which promotes epidermal differentiation and stratification, mimicking human skin. The culture method used in these models allows for topical application of chemicals to the stratum corneum, simulating human exposure scenarios where

chemicals can penetrate the stratum corneum and interact with the underlying skin layers. One commonly used organotypic skin model for allergenicity testing is reconstructed human epidermis (RHE), which is generated by culturing human keratinocytes at the air-liquid interface to create a stratified, epidermis-like structure (Netzlaff et al., 2005). In addition to RHE, more advanced organotypic skin models with immune competence have been developed, incorporating immune cells such as Langerhans cells or T-cells. These immune competent skin models provide a sophisticated and physiologically relevant system for studying the immunotoxicity and allergenicity of chemicals (Bergers et al., 2016). Furthermore, novel microphysiological systems (skin-on-a-chip) are being developed, offering the possibility of culturing human skin models in a systemically controlled microenvironment able to create and maintain homeostasis (Risueño et al., 2021).

1.6 Limitations in knowledge about mechanism of ACD

Although there has been a substantial amount of research on ACD, there are still limitations in the understanding of its underlying mechanisms. These limitations emphasize the need for further research to gain a more comprehensive understanding of the causes and triggers of ACD, and to develop effective prevention and treatment strategies.

One of the major limitations is the insufficient knowledge of the molecular mechanisms involved in the development of ACD. While the role of immune system activation and the formation of hapten-protein conjugates is known, the exact processes leading to skin sensitization are yet to be fully understood. The identity of the proteins involved in the initiation event, the nature of the hapten-protein conjugate triggering the immune system, and the identification of hapten-modified and/or non-modified peptides bound to immunological receptors and recognized by lymphocytes are some of the aspects that require further exploration. Additionally, the role of other biomolecules, such as lipids, in triggering the immune response in ACD is not yet fully established. This lack of understanding makes it challenging to fully comprehend the complex interactions between the skin and immune system in ACD.

Another challenge is the difficulty in identifying and characterizing allergens due to their heterogeneity. There is a wide range of substances that can cause ACD, and the mechanisms of sensitization can vary greatly depending on the type of sensitizer involved. This added layer of complexity makes it difficult to develop a

general understanding of the mechanisms underlying ACD. Thus, further research is needed to understand the properties of allergens that make them capable of inducing ACD.

Increased knowledge of these mechanistic aspects can lead to the identification of specific biomarkers for contact allergy, which could be utilized for development of better diagnostic tools based on blood samples. These advancements will ultimately contribute to the development of more effective strategies for the prevention and treatment of ACD. A reliable biomarker can help identify individuals at risk of developing ACD before symptoms appear, enabling early intervention, such as avoiding exposure to the allergen, implementing preventive measures, and educating patients on allergen avoidance strategies. Biomarkers can serve as targets for drug discovery and development, leading to the creation of new drugs or therapies for the treatment of ACD.

1.7 Mass spectrometry

The history of Mass Spectrometry (MS) can be traced back to the late 19th century, with the principles of MS being first established and the first mass spectrometer built by J.J. Thomson in 1912. He was awarded the Nobel Prize in 1906 for his contributions to the field of electron physics, which is considered the foundation of MS. The 1940s and 1950s saw significant developments in MS with the introduction of magnetic sector MS and time-of-flight MS, which greatly expanded the capabilities of the technique.

In recent decades, the introduction of new ionization methods and mass analyzers has led to the widespread use of MS in various fields, including chemistry, biology, medicine, environmental analysis, and forensic science, among others. Today, MS is considered an essential tool for the analysis of complex mixtures and is highly effective for qualitative and quantitative applications.

The components of a typical mass spectrometer include an ionization source, a mass analyzer, a detector, and a processor. The ionization source and mass analyzer play a critical role in determining the performance and capabilities of the mass spectrometer in terms of detectability and selectivity.

1.7.1 Ion sources

Regardless of the method chosen for sample introduction into the mass spectrometer, the generation of gas-phase ions is required. Ionization of analytes takes place in the ion source of a mass spectrometer. There are several types of ion sources that can be used in MS, each with its own unique capabilities and limitations and the choice of ion source depends mostly on the type of sample being analyzed and the separation technique used prior to the MS analysis. When gas chromatography systems are used, the ion sources employed operate at high vacuum with the most common type being electron ionization (EI). When liquid chromatography (LC) is used, the ionization process is typically at atmospheric pressure, known as atmospheric pressure ionization (API), due to the liquid mobile phase. In this case, methods such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI) are used. Other common off-line ionization techniques include matrix-assisted laser desorption ionization (MALDI) and desorption electrospray ionization (DESI).

ESI is a soft ionization technique that enables the delivery of ions from liquid solutions into the gas phase and it is commonly used for a wide range of compounds, from small molecules to large biomolecules, such as proteins. ESI was initially described by Masamichi Yamashita and John Fenn in 1984 (Yamashita and Fenn, 1984) and independently by Lidia Gall and her colleagues in Russia during the same year (Alexandrov et al., 2008). The first step during ESI is the formation of charged droplets, occurring when the liquid sample solution is sprayed across a high potential difference from the electrospray needle, resulting in a charged spray. In a next step, the spray droplets are dissolvated by nitrogen and heat, causing the droplets to decrease and ultimately release the charged ions. There are two different mechanisms suggested regarding ESI ionization, namely ion evaporation mechanism and charge residue mechanism (Dole et al., 1968), (Iribarne, 1976), (Wilm, 2011). The ion evaporation mechanism suggests that the constant evaporation of the spray droplets will lead to an increase of the field strength at their surface, which will eventually cause the splitting of the droplets and expel solvated ions from them. This mechanism is suggested to be more valid for smaller molecules (Iribarne, 1976), (Wilm, 2011). On the other hand, the charge residue mechanism, which is believed to be more valid in the case of macromolecules, proposes the existence of one analytical ion per droplet formed, and

that the charge excess is accommodated by the molecule itself, upon complete solvent evaporation from the droplet. The charge residue mechanism also involves the splitting of droplets but no expulsion of them (Dole et al., 1968), (Wilm, 2011). In the case of unfolded proteins though, the chain ejection model has been proposed. In the chain ejection model, the presence of hydrophobic residues exposed to the solvent causes the unfolded protein to move towards the surface of the droplet. Subsequently, the protein chain is gradually expelled from the droplet, progressing through different stages where a growing polypeptide tail extends outward. This ejection process is primarily driven by electrostatic repulsion between the droplet and the protein tail (Pimlott and Konermann, 2021).

APCI is another soft ionization technique that is very similar to ESI. The origins of atmospheric pressure chemical ionization sources combined with MS can be found in the 1960s in studies of ions in flames and of ion chemistry in corona discharges up to atmospheric pressure. The first application of APCI combined with MS for trace chemical analysis was by the Franklin GNO Corporation who in 1971 developed an instrument combining APCI with ion mobility and MS (Collins and Lee, 2002). The main difference between the two is that in ESI ions may be formed in both the droplets and in the droplet/gas phase interface, whereas in APCI the ions are formed only in the gas phase. During APCI the voltage is not applied on the inlet capillary, but the mobile phase is evaporated by heat and the atmosphere with nitrogen and water is ionized with a corona discharge. Interaction of the sample molecules with created gas phase protons and hydroxyl groups causes ionization of the former. In the case of APPI, which is a complementary ionization technique to ESI and APCI, photons from a xenon lamp are used as the source of ionization. Robb, Covey, and Bruins were credited with the initial advancement of atmospheric pressure photoionization (APPI) for LC-MS (Robb et al., 2000).

Electron Ionization (EI), is a hard ionization technique that is commonly used for gas chromatography-MS applications involving volatile, small organic molecules. The technique was first introduced in 1918 by Canadian-American Physicist Arthur J. Dempster (Dempster, 1918). In EI the pressure must be around 10^{-5} to 10^{-6} torr and the ionization occurs through interaction of the gas-phase sample molecules with high-energy electrons originating from a heated filament. This electron beam leads to the ejection of an electron from the gas phase molecule producing a positive radical ion.

Another common ionization technique is the Matrix-Assisted Laser Desorption Ionization (MALDI), coined in 1985 by Hillenkamp, Karas, and colleagues. However, the breakthrough of the technique is dated in In 1987, when Tanaka and team used cobalt particles and a nitrogen laser for ionization, achieving ionization of large molecules (Tanaka et al., 1988). A soft ionization technique that is used for the analysis of large biomolecules and non-volatile small molecules, which has found its major application in imaging MS. In MALDI the sample is typically mixed with a matrix and bombarded with a laser. The matrix should contain a chromophore that absorbs at the laser wavelength. The matrix absorbs the laser energy and transfers a proton to the sample resulting in the ionization. MALDI leads to mostly singly charged ions (Knochenmuss, 2006).

Recent advances in ionization techniques, especially for biomolecules, include surface-assisted laser desorption ionization (SALDI), desorption electrospray ionization (DESI), laser ablation electrospray ionization (LAESI), and laser-induced liquid bead ion desorption (LILBID). Among those, DESI and LAESI are ambient ionization techniques enabling direct analysis of samples in their native state.

1.7.2 Analyzers

Mass analyzers are instrumental in separating ions based on their *m/z* ratio prior to their detection. The mechanism of separation is a result of the interactions between charged ions and electric and magnetic fields within a vacuum. Mass spectrometers are capable of detecting not only intact ions but also fragment ions, which are produced by the transfer of energy to the analyte and subsequent breaking of covalent bonds. These fragment ions play a crucial role in structural elucidation and determination of the intact ion.

Mass analyzers can be further classified into two categories: beam-type and trapping-type instruments. In the former category, ions are delivered to the mass analyzer in a single beam, while in the latter, ions are temporarily confined in a spatially restricted region of the mass analyzer before being sent to the detector. Examples of beam-type mass analyzers include the quadrupole and time-of-flight (ToF), while trapping-type mass analyzers include the quadrupole ion trap, linear ion trap, ion cyclotron resonance, and orbitrap.

The choice of mass analyzer is dependent on the analytical task at hand. Triple quadrupoles are commonly used for routine qualitative and quantitative analysis, while high-resolution mass analyzers such as ToF and orbitraps are preferred for

the determination of molecular formulas of *a priori* unknown compounds and screening purposes.

Quadrupole (Q)

A quadrupole (Q) is composed of four parallel rods connected in pairs, with the opposing pairs of rods being electrically connected to one another. The separation of ions is achieved by their stability in an oscillating electric field created within the quadrupole. This electric field is generated by the application of a radio frequency voltage on top of a direct current voltage. One of the pairs have a negative direct current and the other pair a positive direct current. Only ions with a specific *m/z* ratio will pass through at a given setting of radio frequency and direct current. The most commonly used instrument utilizing this type of mass analyzer in combination with LC is a tandem quadrupole mass spectrometer, also known as a triple quadrupole. It consists of two quadrupole mass analyzers separated by a collision cell. In these instruments, mass analysis is performed in the first and third quadrupoles, which can operate as a mass filter for the selection of specific ions based on their *m/z* or in transmission mode where all ions are transmitted. The second device is usually a hexa- or octapole and functions as a collision cell where fragmentation of the ions takes place through collision-induced dissociation with an inert gas such as argon or nitrogen. The resulting fragment ions are then separated and detected based on their *m/z* in the third quadrupole.

