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Exploring the Mechanisms of Allergic Contact Dermatitis: Lipid Peroxidation, CD1 Antigen Presentation and Effects on Skin Lipids

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Philosophy

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Declaration

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Abstract

Allergic contact dermatitis (ACD) affects around 20% of the population and significantly impairs quality of life. While protein-based mechanisms of ACD are well characterised, the contribution of lipids remains poorly understood. Lipids are abundant and highly reactive skin components, implicated in multiple inflammatory dermatoses, yet their role in ACD has been underexplored. This thesis investigates the involvement of lipids in ACD, focusing on the ability of metal allergens to induce lipid peroxidation, the potential contribution of lipid peroxidation to cell death, the role of lipid antigen presentation molecules CD1a and CD1d, and the penetration and lipidome-modifying effects of small organic allergens in skin.

Chapter 2 examined the ability of nickel (II) sulfate (NiSO_4), cobalt (II) chloride (CoCl_2), and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) to induce lipid peroxidation in both a linoleic acid single-lipid system and HaCaT keratinocytes, using the C11-BODIPY 581/591 assay. All three metals induced peroxidation in the lipid model. In HaCaTs, chromium triggered strong peroxidation across all concentrations, while nickel and cobalt did so only at higher levels, coinciding with reduced cell viability. These findings raised the key question of whether lipid peroxidation is a cause or a consequence of toxicity.

Chapter 3 investigated the mechanism of metal-induced cell death. Annexin-V/PI staining and cell cycle analysis indicated necrotic, rather than apoptotic, pathways. Ferroptosis inhibitors ferrostatin-1 (Fer-1) and deferoxamine (DFO) were tested to assess the role of ferroptosis, a lipid peroxidation-driven form of death. Neither inhibitor rescued cells from metal-induced toxicity. However, limitations in Fer-1 performance reduced the strength of these conclusions, and further work is needed to further characterise metal induced cell death.

Chapter 4 explored whether CD1a and CD1d antigen-presenting molecules contribute to ACD through recognition of metal-induced lipid changes. NiSO_4 enhanced T cell activation and TH1 cytokine production in nickel-allergic donors in both a CD1a- and CD1d-dependent manner. Cobalt and chromium produced little CD1-dependent activity. Notably, lipid peroxidation products showed molecule-specific effects: 4-hydroxynonenal (4-HNE) activated

cytokine responses through CD1a, whereas malondialdehyde (MDA) did so via CD1d. These findings identify novel pathways by which lipids and CD1 molecules may contribute to nickel ACD.

Chapter 5 examined the penetration of low molecular weight organic allergens—methyli-sothiazolinone (MI), linalool hydroperoxide (LinOOH), and limonene hydroperoxide (LimOOH)—into ex vivo human skin using Time-of-Flight Secondary Ion Mass Spectrometry. MI penetrated into the viable epidermis, while LinOOH and LimOOH remained mainly in the stratum corneum with limited deeper penetration. All allergens significantly altered skin lipid composition, reducing or modifying key lipids such as cholesterol, monoacylglycerols, diacylglycerols, and phosphatidylcholine, indicating a broader impact of allergens on skin barrier lipid integrity.

Overall, this work highlights metals as inducers of lipid peroxidation, identifies roles for CD1a/CD1d in nickel ACD, and shows organic allergen penetration and lipidome disruption in skin. These findings provide new insights into lipid contributions to ACD and suggest lipid pathways and antigen presentation molecules as potential therapeutic targets.

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List of Abbreviations

μm	micrometre
μM	micromolar
4-HNE	4-hydroxy-2-nonenal
5-FU	5-fluorouracil
ACD	Allergic Contact Dermatitis
AD	Atopic Dermatitis
APC	Antigen presenting cell
Ar	Argon
Ar-GCIB	Argon gas cluster ion beam
BODIPY	C11-BODIPY 581/591
CER	Ceramide
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CL	Cardiolipin
CO₂	Carbon Dioxide
CoCl₂	Cobalt (II) Chloride
CoQ₁₀	Coenzyme Q ₁₀
Cr³⁺	Trivalent Chromium
Cr⁶⁺	Hexavalent Chromium
CRMP2	Collapsin response mediator protein 2
CumOOH	Cumene Hydroperoxide
Cys	Cysteine
DAGs	Diacylglycerols
DCF	Dichlorofluorescein
DCFH	2',7'-dichlorodihydrofluorescein
DCs	Dendritic cells
DFO	Deferoxamine
DHSM	Dihydrosphingomyelin
DMEM	Dulbecco's modified eagles' medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNCB	Dinitrochlorobenzene
EC₃	Effective concentration 3
ER	Endoplasmic Reticulum
ESCD	European Society of Contact Dermatitis
EU	European Union
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
Fer-1	Ferrostatin-1
FFAs	Free fatty acids
FMO	Fluorescence minus one
FSC	Forward Scatter
FSP1	Ferroptosis suppressor protein 1

FSP1/CoQ	Ferroptosis suppressor protein 1/coenzyme Q10
GH	Growth hormone
Glu	Glutamate
GPX4	Glutathione peroxidase 4
GSH	Glutathione
H2DCFDA	2, 7-dichlorodihydrofluorescein diacetate
H₂O₂	Hydrogen Peroxide
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSPB1	heat shock protein beta-1
HUVECs	Human umbilical vein endothelial cells
IBTS	Irish blood transfusion centre
ICD	Irritant contact dermatitis
IFN-γ	Interferon- γ
IFRA	International Fragrance Agency
IgE	Immunoglobulin E
IGF-1	insulin like factor-1
IL-13	Interleukin-13
IL-1α	Interleukin-1 α
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-8	Interleukin-8
IMS	Imaging mass spectrometry
iNKT cells	Invariant natural killer T cells
K₂Cr₂O₇	Potassium Dichromate
KEAP1	Kelch-like ECH-associated protein 1
KeV	Kil-electron volt
kg	Kilogram
LC-MS	Liquid chromatography - mass spectrometry
LC	Langerhans's Cells
LimOOH	Limonene Hydroperoxide
LinOOH	Linalool Hydroperoxide
Lip-1	Lipoxstatin 1
LLNA	Local lymph node assay
LPS	lipopolysaccharides
MAGs	Monoacylglycerols
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MCI	methylchloroisothiazolinone
MD2	myeloid differentiation protein 2
MDA	Malondialdehyde

mg	Milligram
MHC II	Major histocompatibility complex 2
MI	2-Methyl-4-isothiazolin-3-one
mL	millilitre
mM	millimolar
MSI	Mass spectrometry imaging
nA	nanoamps
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	Nuclear factor erythroid 2-related factor 2
NIH	National Institute of Health
NiSO₄	Nickel (II) Sulfate
NKT cells	Natural Killer T cells
nm	nanometre
OECD	Organisation for economic cooperation and development
OH	Hydroxyl Radicals
OrbiSIMS	Orbitrap secondary ion mass spectrometry
PA	Phosphatidic acid
pA	Picoamps
PASI	Psoriasis area and severity index
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered Saline
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI	Propidium Iodide
PLS-DA	Partial least squares - discriminant analysis
PMA	Phorbol 12-myristate 13 acetate
PPG	Periplogenin
PS	Phosphatidylserine
PUFAs	Polyunsaturated Fatty Acids
REDOX	Oxidation-reduction
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSL3	RAS synthetic lethal 3
SAR	Structure activity relationship
SB	Stratum Basale
SEM	Standard Error Measurement
SG	Stratum Granulosum
SM	Sphingomyelin
SQS	Squalene synthase
SS	Stratum Spinosum

SSC	Side Scatter
STAT6	Signal transducer and activator of transcription 6
TAGs	Triacylglycerols
<i>t</i>-BuOOH	<i>tert</i> -butyl hydroperoxide
TCR	T cell receptor
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
ToF	Time-of-flight
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
USA	United States of America
UV	Ultraviolet
VE	Viable epidermis
z-VAD-FMK	Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone
α-GalCer	α -Galactosylcermaide

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Chapter 1: Introduction

This thesis investigates the role of lipids in the skin in the pathophysiology of allergic contact dermatitis (ACD). It will be mainly focused on the interaction between the common allergenic metals nickel, cobalt and chromium, and lipids within the skin, and how this interaction may lead to the activation of T cells. To do this, a strong understanding of the structure and functions of the skin, the skin lipidome, as well as the pathophysiology of metal ACD is necessary.

1.1 Overview of Skin

The skin is the largest organ of the body and performs essential physiological functions, including acting as a barrier against pathogens and physical injury, regulating body temperature through sweat production and vascular adjustments, and providing sensory input such as touch and thermal perception¹. It is comprised of three principal layers, the outer epidermis which is composed of a stratified epithelium, and the inner dermis layer which consists of connective tissue with numerous different cell types, an extensive network of vessels, as well as nerves, sweat glands and receptors². Below the dermis, a third layer of subcutaneous fat termed the hypodermis is found, which consists of adipose tissue, blood vessels and nerves³ (Figure 1.1). The thickness of the skin varies across the body depending on location, for example the thickest skin is found on the palms of the hands and soles of the feet, because the epidermis contains an extra fifth layer in comparison to the four layers found in the epidermis in the rest of the body⁴ and the thinnest skin is found on the eyelids to allow for the rapid movement associated with blinking and thus keeps the eyes healthy⁵.

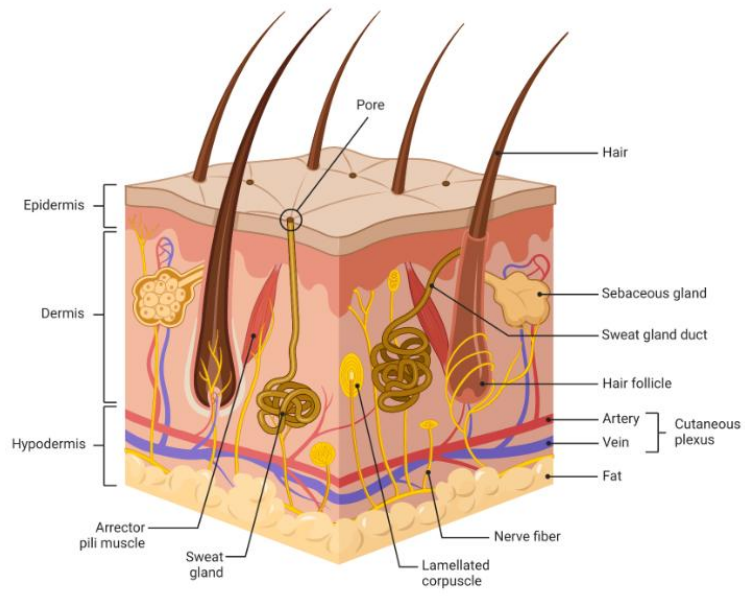


Figure 1.1 Anatomy of the skin⁶.

1.2 Structure and Functions of the Skin

1.2.1 The Epidermis

The epidermis is the outermost layer of the skin and is comprised of four different layers; the stratum basale (SB) which is the deepest layer of the epidermis, the stratum spinosum (SS), the stratum granulosum (SG), and the most outermost stratum corneum (SC) layer, with an extra stratum lucidum layer in areas of thicker skin on the palms of the hands and soles of the feet⁷ (Figure 1.2). The epidermis acts as a barrier between the environment and the body and is the first point of interaction between contact allergens and the skin⁸.

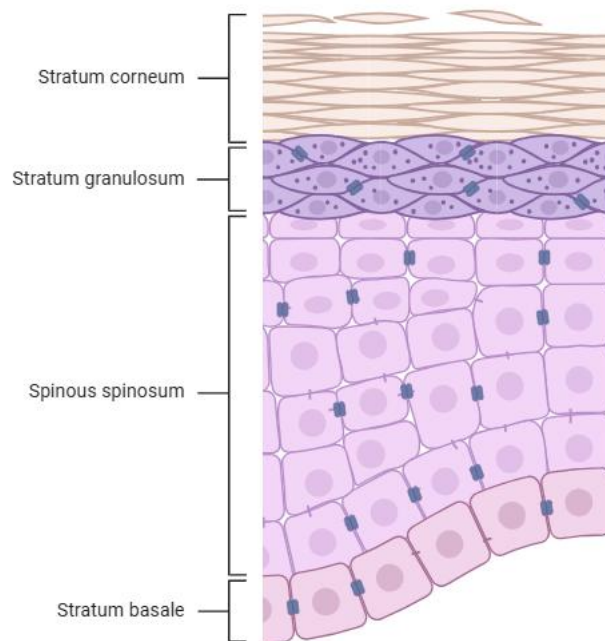


Figure 1.2 Anatomy of the Epidermis

The SC is the outermost layer of cells, made up of keratin and dead keratinocytes, known as anucleate squamous cells⁴. It measures 10-20 μm thick and its principal functions include involvement in the innate immune system and formation of a physical barrier between the external environment and the deeper layers of the skin and rest of the body⁹. The SG contains diamond-shaped cells with keratohyalin granules and lamellar bodies. The lamellar bodies secrete glycolipids to the surface of the cells which act like a glue, ensuring that the cells remain stuck together. Overall, the function of the SG is to act as a transitional layer where keratinocytes develop into their final form and die¹⁰. The SS is also known as the prickly cell layer and measures 8-10 cell layers thick. This layer of the epidermis contains polyhedral shaped keratinocyte cells with spines, or cytoplasmic processes, that extend outward and contact neighbouring cells by desmosomes which maintain the structural integrity of skin¹¹. Additionally, dendritic cells (DCs) are found in this layer. The SS is partly responsible for the skin's strength and flexibility. Finally, the SB is the innermost layer of the epidermis and is separated from the dermis by the basal lamina, which it is attached to

by hemidesmosomes. Stem cells are located in this layer and constantly produce keratinocytes that are continuously pushing upward to replenish the epidermal cells in the layers above¹².

The main cells of the epidermis are keratinocytes, which secrete lipids for formation of the epidermal water barrier and have functions in calcium absorption. Melanocytes are also present in the epidermis and are found between cells in the SB. Their main function is to produce melanin which gives the skin its pigment. Langerhans cells are immune cells that are mainly found in the SS and are the skin's first line defenders against pathogens. They have a significant role in antigen presentation via the major histocompatibility complex I (MHC I), major histocompatibility complex II (MHC II) and CD1a molecules that they express¹³. Finally, Merkel cells are found in the SB and have mainly sensory functions as mechanoreceptors for light touch and are most populous in the fingertips.