Different types of scan modes can be performed on this type of instrument, including full scan, selected ion monitoring, selected reaction monitoring, product ion scan, precursor ion scan, and neutral loss scan. Tandem quadrupole mass spectrometers are ideal for the identification and quantification of known analytes with available reference standards.

Time-of-flight (ToF) mass analyzers

In time-of-flight (ToF) MS, the generated ions are subjected to an electric field that accelerates them into a field-free flight tube, ensuring that all ions possess equal kinetic energy. The ions are then detected at the end of the drift region by measuring the time it takes them to travel the defined distance. The time of travel is proportional to the *m/z* of the ion, with lighter ions reaching the detector earlier than heavier ions due to their comparatively higher velocity. This relationship can be represented as kinetic energy being equal to $\frac{mv^2}{2}$, where m represents the mass

of the ion and v represents its velocity. ToF mass analyzers are highly versatile and can be used for a wide range of analytical purposes (Cotter, 1997).

Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap mass analyzers

Fourier transform ion cyclotron resonance (FT-ICR) is a trapping technology using a high magnetic field to achieve very high mass resolution and accuracy. The FT-ICR process involves capturing the ion signals in the trap, recording their secular frequencies, which are then transformed into a mass spectrum through Fourier transformation. The high stability of the ion orbits in the trap enables the acquisition of high-resolution mass spectra, typically with mass resolution in the range of hundreds of thousands to millions, and unsurpassed mass accuracy. The drawback of FT-ICR is the high instrumental and running costs.

The Orbitrap instrument has largely replaced FT-ICR and is comprised of an outer barrel-like electrode and a central coaxial spindle-electrode. Ions are injected into the trap and follow a stable helical path in a co-axial motion when a voltage is applied between the outer and central electrodes. The frequency of the co-axial motion around the inner electrode is inversely proportional to the *m/z* of the ion, and the motion of the ions is then measured through the induction of a current in a detector located at the center of the trap. The resolution (R) is dependent on the frequency and thus a higher R=m/Δm m is higher for smaller ions. R for ions with *m/z* 200 is around 200 000.

Orbitrap mass spectrometers use, as FT-ICR, Fourier transformation to convert the secular frequencies of the ions into *m/z*. The mass accuracy of Orbitrap mass spectrometers is typically better than 1 ppm, making them an ideal tool for highly accurate quantification and identification of components in complex samples (Zubarev and Makarov, 2013).

Hybrid tandem mass analyzers

Hybrid instruments are characterized by the combination of two or more mass analyzers with distinct designs. A notable example of such an instrument is the ‘hybrid’ Q-ToF, which integrates two quadrupoles with a ToF mass analyzer. The first quadrupole serves either as a mass filter or as a scanning device, while the second quadrupole can function either as a collision cell, where ions are subjected

to collisions with neutral gas molecules, or as a device in which ions are just focused without inducing further fragmentation (Allen and McWhinney, 2019).

Another example of a hybrid instrument is the Q-Orbitrap, which is widely used in the present thesis. This instrument comprises a quadrupole mass analyzer and an Orbitrap analyzer, along with a C-trap, an external ion storage device utilized for accumulating ions prior to injection into the Orbitrap. The fragmentation method employed is known as Higher-Energy Collisional Dissociation (HCD), which takes place in a separate cell situated near the C-trap, as depicted in **Figure 7**. HCD is a term used commercially and should not be confused with the high-energetic collision induced dissociation used in some instruments and that are able to fragment side chains of peptides. The ions are transferred from the C-trap into the HCD cell, where fragmentation and dissociation occur, and then returned to the C-trap before being injected into the Orbitrap. This type of instrument offers several acquisition methods, including full scan, Parallel Reaction Monitoring (PRM), Data Dependent Acquisition (DDA), and Data Independent Acquisition (DIA) (Kaufmann, 2018). The two acquisition methods most relevant for this thesis are DDA and PRM. During DDA a full scan is performed, followed by a selection of precursor ions based on their abundance and fragmentation of them in the MS/MS stage. This method is useful for identifying unknown compounds and is commonly used in metabolomics and proteomics experiments. PRM is a targeted acquisition method that allows the selection and monitoring of specific precursor ions and their corresponding product ions. This method provides higher sensitivity and selectivity for targeted analytes and is commonly used in quantification experiments.

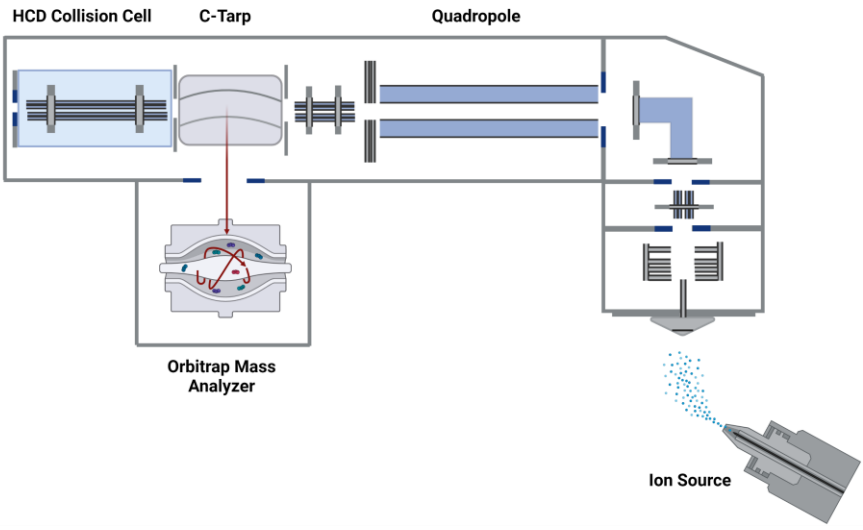


Figure 7: Schematic Diagram of the Q Exactive Orbitrap mass spectrometer. [created with BioRender.com]

2. MS-based methods for the analysis of hapten-protein conjugates (Papers I and II)

2.1 Background

Protein adducts

Adducts to proteins in blood are frequently employed as biomarkers of exposure to electrophiles, or their precursors, owing to their high stability as compared to DNA adducts. Proteins, as serum albumin and hemoglobin are found in high abundance, typically present in concentrations of milligrams per milliliter (mg/mL) in blood, in contrast to DNA that is commonly present in concentrations of approximately micrograms per milliliter (µg/mL). Moreover, protein adducts have a long lifespan and are not repaired by the body's mechanisms, rendering them a more dependable biomarker. Consequently, considerable research has been undertaken to explore Hb and HSA adducts, e.g. for monitoring exposure to carcinogens and neurotoxic chemicals (Törnqvist et al., 2002) (Rubino et al., 2009).

Human serum albumin (HSA)

HSA is the predominant protein present in blood plasma, with concentrations ranging from 30 to 50 mg/mL and a half-life of approximately 20 days. Cys_{34} (Chung et al., 2014), (Li et al., 2011) has been the focal point of numerous studies investigating HSA adducts due to its unusually low pKa, which makes it a highly reactive nucleophilic site. Additionally, HSA is present in significant quantities in most tissues, including the skin, and is primarily utilized as a carrier protein. Consequently, HSA is the preferred protein for exploring the reactivity of contact allergens in research studies. The adductome of HSA has been studied previously for some electrophilic contact allergens (Aleksic et al., 2007), (Parkinson et al., 2014), (Jenkinson et al., 2010a).

Hemoglobin (Hb)

Hb is predominantly found in erythrocytes, with a concentration in blood ranging from 120 to 160 mg/mL and has an estimated lifetime of approximately 126 days, same as erythrocytes (Zhang et al., 2018). Although the study of Hb adducts has been the subject of extensive research and is used for biomonitoring environmental and occupational exposure (Boogaard, 2002) (Bryant et al., 1988), (Hagmar et al., 2001) the formation and role of Hb adducts with contact allergens have not been previously investigated. Many of the applications and further analytical work regarding Hb adducts are directed toward the analysis of adducts formed at the N-terminal Val. One reason is that the substituted N-terminal Val could be detached from the rest of the protein using developed procedures based on modified Edman degradation.

FI*R*E method

The original Edman procedure, which was first developed at Lund University in 1949 by Pehr Edman (Edman, 1949), is the first established method for routine protein sequencing. During the original approach, the N-terminal amino acids were derivatized with phenyl isothiocyanate and subsequently detached under acidic conditions without disrupting the remaining peptide bonds within the protein. The potential of this method for conducting adduct measurements was explored by Ehrenberg's group, who demonstrated that detachment of the modified N-terminal Val of Hb proceeded spontaneously without the need for acidification (Jensen et al., 1984) and was mechanistically favored compared to the detachment of non-modified N-terminal Val (Rydberg et al., 2002). This led to the development of the N-alkyl Edman procedure (Törnqvist et al., 1986), which was the basis for the FI*R*E procedure utilized in this thesis. The FI*R*E procedure employs a fluorescein isothiocyanate (FITC) reagent to derivatize adducts denoted as "*R*" in a modified Edman procedure. It is an LC-MS/MS version of the N-alkyl Edman procedure that utilizes the LC-MS compatible reagent FITC for direct derivatization in the hemolysate of blood and subsequent clean-up using Solid Phase Extraction (SPE) (Rydberg et al., 2009), **Figure 8**. The LC-MS/MS approach utilized to analyze the FTH derivatives depends on the specific analytical task at hand. It can either be targeted for quantification purposes or untargeted for screening N-terminal Val adducts in blood (Carlsson et al., 2017).

Bottom-up proteomics

Bottom-up proteomics is a widely used approach for protein identification and characterization that involves enzymatic digestion of proteins into smaller peptides, followed by their separation and analysis using MS. The development of bottom-up proteomics can be traced back to the early 1990s when advancements in enzymatic digestion methods, peptide separation techniques, and MS instrumentation paved the way for high-throughput protein analysis. Over the years, bottom-up proteomics has become a powerful tool for studying complex proteomes and has revolutionized the field of proteomics, enabling advancements in areas such as protein identification, post-translational modification analysis, and quantitative proteomics (Dupree et al., 2020).

Limited proteolysis (LiP-MS)

While classical bottom-up proteomics and the analysis of N-terminal adducts to Hb by modified Edman methods as FI*R*E, are frequently employed to investigate protein interactions and modifications, identifying protein structural changes through proteomic analysis has not been feasible on a broad scale and with high throughput until recently. A method called limited proteolysis-coupled MS (LiP-MS) has been developed to tackle this issue (Feng et al., 2014). LiP-MS is a powerful technique that allows for the identification of even subtle changes in protein structure, including alterations in secondary structure content, shifts induced by allostery, and transitions between folded and unfolded states. Moreover, LiP-MS provides peptide-level resolution, enabling precise pinpointing of the protein regions undergoing structural transitions. This method has been successfully applied to detect protein structural rearrangements, study protein aggregation, and investigate protein-small molecule interactions leading to changes in the structural properties of the protein. LiP-MS supports both targeted and unbiased analyzes of protein structure, making it applicable for examining individual proteins of interest as well as the entire proteome. (Feng et al., 2014), (Leuenberger et al., 2017), (Stauffer et al., 2014), (Geiger et al., 2016).

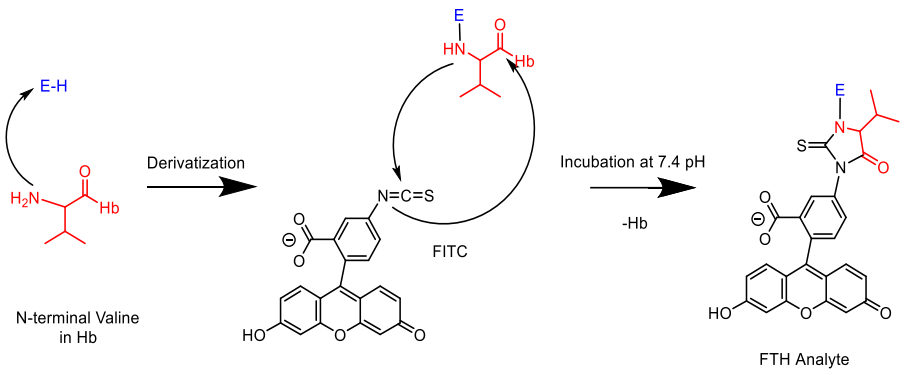


Figure 8: General illustration of the FI*R*E procedure. The N-terminal Val adducts, formed after reaction with electrophilic compounds (E-H) are derivatized using FITC leading to the FTH derivatives being formed.

2.2 Aim

The initial two papers outlined in this thesis focus on the domain of hapten-protein conjugates and hapten-protein interactions. The primary aim of **Paper I** was to investigate the specific locations on the two major blood proteins in humans, namely HSA and Hb, that are susceptible to haptenation upon exposure to electrophilic compounds. Identification of these sites may serve as potential adduct biomarkers for biomonitoring purposes and potentially facilitate the development of improved diagnostic tools based on blood samples. The objective of **Paper II** was to introduce and contrast three different MS-based methodologies, namely bottom-up proteomics, FI*R*E, and LiP-MS, for assessment of exposure through Hb. Both studies share three common haptens, namely 1-chloro-2,4-dinitrobenzene (DNCB), 1,2-epoxy-3-phenoxypropane (PGE), and 2-methyleneglutaronitrile (2-MGN), while **Paper II** extends its analysis to include nickel (Ni) among other compounds, and examine the impact of this prevalent contact allergen on the structure of Hb with implications for contact allergy.

2.3 Experimental approach

In **Paper I**, the adductome of HSA and Hb was investigated *in vitro* using three haptens with varying chemical properties and different reaction mechanisms, namely DNCB, PGE and 2-bromo-2-(bromomethyl)glutaronitrile (MDBGN) (the case of MDBGN is discussed separately in Section 4 of this thesis), **Figure 9**. Fresh blood was used as a source for the two proteins and incubations were carried out for 24 hours at 37°C with 0.1-, 0.5-, 1-, and 5-fold molar ratio of the hapten. The study aimed to explore haptenation at different nucleophilic sites by screening for modification of His, Lys, Cys, Ser, Thr, Met, Trp, and Tyr in both HSA and Hb. Modification of the N-terminal Val was also included for Hb. A bottom-up proteomic approach was carried out using an Orbitrap mass spectrometer to initially screen for adducted sites in DDA mode, followed by targeted confirmation in PRM mode. The most reactive sites were identified by estimating the relative quantities of the adducted peptides, which exhibited an increase in the amount relative to the concentration of the hapten. The sites modified at the lowest ratio of hapten to protein used, namely 0.1-fold, were considered the most reactive.

In **Paper II**, human blood was exposed to six electrophilic compounds, namely acrylamide, acrylic acid, glycidic acid, and three contact allergens (DNCB, PGE, 2-MGN) at different concentrations (0.1- or 5-times molar ratio) and exposure times (1 or 21 hours). For the samples analyzed using the FI*R*E method, two LC-MS/MS analyses were performed. Initially, untargeted DDA acquisition was used to determine the precursor and product ions for each analyte. This was followed by targeted detection of FTH analytes of adducted Val (*R*-Val-FTH) in the FI*R*E samples, once the appropriate transitions were determined. A mixture of deuterated *R*-Val-FTHs served as the internal standard. For the bottom-up proteomics method, a similar approach as in **Paper I** was used. Samples were initially screened for adducted peptides in DDA mode, followed by targeted confirmation in PRM mode. In the final step, a direct quantitative comparison between FI*R*E and targeted bottom-up proteomics was attempted using AA-Val(D_7)-FTH internal standard for FI*R*E and V(+7)LSPADKTNVK for bottom-up proteomics.

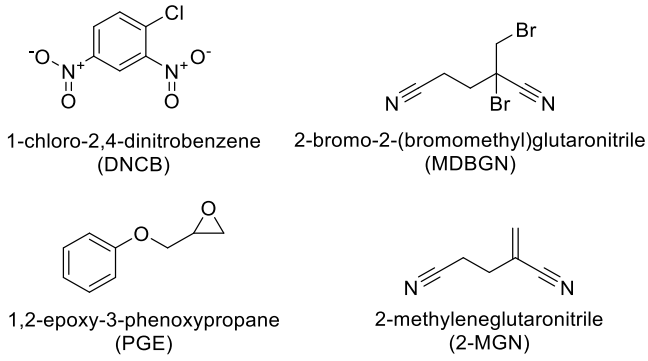


Figure 9: Chemical structure and name of the contact allergens DNCB, PGE, MDBGN and 2-MGN.

Furthermore, separate Hb samples were incubated with a 5-fold molar excess of DNCB, PGE, 2-MGN, and $NiCl_2$ or a DMSO control for 4 hours at 37 °C and subjected to the LiP-MS procedure. LC-MS/MS analysis was carried out in DDA acquisition mode. The obtained data were analyzed to evaluate the potential of the method to detect DNCB adduct formation relative to FI*R*E and bottom-up proteomics. Changes in the Hb structure between the groups treated with hapten and the DMSO control were assessed.

2.4 Results and discussion

In **Paper I**, the initial untargeted analysis led to the identification of a large number of potentially modified side chains, and increasing molar ratios of haptens resulted in increasing numbers of potentially adducted sites for both haptens. For HSA, the targeted analysis enabled confirmation of 11 adducted sites after treatment with DNCB and five adducted sites after treatment with PGE at a 5-fold molar excess of hapten. For Hb, the targeted analysis confirmed a total of 17 reactive sites, 5 of them were modified by DNCB and 12 were modified by PGE.

However, only some of the adducted peptides confirmed in PRM analysis exhibited an increase in relative amount with increased hapten concentration. The relative quantifications are not an accurate quantification of the level of protein modification, but additional support for the reactivity of the haptenated sites. For HSA, seven confirmed sites showed a correlation between increased hapten concentration and increased relative amount of adducted peptide, while for Hb, eight

adducted sites exhibited this correlation. From these, it is noteworthy that three HSA sites (Cys_{34}, Cys_{62}, and Lys_{190}) and six Hb sites (subunit α: Val_1, His_{45}, His_{72}; subunit β: Cys_{93}, His_{97}, and Cys_{112}) were already modified at a low ratio of 0.1 of hapten to protein, **Table 1**. This indicates that these sites are highly reactive and are the most probable targets for modification *in vivo*.

Table 1: Protein sites modified at 0.1-molar ratio of hapten to protein.
Blood protein, hapten responsible for the modification, peptide sequence, confirmed adducted site.

Protein	Hapten	Peptide Sequence	Adducted Amino acid
HSA	PGE	LQQ**C**PFEDHVK	Cys_{34}
HSA	DNCB	TCVADESAEN**C**DK	Cys_{62}
HSA	DNCB	DEG**K**ASSAK	Lys_{190}
Hb	PGE	**V**LSPADKTNVK	Val_1/α
Hb	PGE	TYFP**H**FDLSHGSAQVK	His_{45}/α
Hb	PGE	VADALTNAVA**H**VDDMPNAL-SALSDLHAHK	His_{72}/α
Hb	DNCB	GTFATLSELH**C**DK	Cys_{93}/β
Hb	PGE	L**H**VDPENFR	His_{97}/β
Hb	DNCB	LLGNVLV**C**VLAHHFGK	Cys_{112}/β

Adapted from **Paper I* (Ndreu et al., 2020)

The correlation between the number of adducted sites in HSA and the sensitizing capacity of the different haptens used in the present study with data from earlier LLNA studies is evident. DNCB, which is an extreme sensitizer, produces 12 adducted sites at a 5-fold molar excess, while PGE, a strong sensitizer, produces six adducted sites at the same concentration. However, such a correlation cannot be observed in Hb, as PGE modifies more sites than DNCB. The strong correlation between HSA and the data from LLNA studies suggests that haptenation of HSA could lead to the formation of immunogenic hapten-protein conjugates capable of activating T-cells. This hypothesis is supported by a study by Jenkinson and co-workers that demonstrated the ability of HSA-hapten conjugates to induce T-cell proliferation in contact allergic patients (Jenkinson et al., 2010b). Moreover, the

detection of IgG and IgM antibodies against xenobiotics conjugated to HSA in 8-22% of healthy human subjects further supports the idea that hapten-HSA conjugates may activate the immune system (Vojdani et al., 2015). Despite not being considered immunogenic in the context of contact allergy, Hb is still valuable for monitoring exposure to various substances. This was the first time that the adductome of contact allergens with Hb was investigated, which will open for development toward biomonitoring of contact allergens. Previously the Hb adductome has been utilized for monitoring exposure to different reactive compounds, especially those with genotoxic properties (Rubino et al., 2009). In these analyses, Cys93 in the beta subunit of Hb has been identified as one of the most reactive sites (Cai et al., 2017), while N-terminal Val also have been utilized for monitoring exposure to several compounds (Carlsson et al., 2014).

In **Paper II**, we sought to evaluate and contrast the advantages and limitations of bottom-up proteomics, the FI*R*E method, and LiP-MS in characterizing exposure-derived protein adducts of Hb, **Figure 10**.