1.2.2 The Dermis

The dermis is a connective tissue layer which lies between the epidermis and hypodermis/subcutaneous tissue (Figure 1.1). It is comprised of collagen, elastic tissue, vessels, nerves, hair follicles and glands. The dermis is divided into two layers: the papillary dermis and the reticular dermis. The papillary layer comprises of relatively thin collagen fibres, in comparison to the compact layer of thick collagen fibres that makes up the reticular dermis layer¹⁴. The primary cell type in the dermis is the fibroblast, which produces the extracellular structural proteins, collagen and elastin fibres. Other cell types found include histocytes and mast cells (both immune cells), and dermal adipocytes. The dermis has various functions including skin support and protection, thermoregulation and sensation. The structure of the dermis provides a connective tissue framework for strength, flexibility and protection of deeper anatomical structures. The extensive vasculature present in the dermis contributes to thermoregulation, and the several mechanoreceptors and nerve endings within the dermis and surrounding hair follicles contribute to sensation and allow sensation to extend beyond the skin's surface.

1.2.3 The Hypodermis

The hypodermis is a subcutaneous layer of fat that is highly vascularized and innervated and is situated just below the reticular dermis (Figure 1.1). This layer cushions the upper epidermal and dermal layers. The fat rich hypodermis acts a reservoir of energy and is also where fat-derived mesenchymal stem cells are produced which can regenerate the cellular constituents of the upper layers. Finally, the fat layer of the hypodermis provides significant heat insulation¹⁵.

Understanding the makeup and functions of the different layers of skin is key to unlocking and understanding the pathophysiological mechanisms behind ACD, particularly with the epidermis which acts as the first obstacle that metal allergens must overcome to exert an allergenic effect.

1.3 The Skin Lipidome

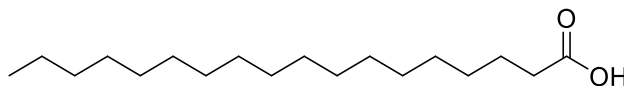
Lipids are an essential component of skin and offer many different functions including acting as sources of energy via fatty acid oxidation, acting as a barrier, acting as immunological mediators, acting as growth regulators (e.g. arachidonic acid and prostaglandins), and as structural support in cell membranes¹⁶. Lipids are a highly reactive and highly diverse class of macromolecule, comprised of many different subclasses of different lipids, all with different structural characteristics and functions¹⁷.

1.3.1 Fatty Acyls

The fatty acyl class is primarily made up of saturated and unsaturated fatty acids and their corresponding conjugates and is therefore one of the most fundamental categories of biological lipids. Fatty acids have several key functions in metabolism, structure, gene regulation, thermal regulation and protection. The structure of fatty acids consists of a carbon chain with a methyl group at one end, and a carboxyl group at the other. They can be saturated or unsaturated. Saturated fatty acids, like stearic acid (C18:0) are named as such because they are saturated with hydrogen i.e., there are hydrogen groups on every available carbon position. Unsaturated fatty acids contain one double bond (monounsaturated), like oleic acid (C18:1), or more than one double bond

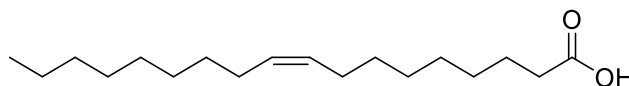
(polyunsaturated), like linoleic acid (C18:2). Most commonly, the double bonds are in *cis* configuration. Polyunsaturated fatty acids (PUFAs) have more than one double bond in their structures, with all double bonds separated by a methylene grouping¹⁸ (Figure 1.3).

Saturated Fatty Acids

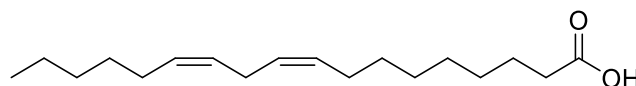


C18:0 Stearic Acid

Unsaturated Fatty Acids



C18:1 Oleic Acid



C18:2 Linoleic Acid

Figure 1.3 The structures of a selection of saturated and unsaturated fatty acids.

Palmitic acid (C16:0) and stearic acid (C18:0) make up the most abundant saturated lipids in the epidermis, and oleic acid (C18:1) and linoleic acid (C18:2) the most abundant unsaturated lipids¹⁹. Unsaturated fatty acids are highly reactive and are very susceptible to attack by reactive oxygen species (ROS) and subsequent breakdown into aldehydes and ketones.

Also present in the fatty acyl group of lipids are the eicosanoids which include prostaglandins and leukotrienes. Eicosanoids are oxidised derivatives of 20-carbon polyunsaturated fatty acids, usually arachidonic acid, formed from various enzymatic pathways including the cyclooxygenase pathway to form prostaglandins and thromboxane,

and the lipoxygenase pathway to form hydroperoxides and leukotrienes and cytochrome P450²⁰ (Figure 1.4).

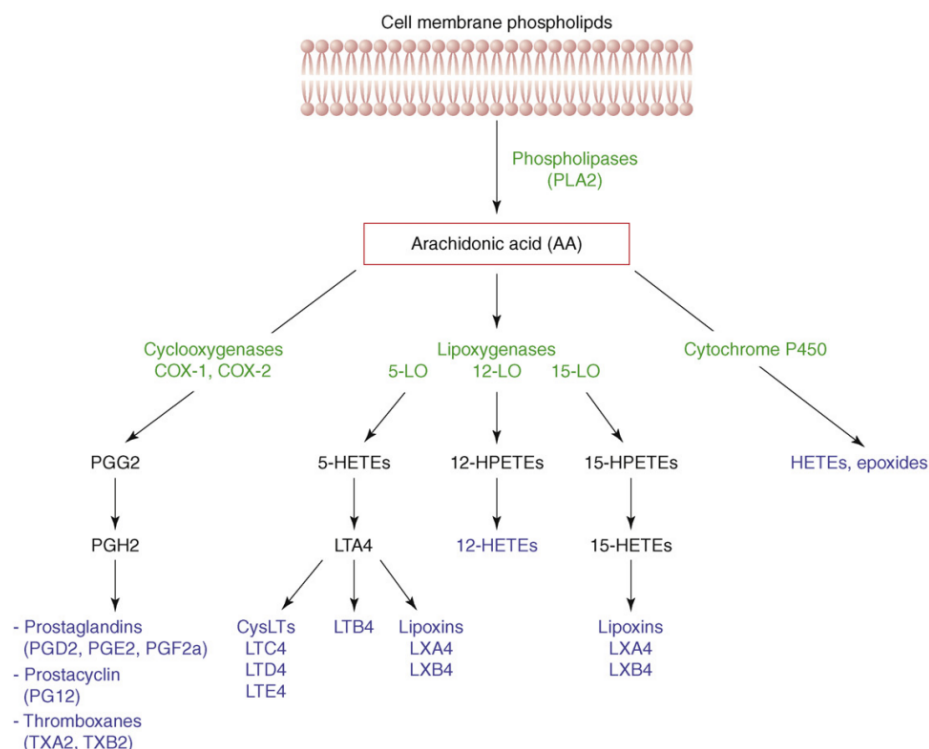


Figure 1.4 Outline of different enzymatic pathways of synthesis of eicosanoids from arachidonic acid²¹
 Arachidonic acid is released from membrane phospholipids by phospholipases including phospholipase A2 (PLA2). Arachidonic acid is converted to eicosanoids via the cyclooxygenase (COX) pathway, the lipoxygenase (LO) or cytochrome P450 pathways. LOX enzymes catalyse the formation of leukotrienes (LTs), hydroperoxyeicosatetraenoic acids (HPETEs), and lipoxins (LXs). COX-1 and COX-2 catalyse the formation of prostaglandins (PGs). The cytochrome P-450 pathway catalyses the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxides. Eicosanoids are highly reactive and have significant roles in the regulation of the vascular, renal, gastrointestinal and reproductive systems. However, they are often associated with disease and the enzymes responsible for their synthesis are targets for different types of drug therapies. For example, prostaglandins promote inflammation and pain, and non-steroidal anti-inflammatories including ibuprofen were developed to inhibit their synthesis via blocking of cyclooxygenase enzymes and therefore induce a reduction in inflammation²².

1.3.2 Sphingolipids and Ceramides

Sphingolipids are a class of lipids defined by their eighteen-carbon amino-alcohol backbones which are synthesised in the endoplasmic reticulum (ER) from non-sphingolipid

precursors. Modification of this structure gives rise to a vast family of sphingolipids, including ceramides²³. Sphingolipids have an important structural role in cells, as well as contributing to key biological activities such as cell motility, growth and differentiation²⁴. Ceramides are the basic structural units of all sphingolipids and are formed through the union of very long-chain fatty acids with sphingosine. Fifteen classes of free ceramides have been identified, along with three classes of covalently bound ceramides²⁵ (Figure 1.5).

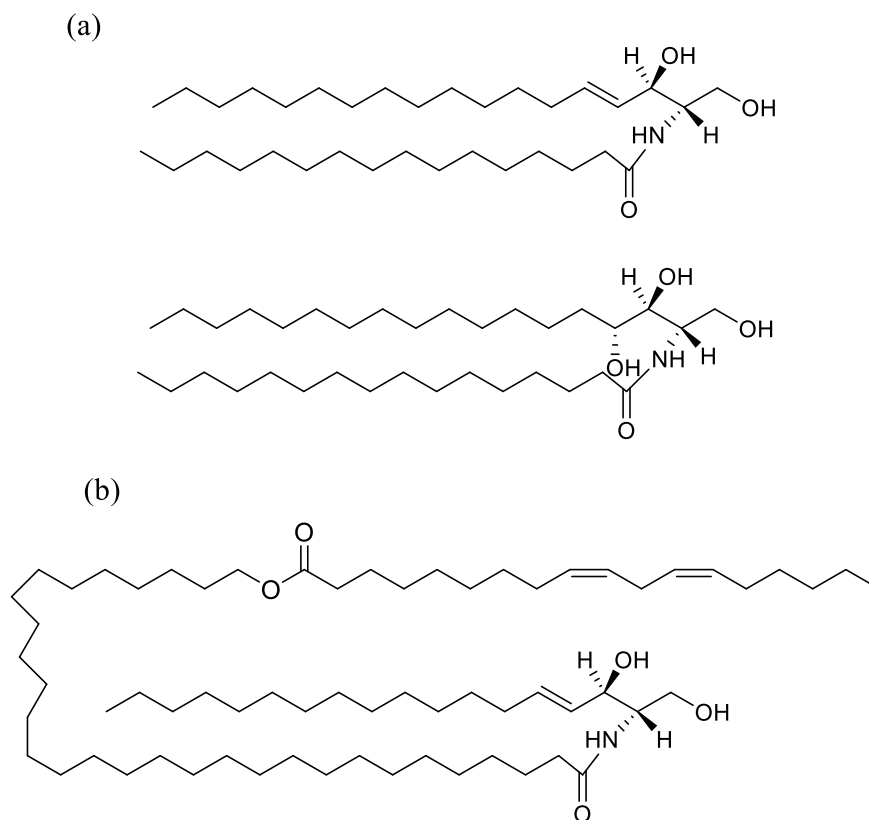


Figure 1.5 Molecular structures of (a) free ceramides and (b) covalently bound ceramides²⁵

The nomenclature for ceramides was established by Motta et al. (1993), and is based on the molecular structures corresponding to the sphingoid base chain, which includes sphingosine (S), 6-hydroxy sphingosine (H), dihydrosphingosine (DS), phytosphingosine (P), dihydroxy-sphingosine (T), and the fatty acid chain which includes alpha-hydroxy acid (A), non-hydroxy fatty acid (N) and omega-hydroxy fatty acid (O)²⁶. For ceramides covalently bonded to the corneocyte protein envelope via an omega-hydroxy fatty acid the letter P

precedes the base and fatty acid chain letters. Ceramide [NP] is the class of ceramide species present in the highest abundance in the SC, followed by ceramide [NH].

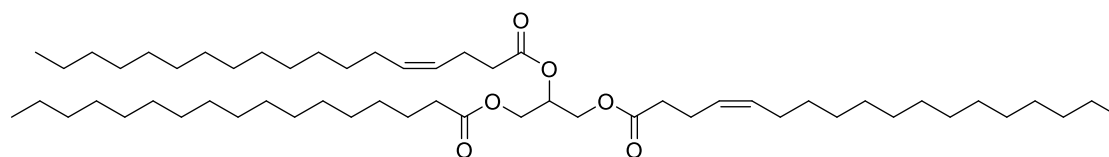
Ceramides are present in high amounts in the skin and make up the highest percentage of lipids in the SC, comprising approximately 50% of the intracellular lipids, and as such they have very important roles in skin barrier function²⁷. They are most effective at restoring barrier function and increasing skin hydration. Ceramides are produced by two methods: the de novo pathway and the salvage pathway²⁸. The de novo pathway is the predominant method of ceramide production in skin²⁹. The amino acid serine and palmitoyl co-enzyme A are condensed to make 3-keto dihydrosphingosine, which is then N-acetylated via fatty acetyl-CoA by the one of the six types of different ceramide synthase enzymes to form dihydro-ceramide. Dihydro-ceramide can then be further modified through desaturation or hydroxylation to produce the various other sphingoid bases found in skin ceramides. Recent LC-MS analysis quantified 1,327 unbound ceramides and 254 protein bound ceramides in the human SC, with a range of different chain lengths. C18 ceramides were the most abundant (28.6%) followed by C20 ceramides and C22 ceramides³⁰.

Due to their important skin barrier functions, ceramides have routinely been added to skin care formulations, to aid in restoration of ceramides that may have been damaged or altered in different skin conditions like psoriasis or dermatitis, and to help keep skin hydrated by preventing trans epidermal water loss. Numerous ceramides including hydroxypalmitoyl sphinganine³¹ and ceramides 1 and 3³², have been added to formulations in many common moisturizers, lotions and body washes, including in popular skincare brands.