While DNCB has been found to be effective in treating warts (Word et al., 2015), it is not a commonly encountered compound, and therefore, it is not considered a significant clinical contact allergen. However, DNCB is among the most commonly used haptens in studies of contact allergy. DNCB is classified as an extreme hapten in the LLNA (Basketter et al., 2000) and reacts with nucleophilic sites via an S_NAr mechanism, **Figure 3c**. PGE serves as a representation of the widely used epoxy resin monomer, DGEBA, which is commonly employed in the production of epoxy resin systems. Epoxy resin monomers are among the primary causes of occupational ACD (Dickel et al., 2001). PGE is classified as a strong sensitizer by the LLNA, and its reaction occurs via a S_N2 mechanism, **Figure 3a** (Niklasson et al., 2009). MDBGN was initially introduced in the 1980s for use in cosmetic and industrial products. However, due to an increased incidence of contact allergy, it was prohibited in the European Union for leave-on products in 2003 ("Commission of the European Communities. Commission Directive 2003/83/EC of 24 September 2003") and rinse-off products in 2007 ("Commission of the European Communities. Commission Directive 2007/17/EC of 22 March 2007"). MDBGN is classified as a moderate sensitizer (Loveless et al., 2010) and is believed to react via a typical S_N2 mechanism.

Using the FI*R*E method, a positive dose-response relationship was observed between the majority of electrophiles incubated with blood and the resulting adduct formed at the N-termini of hemoglobin. Among these, PGE exhibited among the

highest levels of reactivity, as evidenced by the substantial amounts of adduct formation observed. On the other hand, 2-MGN formed significantly lower levels of adducts, while notably, FTH derivatives of DNCB-adducted Val could not be detected at any concentration, nor for any length of incubation time. This is a surprising finding that can be attributed to the large surface area of the 2-MGN adduct partially blocking the electron pair's attack on the bulky fluorescein isothiocyanate molecule. Additionally, there is evidence on the preferential modification of Cys-residues by 2-MGN (Bao et al. 1998). In the case of DNCB, the aromatic nature of the adduct could lead to the partial delocalization of the electron pair in the N-terminal nitrogen within the adduct, which theoretically makes the DNCB-Val adduct less nucleophilic and thereby the derivatization reaction with FITC is less likely to occur. These considerations suggest that although the FIRE method is a powerful tool for Hb adductomics, it may not be suitable for all compounds.

In contrast to the FI*R*E method, bottom-up proteomics successfully detected N-terminal adduct formation of all six electrophiles in Hb, with the Cys_{93} residue on the beta chain demonstrating the highest reactivity, evidenced by the formation of five adducts. For DNCB and PGE, the findings were in good agreement with Paper **I**. The most reactive sites presented in **Paper I** for DNCB, namely βCys_{93} and βCys_{112} in Hb, were also found to be modified by DNCB in **Paper II**. Same is the case for the most reactive sites presented for PGE, namely αHis_{45}, βCys_{93}, βHis_{97} and βCys_{112}. The comparative effectiveness of the FIRE method and bottom-up proteomics in detecting low levels of adduct formation was investigated by analyzing blood samples exposed to low concentrations (ranging from 5 µM to 100 µM) of PGE and 2-MGN. For bottom-up proteomics, both N-terminal peptides and the beta chain peptide containing Cys_{93}- GTFATLSELHCDK- were selected as targets for analysis, and the detected analytes were normalized using an internal standard (AA-Val(D7)-FTH for FI*R*E and V(+7)LSPADKTNVK for bottom-up proteomics) for semi-quantitative analysis. The results from PGE exposure indicate that the FI*R*E method delivered higher levels of Val adduct formation than the peptide targets, whereas exposure to 2-MGN consistently resulted in higher levels of adduct formation in the peptides detected by bottom-up proteomics. This suggests that the optimal methodology for detecting low levels of adducts may be dependent on the specific nature of the electrophiles of interest.

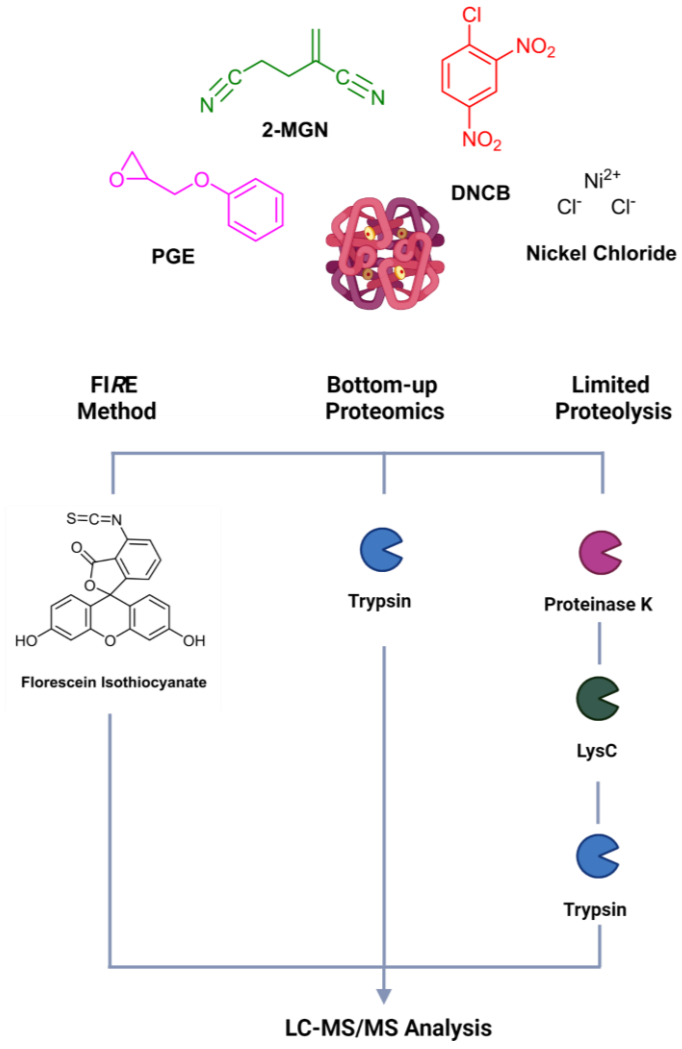


Figure 10: Experimental overview of the three methods presented in Paper II, namely FI*R*E, bottom-up proteomics and LiP-MS. [created with BioRender.com]
*(Adapted from **Paper II**)*

LiP-MS was conducted on Hb exposed to DNCB, PGE and 2-MGN to compare the ability of this technique to detect adduct formation relative to FI*R*E and conventional bottom-up proteomics. The results showed that LiP-MS was able to detect adduct formation in Hb exposed to DNCB, detecting changes in the abundance of certain peptides in the alpha and beta chains of Hb. The regions of the alpha

chain that were significantly altered by DNCB exposure were between the 20^{th}-30^{th} residues, as well as the 40^{th}-50^{th}, 80^{th}-90^{th}, 110^{th}-130^{th}, and 130^{th}-140^{th}. The regions of the beta chain that were significantly altered by DNCB exposure were 10^{th}-30^{th}, 50^{th}-60^{th}, 70^{th}-80^{th}, 100^{th}-105^{th}, and 110^{th}-150^{th} residues. These regions corresponded closely with the residues of αTyr_{24}, αHis_{45}, αCys_{104}, βSer_{73}, βCys_{112}, and βLys_{144}, which were observed to have DNCB adducts from the bottom-up proteomics experiments. Importantly, the N-termini and βCys_{93}, which were identified as adducted sites in other experiments, did not display any changes in abundance following exposure to DNCB and were not identified as adducted sites with this method. Interestingly, no changes in Hb exposed to PGE and 2-MGN could be observed with LiP-MS. In contrast to the electrophiles, $NiCl_2$ only forms non-covalent interactions with the sidechains of Hb. LiP-MS analysis of Hb exposed to $NiCl_2$ showed changes in the abundance of certain peptides in both the alpha and beta chains, with a distinct region of increased abundance between residues 50 and 60 on the beta chain, **Figure 11**. This site was identified as an adduct formation site due to the presence of four lysine residues and a histidine residue, which could coordinate with the Ni^{2+} ion. LiP-MS excels over FI*R*E and bottom-up proteomics in detecting non-covalent adducts, as demonstrated by exposure of Hb to $NiCl_2$, where several coordination sites for non-covalent binding were identified.

Overall, the study showed that each methodology has different strengths and limitations in detecting adducts stemming from different compounds and determining their locations within the Hb molecule. Both the FI*R*E methodology and bottom-up proteomics can be made quantitative with appropriate internal standards, while LiP-MS can detect both covalent and noncovalent adducts.

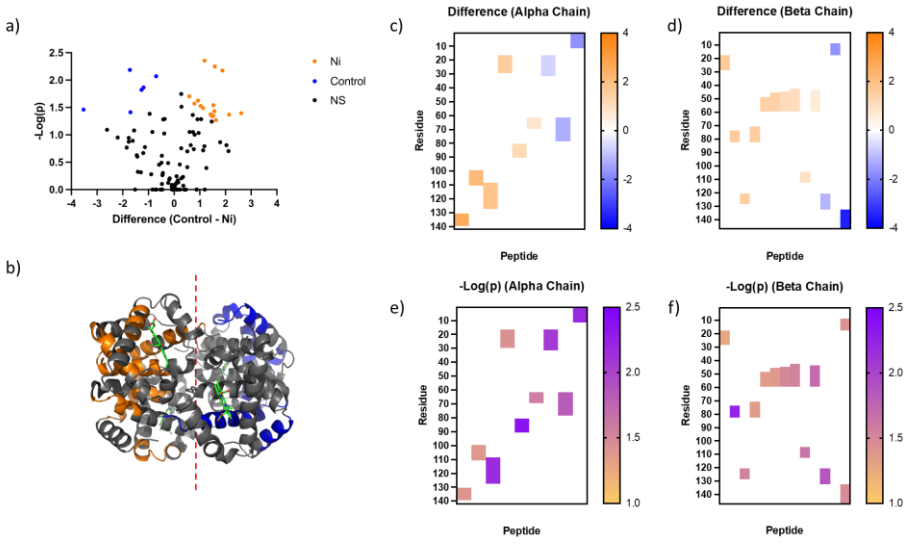


Figure 11: LiP-MS results of $NiCl_2$ treatment. a) Volcano plot of Hb peptide label-free quantification (LFQ) values of identified following Hb (7.8 µM) treatment with NiCl2 (39 µM) for 4 hours in PBS buffer (pH 7.4) and iterative digestion with proteinase K and then a mixture of trypsin and LysC. Peptides highlighted in orange or blue were significantly enriched in the NiCl2 or DMSO treatment, respectively, using a statistical cut-off of a 0.05 FDR and a 0.1 minimal coefficient of variation (S0). Data are representative of 3 independent replicates from each treatment condition. b) Crystal structure of Hb (PBD: 1A3N) with significantly enriched LiP peptides for NiCl2 or DMSO treatment shown in orange or blue respectively. c,d) Heat map of fold change difference of significantly enriched Hb peptides for the alpha or beta chain respectively. e, f) Heat map of -log(p) of significantly enriched Hb peptides for the alpha or beta chain respectively. (*Adapted from* ***Paper II***) **NS: Not Significant*

3. Protein conjugates with immunological relevance (Paper III)

3.1 Background

The recognition of immunogenic hapten-protein conjugates as the initiation event in contact allergy or contact hypersensitivity, has been established since 1936 (Landsteiner, 1936). However, many aspects of the underlying mechanism related to these immunogenic complexes remain unknown, such as which skin proteins are targeted by haptens *in vivo*, what hapten-protein conjugates trigger the immune system, and what is presented to naïve T-cells during sensitization. Due to the lack of understanding, most potential biomarkers for ACD represent general inflammatory conditions. Studies investigating hapten-protein adducts include exposure of complex protein lysates from a keratinocyte cell line and human skin tissues to contact allergens (Parkinson et al., 2018), specific skin proteins such as human cytokeratin 14 and human cofilin (Aleksic et al., 2008), different skin cell lines (Parkinson et al., 2020) and *ex vivo* skin (Simonsson et al., 2011). Despite the extensive mapping of the adductome of HSA and of other skin proteins with haptens, no protein-conjugates have been found *in vivo*.

Karlsson and co-workers were the first to identify a modified protein in the local lymph nodes of mice topically treated with the synthetic hapten tetramethyl rhodamine isothiocyanate (TRITC). The protein identified was the macrophage migration inhibitory factor (MIF) and the modification was shown to occur on the N-terminal Pro residue (Karlsson et al., 2018).

MIF, a cytokine first identified over 55 years ago, was found to be secreted by T-cells and able to inhibit the migration of macrophages (Bloom and Bennett, 1966), (David, 1966). It is now known that MIF is expressed in most immune cells and tissues that are in direct contact with the external environment, as well as tissues of the endocrine system (Baugh and Bucala, 2002). MIF is a homotrimeric cytokine with two catalytic activities, tautomerase and oxidoreductase activity, and

its N-terminal Pro is required for its tautomerase activity (Faist et al., 1989). Although MIF's structure has been characterized, its complete mode of action is still not fully understood, and it is unclear if the monomer, dimer, or trimer mediates its action. Despite extensive research, no endogenous substrate has been identified for MIF's tautomerase activity (Kudrin and Ray, 2008). However, it is well established that MIF has a critical role in immune and inflammatory responses and is involved in various conditions, such as asthma (Yamaguchi et al., 2000), rheumatoid arthritis (Leech et al., 1999), sepsis (Bozza et al., 2004), atopic dermatitis (Shimizu et al., 1997), and breast cancer (Verjans et al., 2009).

3.2 Aim of the study

The objective of **Paper III** was to build upon Karlsson and co-worker´s previous work from 2018 by expanding the investigation into mapping the adductome of two major murine blood proteins, serum albumin (Alb) and Hb, as well as murine MIF, when treated with TRITC. This effort aimed at identifying the most reactive sites *in vitro,* and examine the presence and quantity of such conjugates *in vivo,* in blood and lymph node samples using a bottom-up proteomic approach.

3.3 Experimental approach

Firstly, the adductome of the two major blood proteins, Alb and Hb, from mouse as well as MIF when treated with TRITC was investigated *in vitro*. All three proteins were subjected to incubations with different concentrations of TRITC for 24 h at 37°C. An untargeted screening with Orbitrap MS was performed to identify potentially adducted sites in all three proteins and the suggested sites were confirmed by targeted analysis and localization of the correct b- and y- ions. These sites were used to build an inclusion list for the Orbitrap MS analysis to screen blood and lymph node samples from mice topically treated with TRITC. The adducted peptides that were present in these samples were quantified using appropriate standards. Lastly, the potential of MIF to react with other common contact allergens, namely DNCB, PGE, cinnamic aldehyde and 5-chloro-2-methyl-2h-isothiazolin-3one (CMIT)/2-methyl-2h-isothiazol-3-one (MIT) mixture was examined, **Figure 12**.

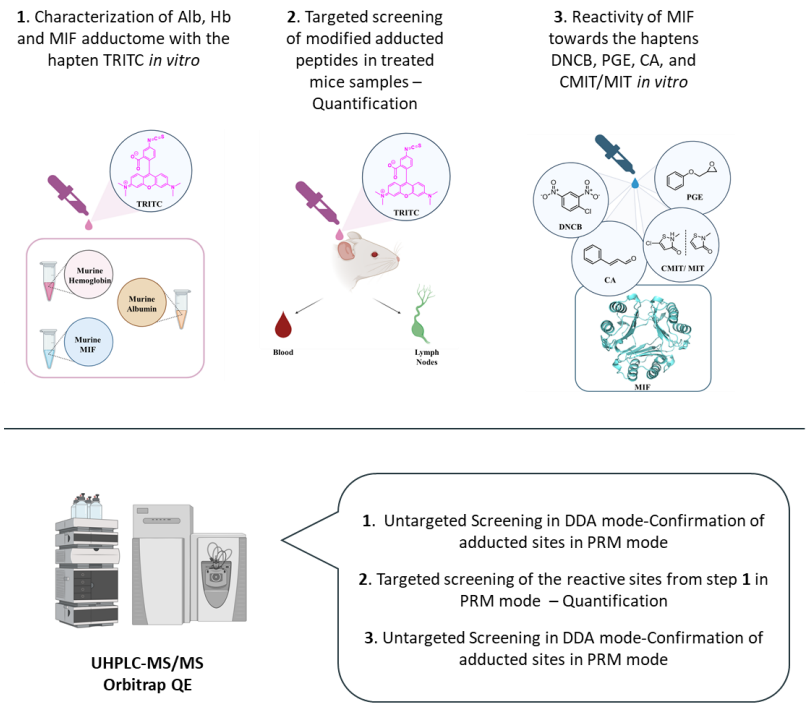


Figure 12: Experimental overview of **Paper III**. [created with BioRender.com]

3.4 Results and discussion

According to the untargeted results, TRITC could potentially modify two sites in Alb, three sites in Hb, and three sites in MIF. These sites were observed to be modified at all molar ratios for Alb and Hb, while for MIF, only the N-terminal Pro was found to be modified at all ratios. After the untargeted analysis, an inclusion list of confident TRITC-modified peptide masses was prepared for the PRM analysis, which confirmed two sites in Alb and three amino acid sites in Hb. For MIF, the targeted analysis confirmed three modification sites, including the N-terminal Pro. The reactivity of the confirmed sites was assessed based on an increase in the area of the TRITC fragment with an increase in molar ratio of TRITC. Amino acid sites that exhibited this trend and were already modified at the lowest molar ratio of TRITC used were considered most relevant for *in vivo* screening. Based on these results, the PRM list for screening adducts in whole blood and lymph node samples of TRITC-treated mice included the two TRITC-modified

peptides of Alb, the two N-terminal- and the one Ser-containing peptides of Hb, and the N-terminal-containing peptide of MIF.

Following the *in vitro* characterization of the most reactive sites, targeted screening was carried out on samples from topically TRITC-treated mice. Alb or Hb adducts were not detected, but the TRITC-MIF peptide was observed in the blood samples from treated mice. This is the first time a hapten-protein conjugate has been found in blood from mice after topical application of a hapten in the context of murine CHS. The concentration levels of the TRITC-MIF peptide varied between different blood samples, while the levels were lower in lymph node samples. Regarding the unmodified MIF peptide, higher levels were observed in the lymph node samples compared to the blood samples, but the levels showed no significant difference between samples from TRITC-treated and control mice. MIF levels have been found to be elevated in various conditions, such as rheumatoid arthritis, cancer, atherosclerosis, and diabetes. However, previous studies have not differentiated between modified and non-modified MIF, and have measured total MIF levels. The study presented in **Paper III** demonstrated that during the initiation of the allergic response in treated mice, total N-terminal MIF peptide levels (modified and unmodified) increased. However, unmodified MIF levels were not significantly different between control and treated mice, **Figure 13**. Therefore, the elevated MIF levels during the initiation of the allergic response were solely caused by hapten-modified MIF.

The inability to detect TRITC-Alb and TRITC-Hb in the blood of TRITC-treated mice, despite being present in *in vitro* incubations, suggests that MIF was most likely haptenated in the skin and transported to the lymph nodes by DCs. Immune cells, including DCs and KC, are known to produce high levels of MIF upon activation. The authors of a recently published article on the role of MIF in DC mobility (Ives et al., 2021) demonstrated that MIF is involved in promoting the migration of DCs. This insight is useful in the context of our study, strengthening the hypothesis that MIF was probably haptenated in the skin and transported to the lymph nodes by DCs. The unmodified MIF peptide is also important in order to investigate the levels of MIF during an allergic response and to assess differences in protein levels between sample types. In lymph node samples, denaturation before sample preparation revealed an approximate 3-fold increase in unmodified MIF for both treated and control groups, indicating the presence of MIF in bound form in the lymph nodes.

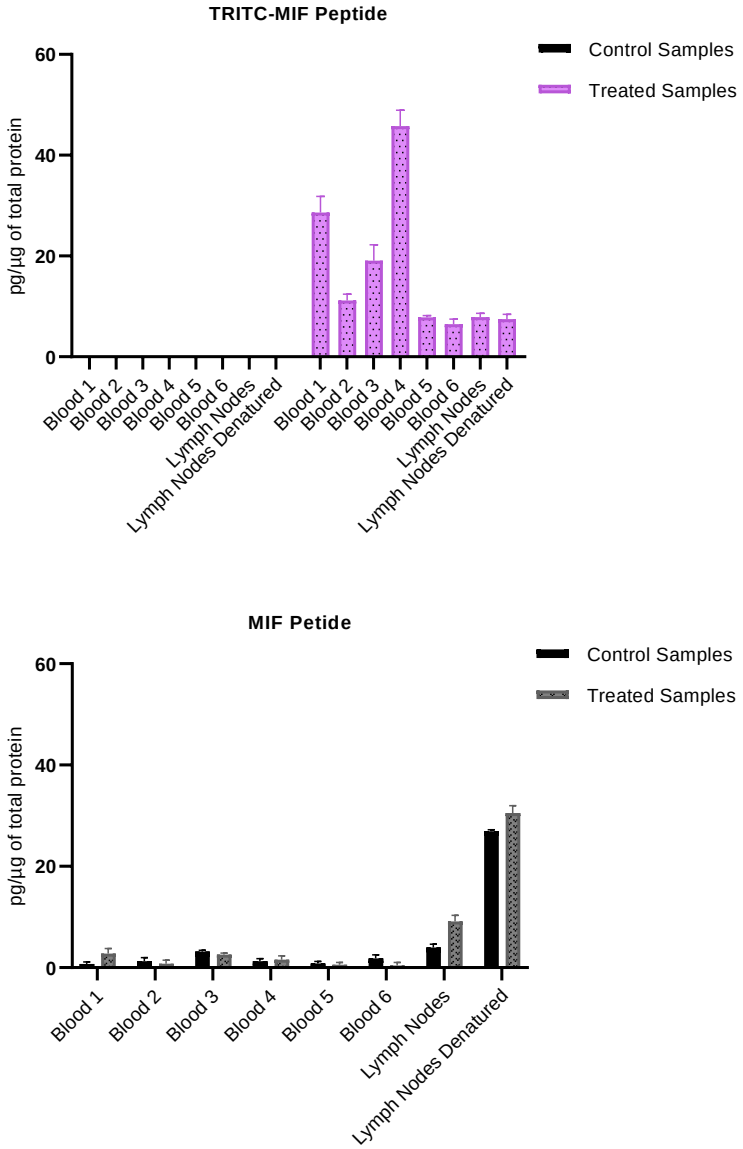


Figure 13: (Top) Amount of TRITC-modified MIF peptide per µg of total protein in the different whole blood and lymph node samples. (Bottom) Amount of MIF peptide per µg of total protein in the different whole blood and lymph node samples. No significant difference ($P < 0.05$) could be observed for the MIF peptide between the treated and control

samples. Values are the mean of three replicate analysis ± STD. (*Adapted from* ***Paper III*** *(Ndreu et al., 2022)*)