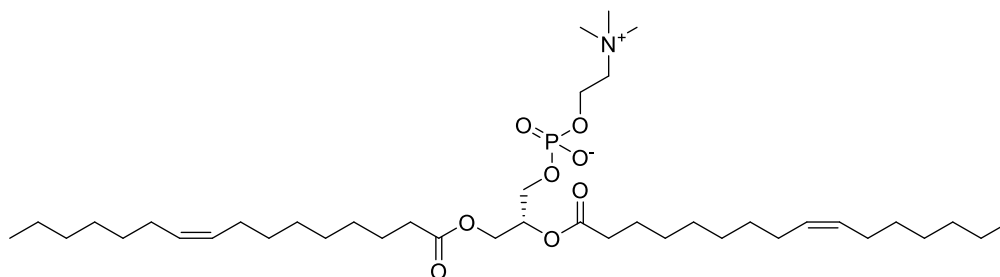
1.3.3 Glycerolipids

Glycerolipids are a large group of lipids necessary for membrane function, caloric storage and intracellular signalling processes³³. Their structure is based upon a glycerol-3-phosphate backbone scaffold with attached long chain acyl and alkyl groups as well as polar alcohols. This class of lipids consists of the mono and disubstituted glycerols, the more well-

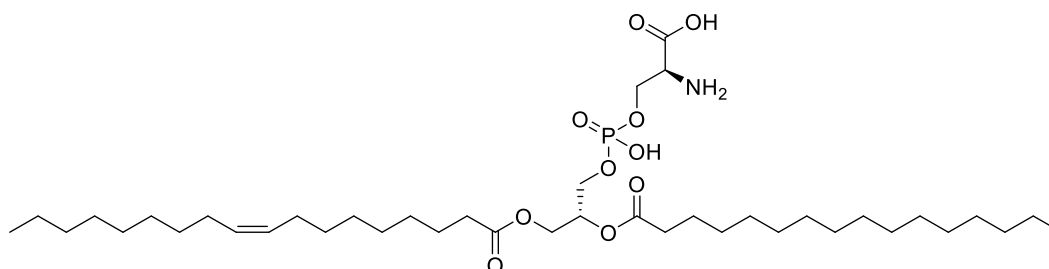
known trisubstituted glycerols, more commonly known as triacylglycerides, and finally phospholipids (Figure 1.6).



Triacylglycerol 17:0, 18:1, 18:1



Phosphatidylcholine C32:2



Phosphatidylserine C34:1

Figure 1.6 Structures of common glycerolipids.

Phospholipids have the ability to form membrane bilayer structures in aqueous environments and are the primary constituent of the membrane bilayer in eukaryotic cells. This membrane is essential for cells to define the boundaries that separate them from their environment and designate intracellular organelles for specific intracellular function. Glycerolipids without phosphate are also of major importance, most importantly triacylglycerols (TAG, also known as triglycerides). TAG is the major storage form of fat in our bodies, serving as an energy source as well as providing insulation to cells and partaking in thermoregulatory functions, as well as aiding in the absorption of fat-soluble vitamins³⁴. Diacylglycerols (DAG, also known as diglycerols) also serve important bodily functions, including activation of protein kinases that regulate cell growth, and serving as a precursor

for the synthesis of triglycerides as well as phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE)³⁵.

1.3.4 Sterol Lipids

Sterols are structural lipids present in the membranes of most eukaryotic cells, and define membrane fluidity and permeability, as well as serving as precursors for a variety of products with specific biological activities such as steroid hormones and bile acids³⁶. Cholesterol is the major sterol lipid in animal tissues and has many important biological functions. It is a key component of the cell membrane, contributing to its mechanical strength as well as modulating its fluidity. In addition, cholesterol also serves as a precursor for vitamin D synthesis, as well as steroids and hormones. Its structure consists of a tetracyclic ring system, with a double bond and a free hydroxyl group. This same fused four ring core structure is present in the steroids, including C18 steroids like oestrogen family of hormones, C19 steroids like testosterone and other androgens, and C21 steroids which include progestogens, glucocorticoids and mineralocorticoids³⁷ (Figure 1.7).

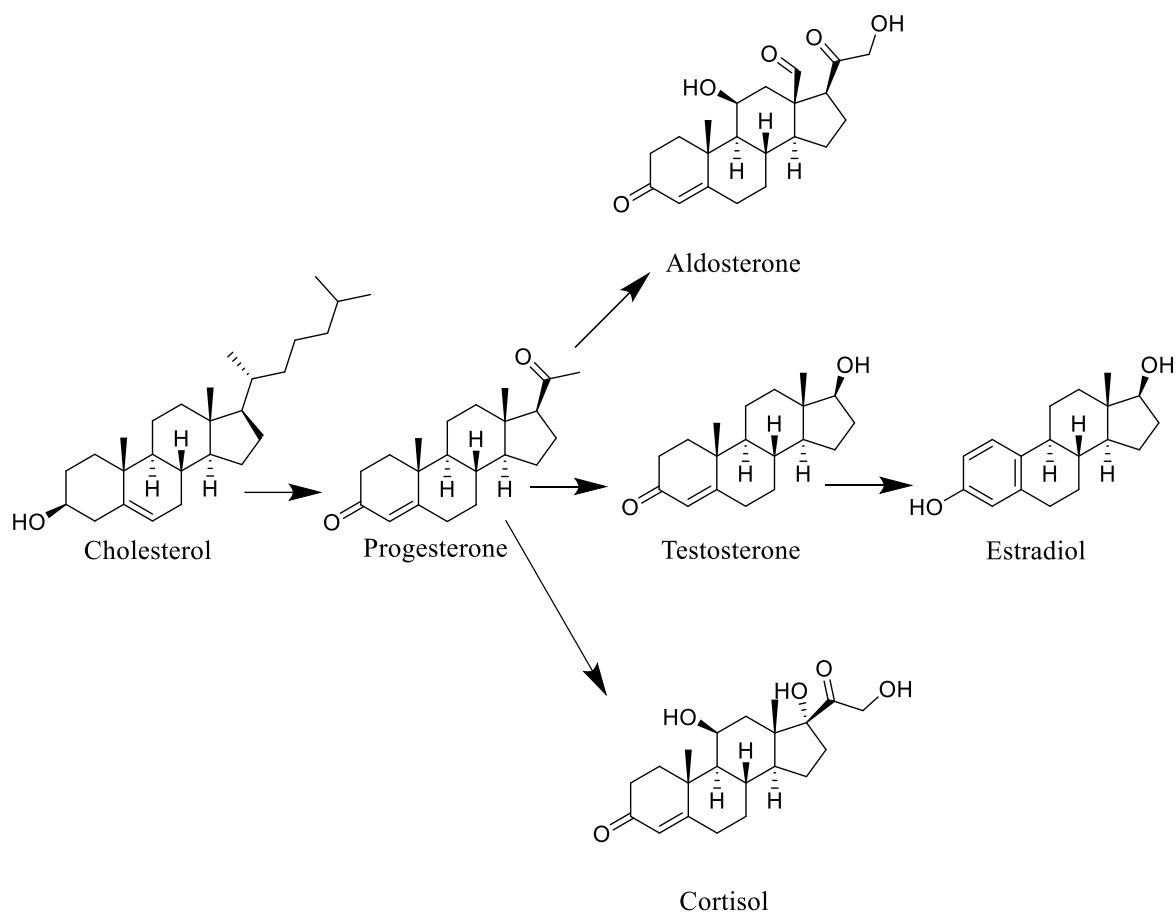


Figure 1.7 The Structure of Cholesterol and steroids and hormones derived from cholesterol ³⁸.