The chemical nature, potency, and preferential reaction mechanism of haptens vary widely. Therefore, to ensure that potential biomarkers for contact hypersensitivity can be haptenated by a broad range of haptens, *in vitro* adduct formation of MIF with four different haptens was investigated. Incubation of MIF with different contact allergens reacting via different mechanisms, namely DNCB, PGE, CA and CMIT/MIT mixture, showed that MIF can react with a broad range of haptens. It was confirmed that Pro_1 and Cys_{80} were modified by DNCB and PGE, with an additional site of His_{88} for PGE. Three sites were confirmed for CA modification, Pro_1, Lys_{77}, and His_{88}, while CMIT was confirmed to modify Lys_{77} and His_{88}.

MIF is a protein that plays a crucial role in various allergic skin disorders, including atopic dermatitis (Yoshihisa et al., 2011), zinc-allergic contact dermatitis (Yanagi et al., 2006), and pollen dermatitis in mice (Nagata et al., 2015). Many studies have shown elevated levels of MIF in patients with inflammatory and autoimmune conditions such as rheumatoid arthritis and systemic lupus erythematosus. MIF-based therapies have shown promise in treating these conditions and cancer (Bloom et al., 2016), (Bilsborrow et al., 2019). Based on the finding of **Paper III** the presence of hapten-modified MIF in ACD patients is worth exploring as a potential disease biomarker. New diagnostic tools based on blood samples could provide a more efficient way to identify the chemical causing the allergic response in each patient.

4. MS-based investigation of pro- and pre-haptens (Papers I, IV and V)

4.1 Background

While haptens can directly modify skin proteins and form conjugates that are immunogenic, some compounds may require chemical modification or enzymatic biotransformation before they can bind to proteins and act as haptens. Such compounds are known as pre- and prohaptens.

Prehaptens are compounds that are non- or weakly sensitizing on their own, but can transform into haptens outside the skin through abiotic activation, through chemical processes like autoxidation or photoactivation. Analogously, prohaptens are compounds that transform into haptens through bioactivation within the skin, usually via enzymatic catalysis (Karlberg et al., 2013). Determining whether an allergen acts as a prehapten or prohapten can be a challenging task, as both abiotic and biotic activation mechanisms can generate similar products, and a single compound can exhibit both modes of action.

An illustrative example, pertinent to the present thesis, is cinnamic alcohol, a natural compound found in Cinnamomum trees and balms, such as styrax and balsam of Peru. Due to its hyacinth-like odor, cinnamic alcohol is commonly utilized in fragrances, shampoos, and other toiletries. Nevertheless, it is imperative to note that cinnamic alcohol is a frequent contact allergen. Consequently, it has been included as one of the eight constituents of the fragrance mix I (FMI), utilized in the baseline series for screening contact allergy in dermatitis patients. It has been observed that cinnamic alcohol, despite lacking inherent structural alerts for protein reactivity, can act both as a prehapten forming cinnamic aldehyde and epoxy cinnamic alcohol (Niklasson et al., 2013), and prohapten after bioactivation (Niklasson et al., 2014) forming the reactive epoxy cinnamic alcohol and epoxy cinnamic aldehyde among others.

Of interest is also propolis, a resinous mixture made by honeybees for hive construction and protection possessing antibacterial, antimycotic, and antiviral properties. Propolis, which is today widely used in consumer products such as biocosmetics, has been identified as a common cause of contact allergy. As a result, it has been added to the test battery of compounds used in routine diagnosis of ACD in Europe (Wilkinson et al., 2018). Studies have shown that cinnamic acid (CA) and its esters, namely caffeic acid 1,1-dimethylallyl ester (CAAE), and caffeic acid phenethyl ester (CAPE), **Figure 14**, are the primary chemical species responsible for the haptenic activity and allergenicity of propolis (Hausen et al., 1992). However, the precise molecular mechanism that underlies the allergenic activity of these compounds is still not fully understood.

There are several challenges associated with the identification of pre- and prohaptens in ACD. The primary challenge lies in accurately predicting which chemicals will act as prohaptens or prehapten and which compounds will serve as the protein reactive specie. This challenge is primarily due to the limitations of *in vitro* methods available for predicting sensitization potency. While methods such as the DPRA can detect prohaptens after oxidation, they cannot be used to classify prohaptens. On the other hand, methods utilizing keratinocytes or other systems with metabolism capabilities may be effective in detecting prohaptens but might not be efficient for prehapten detection. In neither case, insight into the actual culprit that serves as the sensitizer will be provided. Incorporating MS methodologies can provide valuable information about the nature of the autoxidation and/or biotransformation product and the adduct formed in each case.

4.2 Aim of the study

This thesis section includes papers that showcase the application of MS-based analysis for detecting and characterizing pre- and/or prohaptens and their conjugates.

In **Paper I**, MDBGN, a widely recognized contact allergen that has been proposed to react via an S_N2 mechanism, was incorporated, alongside other contact allergens, described in Section 2, in a bottom-up proteomic approach aimed at identifying sites on HSA that are modified by various haptens. Following initial experiments designed to detect MDBGN-modified sites within the protein, the study focused on demonstrating that the biotransformation product of MDBGN,

namely 2-MGN, was responsible for the formation of adducts, thereby characterizing MDBGN as a prohapten.

Paper IV investigated the protein reactivity of three primary contact allergens in propolis, CA, CAAE, and CAPE. The study utilized modified versions of DPRA, along with different abiotic conditions, to characterize the nature and structure of the conjugates formed with synthetic peptides, which allowed for the identification of the specific compounds believed to be responsible for the sensitization potency of propolis.

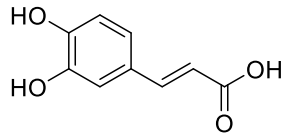
In **Paper V**, bioactivation of cinnamic alcohol was investigated by employing various *in vitro* and *in vivo* systems. The study initially used human liver microsomes (HLM), liver S9 fraction and RHE models to conduct a targeted investigation into the metabolites formed by cinnamic alcohol. Furthermore, LLNA and modified DPRA were utilized to assess the skin sensitization potential of relevant metabolites derived from the initial part of the study.

4.3 Experimental approach

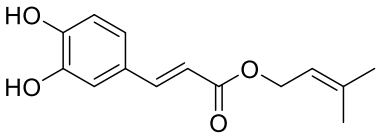
In **Paper I**, the reactivity and adduct formation of MDBGN and its metabolite 2-MGN toward the synthetic peptide Ac-PHCKRM, was investigated. The reaction mixtures were prepared by incubating the peptide with MDBGN, 2-MGN, and reduced GSH under various conditions, a) peptide/MDBGN at a molar ratio of 1:10, b) peptide/MDBGN/reduced glutathione (GSH) at a molar ratio of 1:10:20, respectively, and c) peptide/2-MGN at a molar ratio of 1:10. The depletion of the peptide and the formation of the adduct were monitored for 24 h using a DDA acquisition mode. Additionally, HSA exposed to 0.1-, 1-, 10-, and 100-fold molar ratio of MDBGN was subjected to the same bottom-up approach as described in Section 2 in order to investigate adduct formation with different nucleophilic sites of the protein.

In **Paper IV**, classification of the three compounds investigated, namely CA, CAAE and CAPE, according to their sensitization potency was attempted. Initially, the OECD-approved kDPRA and a modified version utilizing the synthetic peptide Ac-PHCKRM, a different incubation time point, and buffer composition were used. Subsequently, targeted LC-MS/MS analysis was conducted to investigate the nature of the adducts formed between a 5-molar excess of these compounds and the peptide Ac-PHCKRM. The effect of oxygen on adduct formation was studied in three different systems: a closed system in the presence of oxygen,

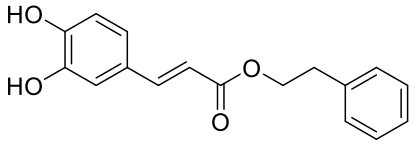
a closed system under inert conditions (oxygen-free), and an open system (ambient air). In addition, NMR was employed to identify the exact position of adduct formation in the aromatic ring of these compounds, and their oxidation potential was demonstrated through a series of UV-experiments.



Caffeic acid (CA)



Caffeic acid 1,1-dimethylallyl ester (CAAE)



Caffeic acid phenethyl ester (CAPE)

Figure 14: Chemical structure and name of CA, CAAE and CAPE.

In **Paper V**, a targeted multiple reaction monitoring (MRM) method was employed to investigate and quantify the metabolites of cinnamic alcohol formed in three *in vitro* systems, namely HLM, liver S9 fraction and RHE. Two sets of RHE experiments were conducted: one where the models were treated with low levels of cinnamic alcohol (2 µl of a 5 mM solution in DMSO), and another where the models were treated with higher levels of cinnamic alcohol (30 µL of a 50 mM solution in DMSO). The decrease of cinnamic alcohol and the potential formation of eight metabolites of interest, namely cinnamic aldehyde, cinnamic acid, 4-hydroxy-cinnamic alcohol (pOH-cinnamic alcohol), 4-hydroxy-cinnamic aldehyde (pOH-cinnamic aldehyde), 4-hydroxy-cinnamic acid, epoxy cinnamic alcohol, epoxy cinnamic aldehyde, epoxy cinnamic acid and cinnamic sulfate, **Figure 15**, were monitored at different time points, 0, 10, 20, 30, 40, 60 min in the HLM and S9 incubations, and at 0, 2, 4, 6 h (and 24h) in the RHE models. Deuterated cinnamic-d_7 acid was used as internal standard. Two metabolites, pOH-cinnamic alcohol and pOH-cinnamic aldehyde, were found to be of particular interest and their sensitizing potential was further investigated through the LLNA protocol and via their reaction toward the model synthetic peptide Ac-PHCKRM.