Cholesterol is present in high amounts in the skin, making up around 27% of lipids in the SC, and is important for maintaining rigidity and controlling membrane fluidity amidst fluctuations in body temperature¹⁹. In addition, cholesterol esters comprise of about 10% of lipid content in the SC, most notably cholesterol sulfate. Cholesterol sulfate is generated in the viable epidermis (VE) by the enzyme cholesterol sulfotransferase. Its functions are believed to include regulation of desquamation and SC cohesion.

1.3.5 Prenol Lipids, Polyketides and Saccharolipids

Other important classes of lipids include the prenyl lipids, the polyketides and the saccharolipids. Prenyl lipids are derived from five-carbon precursors (isopentenyl diphosphate and dimethylallyl diphosphate) and include quinones and isoprenoids³⁰. Carotenoids, a simple class of isoprenoids, act as antioxidants and vitamin A precursors³⁹.

Quinones and hydroquinones play key roles in electron transport and oxygen activation, making them essential for respiration and photosynthesis⁴⁰.

Polyketides are rare lipids, typically produced by bacteria and fungi through condensation of acetyl-CoA with malonyl-CoA. They include compounds such as flavonoids, macrolides, tetracyclines, and possess notable antimicrobial activity. Many are clinically important, serving as antibiotics (tetracycline, nystatin), anticancer agents (doxorubicin), and cholesterol-lowering drugs (lovastatin)⁴¹.

Saccharolipids consist of fatty acids linked to a sugar backbone, forming structures compatible with membranes. A key example is lipid A, a component of lipopolysaccharides in Gram-negative bacteria. These lipids vary structurally across organisms and play complex roles in the gastrointestinal tract⁴².

1.3.6 Lipid Composition in the SC

Lipids have a large presence throughout the different layers of the skin. The SC is the first point of contact between the skin and the environment and is dominated by ceramides, cholesterol and free fatty acids (FFAs)¹⁹ (Figure 1.8). Due to the small headgroups of the ceramides and FFAs and the long hydrocarbon chains, strong hydrophobic forces exist in the SC lipid matrix. These hydrophobic forces play a prominent role in the tight packing of the lipids in a three dimensional arrangement and lead to the formation of crystalline lipid lamellae⁴³. Lipids are supplied to the SC by lamellar bodies which can be found in high concentrations in the SG. Lamellar bodies contain many lipid subtypes and enzymes which are expelled into the interface between the SG and SC via exocytosis⁴⁴. The composition of the lipid matrix is unique to the SC and contains approximately 50% ceramides, 25% cholesterol and 10-20% FFAs (Figure 1.8).

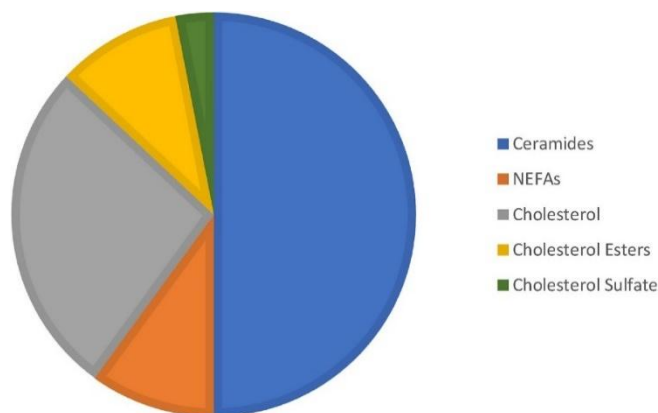


Figure 1.8 Lipid composition in the stratum corneum¹⁹.

There are currently 25 ceramide subclasses identified in SC. The first ceramides were identified in the SC by Gray et al. (1975) who performed lipid analysis of porcine and human skin and found that the SC contained a higher percentage of ceramides than in the viable skin layers⁴⁵. The classes of ceramides found in the SC are depicted in Figure 1.9.

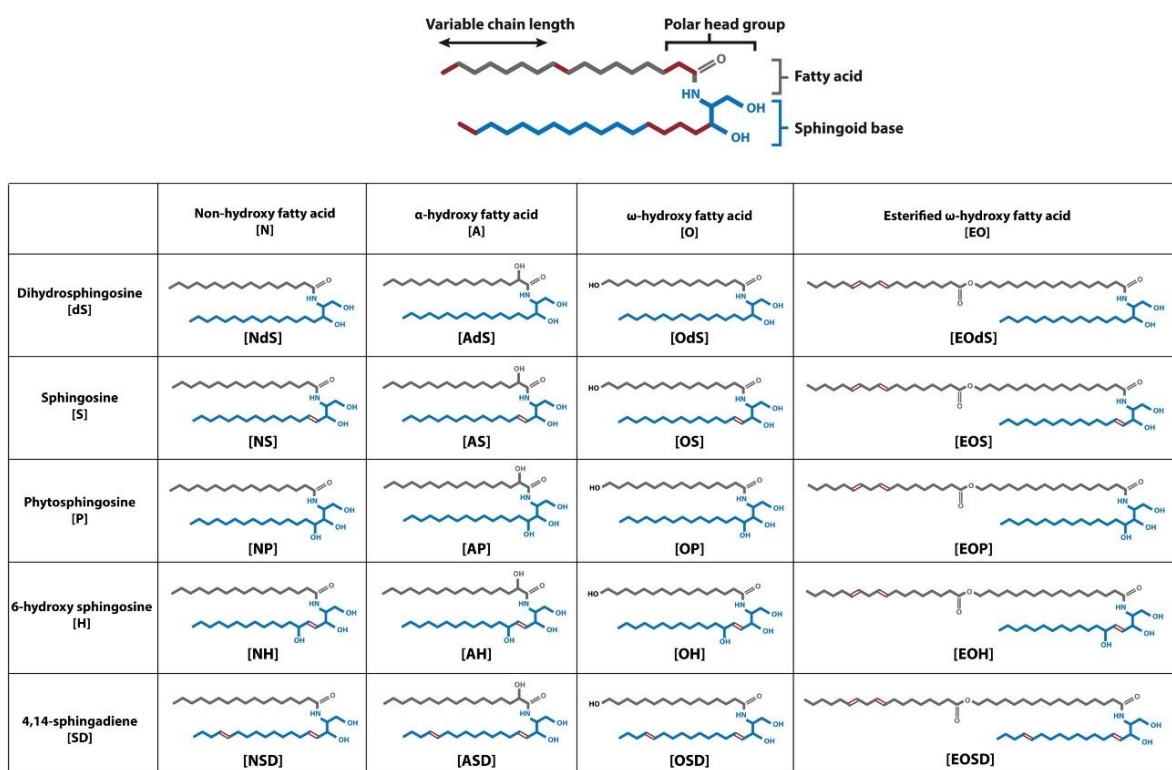


Figure 1.9 Classes of ceramides identified in the stratum corneum⁴³.

FFAs are also abundant in the SC, with the most common chain lengths in porcine and human SC being C24 (lignoceric acid) and C26 (cerotic acid)⁴⁶. High levels of C22 (behenic acid) in the SC have also been reported⁴⁷. Many studies have reported that the levels of unsaturated fatty acids in the SC are low. Van Smeden et al. reported that C18:1 (oleic acid) accounted for 2.3% of FFAs in the SC⁴⁸.

Mass spectrometry imaging showed that ceramides and cholesterol sulfate in the SC had oscillatory distributions, representing the rise and fall of the intensity of the lipid ions in the alternating corneocyte and lipid layers⁴⁹. However, distribution of cholesterol oleate did not display a similar pattern and instead appeared in spot-like irregular locations. This indicated that cholesterol oleate (and potentially other cholesterol esters) possess a different functional role to other skin lipids. 3D OrbiSIMS analysis concluded that cholesterol sulfate intensity steadily increases throughout the SC and reaches a maximum in the SG after which it starts to decline⁵⁰. 3D OrbiSIMS analysis also showed that FFAs such as palmitic acid, oleic acid and linoleic acid are at their highest intensity in the very upper layers of the SC, before decreasing in the deeper layers of the SC⁵⁰. The intensity of these fatty acids began to increase again toward the lower layers of the SC and the upper layers of the VE. This contrasts with the FFAs lignoceric acid and cerotic acid which reach the highest intensity in the SC and do not appear to be present at all in the VE.

1.3.7 Lipid Composition in the VE

As mentioned in section 1.3.6 previously, cholesterol sulfate is abundant in the upper SG layer of the VE, but other lipid species that make up the VE include cholesterol esters, ceramides, TAGs and FFAs. TAGs are found at a high intensity deep in the SB and the dermis layers. Phospholipids are also localised to the VE, with Tokoro et al (2025) using imaging mass spectrometry (IMS) to identify an ion at m/z 184 that correlated to the headgroup of PC⁵¹. PC is the most abundant phospholipid in the VE, but others that have been identified in the VE include phosphatidic acid (PA), PE, cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI) and the sphingolipids sphingomyelin (SM) and

dihydrosphingomyelin (DHSM)⁵². Phospholipids represent approximately 62.3% of lipids in the VE, in comparison to 0.1% in the SC⁵³. Glucosylceramides, precursors of ceramides, are also present in the VE, and accumulate in the SG layer. They are stored in lamellar bodies and as the keratinocytes move upwards towards the SC, they are enzymatically converted to ceramides⁵⁴. Other lipids identified in the VE were glycerophospholipids and sterols⁵¹. The distribution of lipids in the different layers of the epidermis is depicted in Figure 1.10.

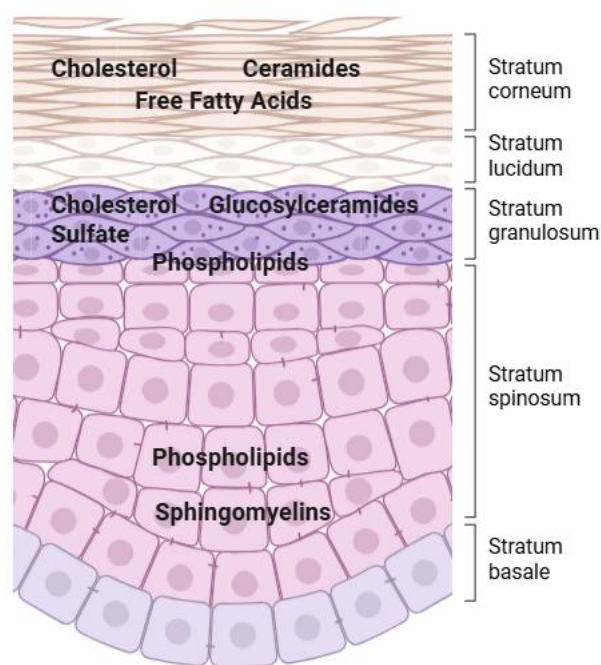


Figure 1.10 Predominant lipids present in the different layers of the human epidermis.

1.4 Lipids in Inflammatory Skin Conditions

Lipids have been implicated in the pathogenesis of numerous inflammatory skin conditions, with alterations in the composition and organization of the skin lipidome documented in disorders such as psoriasis, rosacea, acne vulgaris, and atopic dermatitis.

1.4.1 Psoriasis

The lipid content of the skin has been shown to undergo many changes in different inflammatory skin conditions. Differences in the abundance of FFAs, ceramides and cholesterol levels in the SC of psoriatic skin have been reported. A study by Uchino et al

(2023) reported an increase in total FFA content in psoriatic skin in comparison to healthy skin, with further analysis revealing an increase in unsaturated FFAs and no significant change in the abundance of saturated FFAs⁵⁵. The relative abundance of sapienic acid (C16:1), oleic acid (C18:1) and linoleic acid (C18:2) were increased in psoriatic skin⁵⁵. The increase in unsaturated FFA content could be indicative of lipid peroxidation, via the peroxidation of phospholipids which can release unsaturated FFAs. While no significant changes were observed in the relative abundances of total ceramides in psoriatic skin in comparison to healthy skin, there were significant changes in the abundance of particular ceramide species in psoriatic skin. The relative abundance of CER [NdS], CER [NS], CER [AdS], CER [AS], and CER [AH] were all significantly higher in the SC of psoriatic skin, while the abundances of CER [NP] and CER [AP] were significantly lower⁵⁵. Additionally, the abundance of the [EO] class of ceramides also decreased in the psoriatic SC. Finally, the levels of cholesterol sulfate in the SC of psoriatic skin were significantly increased in comparison to the SC of healthy skin⁵⁵. This increase in ceramide content is indicative of a compensatory response to SC barrier disruption, potentially induced by oxidative damage like lipid peroxidation. Cholesterol levels have also been shown to increase in the SC of psoriatic skin⁵⁶. The reduction in long-chain ceramides such as the [EO] class of ceramides compromises the ability of SC lipids to form well-organized and tightly packed lamellae⁵⁷, while the increase in shorter-chain ceramides and unsaturated fatty acids reduces the hydrophobicity and cohesive strength of the lipid matrix⁵⁸. Both phenomena lead to structural disruption and weakness of the skin barrier function. The authors postulate that the observed lipid composition changes are both a consequence and a driver of psoriatic pathology. Psoriatic inflammation can disrupt enzymes involved in lipid synthesis leading to lipid composition changes, while the compromise of the skin barrier function that results from the lipid changes may further exacerbate immune activation through increased antigen exposure and increase the severity of psoriasis symptoms⁵⁵. In addition to the lipid changes observed in the SC, changes in the phospholipid levels in the VE have also been reported in psoriatic lesions. The levels of PCs and PS are increased in the VE of psoriatic skin, while the levels of PEs are reduced⁵⁹.