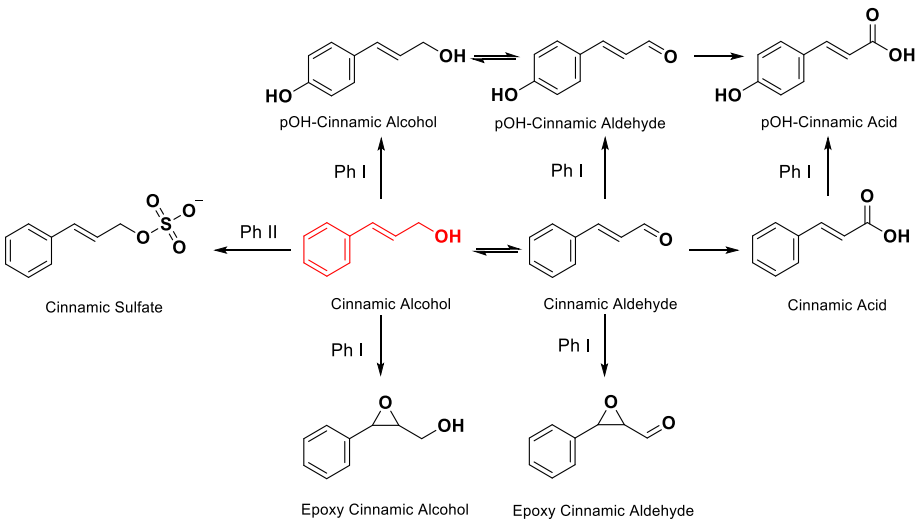


Figure 15: Targeted cinnamic alcohol metabolites formed by phase I (Ph I) or phase II (Ph II) metabolism. (*Adapted from* ***Paper V****)*

4.4 Results and discussion

MDBGN is classified as a moderate sensitizer and is believed to react through a typical S_N2 mechanism, which would result in an added mass of 183.9636 Da. However, biotransformation of MDBGN to 2-MGN has also been reported (Bao et al., 1998), **Figure 16,** resulting in an added mass of 106.0609 Da.

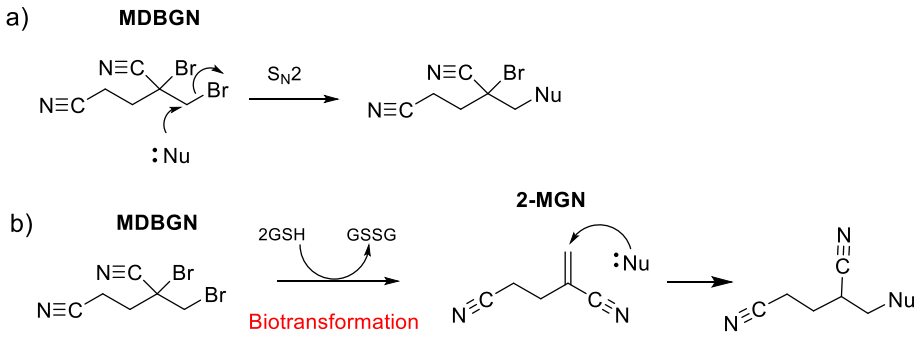


Figure 16: Potential reaction mechanisms of MDBGN: a) via S_N2 nucleophilic substitution, or b) after biotransformation to 2-MGN. (*Adapted from **Paper I** (Ndreu et al. 2020)*)

Incubation of only MDBGN with the model peptide resulted in no adduct formation during any time point of the study. However, complete dimerization of the peptide was observed. Incubation of the peptide with MDBGN and reduced glutathione (GSH), lead to the formation of a 2-MGN adduct, and the site of localization of the adduct was confirmed to be the cysteine residue. The same adduct was formed when the peptide was incubated directly with 2-MGN, **Figure 17**. The adduct formation increased over time and resulted in a depletion of the initial peptide. The depletion was observed to be 69% after 24 h, categorizing 2-MGN as a moderate sensitizer, which is consistent with the sensitization category of MDBGN as assessed *in vivo*.

Furthermore, HSA samples treated with MDBGN and analyzed using Proteome Discoverer to detect potential adducted sites corresponding to the modification of 2-MGN or the direct S_N2 reaction of MDBGN showed that no potential adducted sites corresponding to the modification of MDBGN could be detected. However, when incubated with a 100-fold excess of MDBGN, more than 20 potentially modified sites corresponding to modifications of 2-MGN were detected. This finding supports the hypothesis that biotransformation of MDBGN by free sulfhydryl groups to 2-MGN leads to the binding of 2-MGN to proteins in the initiation event of contact allergy, and thereby categorizing MDBGN as a prohapten.

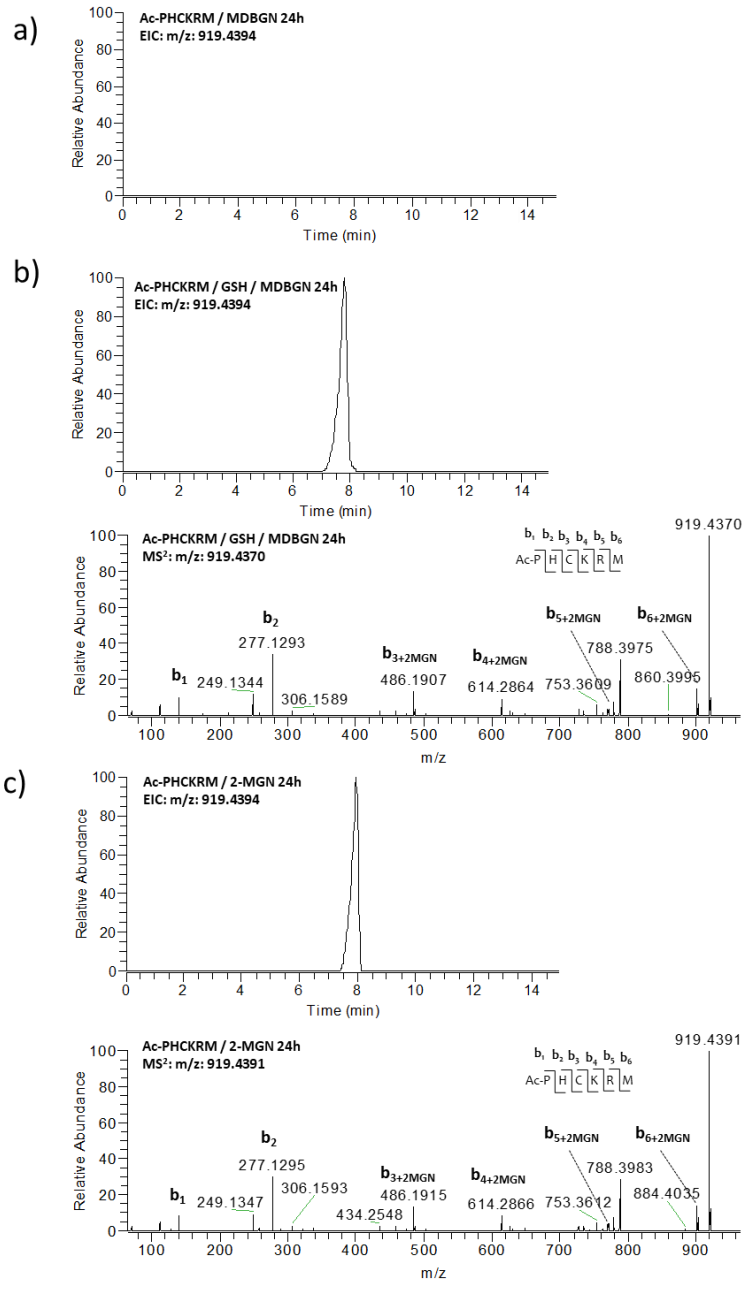


Figure 17: Extracted ion chromatograms (EIC) and MS^2 of the expected adduct formed in the following incubations: a) MDBGN and Ac-PHCKRM, b) MDBGN, GSH and Ac-PHCKRM, d) 2-MGN and Ac-PHCKRM, after 24h.

In **Paper IV**, attempts to classify the propolis components CA, CAAE and CAPE using the UV-based kDPRA, as well as a modified version of it, were unsuccessful. No significant variation in adduct formation with respect to time were observed which was inconsistent with the clinical data, especially for CAPE. Thereafter, a more sensitive targeted LC-MS/MS method was employed to study the adducts formed between the tested compounds and the synthetic peptide AC-PHCKRM under different oxygen conditions. The results obtained showed that in the presence of oxygen two different adducts were formed for each compound, one corresponding to addition to the aromatic ring (structures 1a-c) and the other adduct was formed by addition to the α,β-unsaturated bond next to the carbonyl of the compounds (structures 2a-c), **Figure 18**.

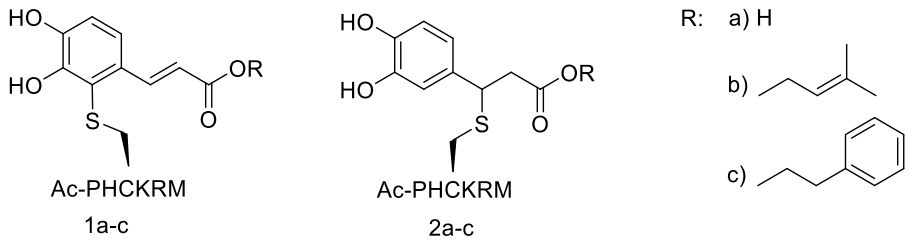


Figure 18: Structures of the expected peptide adducts formed for CA, CAAE, and CAPE.

The rates of formation of the two adducts were lower when limited amount of oxygen was available, and increased with increased access to oxygen. In both cases, the adduct corresponding to addition to the α,β-unsaturated carbonyl was lower than the one corresponding to addition to the aromatic ring. The most interesting finding however, was that no adducts were formed under anaerobic conditions, **Figure 19**. These findings emphasize the necessity of oxygen presence in adduct formation, classifying these compounds as prehaptens. Additional NMR experiments showed that thiol addition to the ring system occurs at the 2-position, a finding in agreement with a previous report (Hansson et al., 1995).

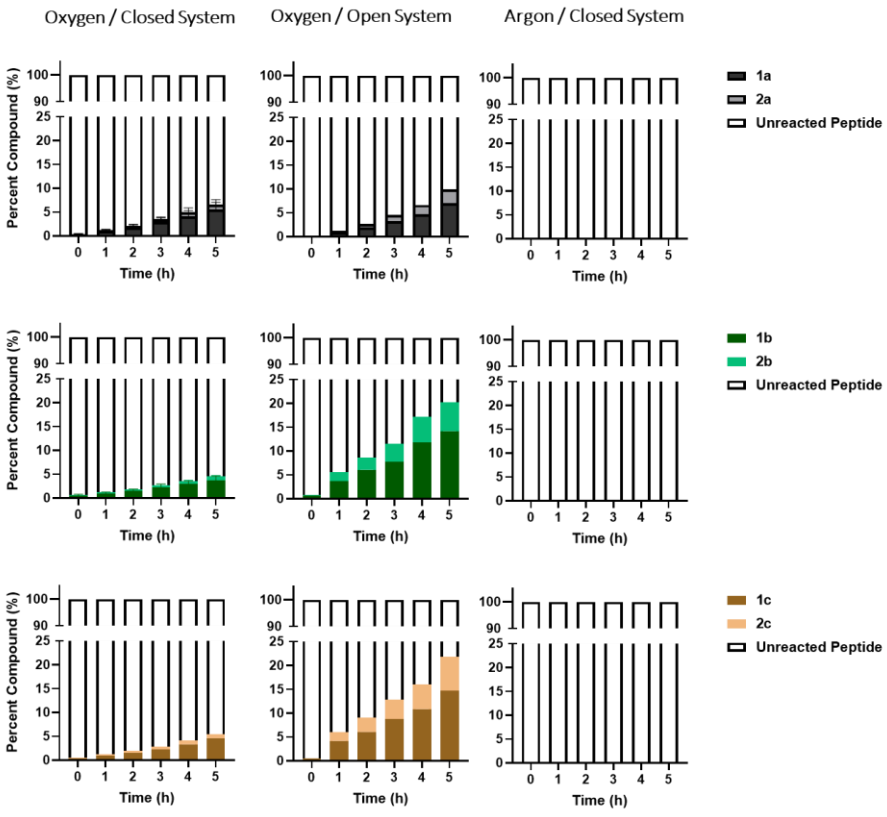


Figure 19: Relative ratios of peptide adducts with CA (upper), CAAE (middle), and CAPE (lower). The model peptide was incubated at 5 times molar ratio of each compound in the presence of oxygen, in a closed system and open system (ambient air), and under an argon atmosphere prior LC-HRMS analysis. Peak areas corresponding to each respective adduct and unreacted peptide were summed and used to calculate the relative percent compound present in each timepoint. (*Adapted from* ***Paper IV****)*

In **Paper V** bioactivation and metabolite formation of cinnamic alcohol in HLM, liver S9 and RHE was investigated with a targeted method including cinnamic alcohol and eight possible metabolites, as shown in **Figure 15**.

HLM incubations of 10 µM cinnamic alcohol led to the formation six different metabolites, including cinnamic aldehyde, pOH-cinnamic alcohol, pOH-cinnamic aldehyde, epoxy cinnamic alcohol, epoxy cinnamic aldehyde, and cinnamic acid. Cinnamic acid and cinnamic aldehyde were formed in higher amounts and their concentration increased over time. The other metabolites were formed at low levels

close to their LoQ, but their concentrations also showed slight increase with longer incubation time. The results from this study are congruent with that of (Niklasson et al., 2014) who showed that HLM incubation of cinnamic alcohol leads to the formation of cinnamic aldehyde, epoxy cinnamic alcohol, epoxy cinnamic aldehyde and cinnamic acid. However, this is the first time that pOH-cinnamic alcohol and pOH-cinnamic aldehyde have been detected as free metabolites.

After bioactivation of low levels of cinnamic alcohol using RHE, the only detected metabolite in all the timepoints investigated was cinnamic acid. Four metabolites could be detected when the RHE models were treated with higher levels of cinnamic alcohol. After 24 hours, an increase in levels of cinnamic alcohol, cinnamic aldehyde, cinnamic acid, and epoxy cinnamic alcohol in the media was observed. Stable levels of pOH-cinnamic aldehyde were observed at all time points. Cinnamic sulfate, potential product of phase II metabolism, previously suggested as a metabolite formed after in situ metabolism of cinnamic alcohol using RHE (Moss et al., 2016), could not be detected in the liver S9 or in the RHE incubations. The low quantities of pOH-metabolites and epoxy-metabolites in the HLM/RHE incubations and complete lack of identified cinnamic sulfate in the S9/RHE incubations, do not necessarily preclude their formation. It is possible that they are formed but immediately react with nucleophilic sites of various proteins, which would decrease the likelihood of their detection as free metabolites. The sulfate has also been shown to hydrolyze rapidly back to cinnamic alcohol in aqueous systems.

pOH-cinnamic alcohol and pOH-cinnamic aldehyde, two compounds of suspected sensitizing potential, were of particular interest. Therefore the LLNA (Gerberick et al., 2007) was utilized to assess the skin sensitizing potency of these two compounds. Our group has found the EC3 value for cinnamic aldehyde to be 0.75% w/v and the EC3 value for cinnamic alcohol to be 13.33 % w/v. From the LLNA assessment of the sensitizing potential of the new detected compounds, pOH-cinnamic aldehyde gave an EC3 value of 6.1% w/v which corresponds to almost a ten-fold decrease in sensitization potency compared to cinnamic aldehyde, while pOH-cinnamic alcohol was tested in a too low concentration to result in an EC3 value (>1.8 M, 27% w/v), **Figure 20**.

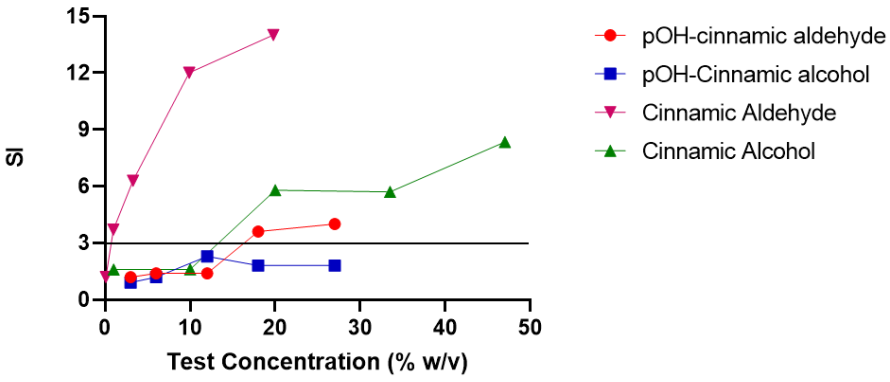


Figure 20: Dose-response curves in the LLNA for pOH-cinnamic aldehyde, pOH-cinnamic alcohol, as well as cinnamic aldehyde and cinnamic alcohol for comparison reasons. (*Adapted from* ***Paper V****)*

5. Concluding remarks and future perspectives

The exposure to hazardous chemicals in our environment, including through the skin, is a growing concern for society. While extensive research has been dedicated to analyzing the health effects of chemicals in the air and in our food, our skin has been under-investigated regarding exposure and uptake of chemicals. This is despite being our largest organ and constantly exposed to high levels of various compounds from our close environment. It is essential to develop methods to evaluate our exposure to skin-permeable compounds and assess their health effects to identify hazardous compounds and determine their effects on people's health. Contact allergy and its possible consequence, ACD, deteriorate the quality of life of the individual and have a negative socioeconomic effect in the form of sick leave and healthcare expenses. Contact allergy is twice as common in women than in men and is observed at high incidence rates in children and young adults. Unless the exposure to these health hazardous compounds can be limited, contact allergy will become an even greater health problem in the future. Therefore, further research and better care for patients could result in substantial financial savings and improve the quality of life for affected individuals.

The utility of MS based methods to characterize contact allergens and their protein conjugates was demonstrated in the present thesis. Bottom-up proteomic analysis of abundant blood proteins in **Paper I**, allowed for the most reactive sites of these proteins with common contact allergens reacting via different mechanism to be determined. Haptenation of these sites has the potential to be explored for monitoring exposure to contact allergens and for development of improved diagnostic methods for contact allergy based on a blood sample. The power of MS analysis in detecting the exact species possessing sensitizing potential was also demonstrated in the same paper, specifically through the case of MDBGN. Despite being widely accepted to react via an S_N2 mechanism, MDBGN was shown to be biotransformed to the metabolite 2-MGN before haptenation of proteins occurred. Moreover, this thesis has highlighted that MS-based proteomic advances can be applied to new areas in the field of contact allergy, as exemplified in the case of Ni in **Paper II**. Methods such as LiP-MS have great potential to be included in the toolbox of methods for assessing skin sensitization to compounds other than electrophilic ones, such as Ni, through a blood sample.

The detection and quantification of hapten-modified MIF in the blood and lymph node samples of treated mice in **Paper III**, represents an important discovery that requires further investigation. *In vitro* studies can lead to better understanding of the role of haptenated MIF in DC migration and its involvement in the mediation of the condition. Furthermore, it is important to determine whether the findings of the study can be directly translated to humans. Although murine and human MIF are different, the N-terminal peptide containing the site of the modification are identical in the two species. Ultimately, it is crucial to investigate if hapten-MIF conjugates are found in the blood of ACD patients, as it may serve as a diagnostic tool and facilitate significant progress in the treatment of contact allergy.

It should also be noted that MS approaches can be of value in comprehending the role of biomolecule adducts beyond proteins. For example, in recent years, significant developments have been made in elucidating the involvement of lipids, which were previously considered inert, in the molecular mechanisms of contact allergy.

Additionally, it was demonstrated in this thesis that the development of protocols to assess the sensitization potential of compounds using MS methods is crucial. These protocols not only increase the likelihood of *in vivo* detection of exposure to contact allergens, but also aid in unraveling the nature of the hapten-protein conjugates formed, especially in the case of prehaptens, as exemplified in **Paper IV**. By implementing this approach in the case of propolis compounds, it was revealed that these compounds are prehaptens and that the process of autoxidation plays a significant role in their activation toward the eventual protein reactive species. These findings carry significant importance in the comprehension of hapten-modified peptides/proteins, which may trigger skin sensitization toward propolis, and are thus of great clinical significance. The simultaneous targeted MS analysis of both Phase I and II metabolism of the common contact allergen cinnamic alcohol in **Paper V**, led to the detection of new free metabolites, namely pOH-cinnamic alcohol and pOH-cinnamic aldehyde, increasing the array of potential contact sensitizers originating from the otherwise non-reactive cinnamic alcohol. However, in order to improve understanding of cinnamic alcohol metabolism, non-targeted analysis should be used to look for other unidentified metabolites and metabolic pathways beyond a suspect list. Additionally, bottom-up proteomic analysis of the RHE treated models could give additional insights into the reactive metabolites that form protein adducts.

In conclusion, MS-based methods have demonstrated their significance as invaluable tools for the identification and characterization of contact allergens and their associated protein adducts in the context of contact allergy. These methods have facilitated advancements in our understanding of the mechanisms underlying

the initiation event of contact allergy, and potential biomarkers have been identified that hold promise for the development of improved diagnostic tools and targeted therapeutics for this health condition.

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