The skin lipid barrier has been investigated as a potential therapeutic target, with one randomised control trial reporting that psoriasis patients experienced a 65.5% reduction in the psoriasis area and severity index (PASI) after treatment with a ceramide containing emollient⁶⁰. Another clinical trial documented that psoriasis patients who were treated with moisturiser containing linoleic acid and ceramides alongside steroid treatment, had a superior response and higher reduction in PASI in comparison to patients treated with steroids alone⁶¹.

1.4.2 Rosacea

Rosacea is a chronic skin disease that is characterised by the presence of erythema, telangiectasia, papules and pustules on the face. There are very few studies that investigate the alterations in skin lipid composition in patients with rosacea. A study by Yang et al. (2025) examined the changes in the skin surface lipids in patients with rosacea, and found that DAGs were downregulated, FFAs were downregulated, and PC was upregulated. There were also changes in TAG levels in rosacea patients. The majority of the TAGs examined were upregulated in rosacea patients in comparison to healthy patients, however the authors noted that there were some TAG lipids that were downregulated⁶². A study by Ní Raghallaigh et al. (2012) investigated the fatty acid profile of the skin surface in rosacea patients⁶³. The myristic acid levels in rosacea patients were significantly higher in comparison to controls, however a downregulation in arachidic acid, behenic acid, tricosanoic acid, lignoceric acid and *cis*-11-eicosenoic acid was observed in rosacea patients.

1.4.3 Acne Vulgaris

Acne vulgaris is a persistent chronic skin inflammatory condition that affects more than 85% of teenagers. It is characterised by non-inflammatory or inflammatory lesions like papules and pustules, observed on the face, neck, upper back and chest. The skin lipidome has been shown to have a role in the development of acne. TAGs, FFAs, DAGs and ceramides were all elevated in moderate acne in comparison to mild acne. The increase in FFA, TAG and DAG content is indicative of membrane phospholipid breakdown, as all three lipid species are products of phospholipid breakdown. Phospholipids are a major substrate for lipid peroxidation, indicating that lipid peroxidation is present in skin affected by severe

acne. Additionally, there was shorter average fatty acid chain length in patients with moderate acne in comparison to mild acne⁶⁴. In comparison with healthy patients, there was an increased abundance of glycerolipids, glycerophospholipids and FFAs in acne patients. Acne patients also exhibited a reduced average ceramide chain length, as well as an increase in the levels of TAGs and PI⁶⁵. In infantile acne, the abundance of TAGs, ceramides and unsaturated fatty acids were increased, while the abundance of DAGs were decreased in comparison to healthy controls⁶⁶. Lipidomic analysis involving 51 acne patients and 38 healthy control patients indicated that there were differences in the skin lipid profile between the two cohorts⁶⁷. Ceramides, CL, PA, PC, PE, PI, SM and TAGs were significantly elevated in patients with acne, while DAGs and phosphatidylglycerol levels were decreased. There were no differences in the levels of cholesterol between acne patients and healthy patients. There was also an increase in the ratio of linoleic acid to linolenic acid in the skin surface lipids of acne patients⁶⁷. Both FFAs contribute to the anti-inflammatory properties in the skin, and it is hypothesised that their imbalance can lead to increased skin inflammation and an increase in the severity of acne.

1.4.4 Atopic Dermatitis

Atopic dermatitis (AD) is a type I hypersensitivity reaction mediated by immunoglobulin E (IgE). It is characterised by pruritus, lichenification and xerosis. Lipid changes in atopic dermatitis are very well characterised. A study by Emmert et al. (2021) reported that there was an increase in total lipid levels in the skin of patients with atopic dermatitis in comparison to healthy skin, with an increase in total ceramides, FFAs and cholesterol sulfate shown⁶⁸. In AD lesions, long chain saturated FFAs were downregulated, while arachidonic acid and other unsaturated fatty acids were increased in AD patients⁶⁹. The increase in arachidonic acid is likely due to the increases in inflammation seen in AD. An increase of short chain unsaturated FFAs is also indicative of phospholipid breakdown, which is characteristic of lipid peroxidation. In addition, an increase in short chain FFAs and a decrease in long chain FFAs has been observed in AD skin¹⁹.

A study by Berdyshev et al. (2018) reported an overall increase in short-chain lipid species in AD lesions, including CER [NS] with short chain fatty acids, sphingomyelins with short

chain fatty acids, and lysophosphatidylcholine species with short chain fatty acids⁷⁰. In addition, long chain ceramide species and SM species were reduced. Further investigation revealed that the lipid changes were driven by Th2 cytokines including interleukin-4 (IL-4) and interleukin-3 (IL-3), which were mediated by signal transducer and activator of transcription 6 (STAT6). STAT6 is a transcription factor and silencing of it reversed the lipid changes observed with IL-4 and IL-13. The increase in short chain lipid species and decrease in long chain lipid species was driven by Th2 cytokines, suggesting that Th2 cytokines contribute to impaired barrier function and increased permeability in atopic dermatitis⁷⁰. Topical application of lipid-based treatments has been used to restore barrier function in mild and moderate AD. These topical treatments included ceramide- fatty-acid- and cholesterol-based formulations, and have been shown to restore the structure of the skin barrier, improve disease severity and reduce pruritus^{71, 72}.

In contrast, the lipid changes in irritant contact dermatitis (ICD) and ACD are not as well characterised. Current research shows that there may be an increase in linoleic acid and arachidonic acid metabolites present in ICD or ACD, with no overall change in free fatty acid levels, as well as a decrease in overall ceramide content^{73, 74}. More extensive research into lipid changes in contact dermatitis is required to characterise these lipid changes further.

1.5 Allergic Contact Dermatitis

ACD is a delayed type IV hypersensitivity reaction, which is brought on by physical contact with a compound or substance that the individual is allergic to, at a concentration that exceeds the individual's threshold⁷⁵. ACD affects approximately 20% of the general population⁷⁶, and its prevalence is estimated at 16.5% in the paediatric population⁷⁷. Over 4000 contact allergens have been identified⁷⁸.

Common contact allergens can be classified into five categories⁷⁹:

1. Natural rubber – most commonly found in rubber gloves
2. Metal based - nickel, cobalt, chromium
3. Fragrance based - linalool, eugenol, limonene and other fragrance terpenes
4. Preservative based – formaldehyde and methylisothiazolinone (MI)

5. Additive/dye based - *p*-phenylenediamine, coal tar

Nickel is the most common contact allergen, affecting approximately 10% of the population, due to its widespread uses in the manufacture of jewellery, silverware and other common metal appliances^{80, 81}. Balsam of Peru is the most common fragrance contact allergen, and is used in food and drink for flavouring, in perfumes and cosmetics for fragrance, and in medicine for its healing properties⁸². Other common fragrance allergens include limonene and linalool, which are present in skin care, fragrance and cleaning products. Limonene and linalool undergo auto-oxidation with air to form their respective hydroperoxides, which have a higher sensitization potency than their parent compounds.

1.5.1 Mechanism of Allergic Contact Dermatitis

ACD is a delayed type IV hypersensitivity reaction which is mediated by antigen specific T lymphocytes. The currently accepted model of ACD involves two different stages: the sensitization phase and the elicitation phase⁸³ (Figure 1.11).

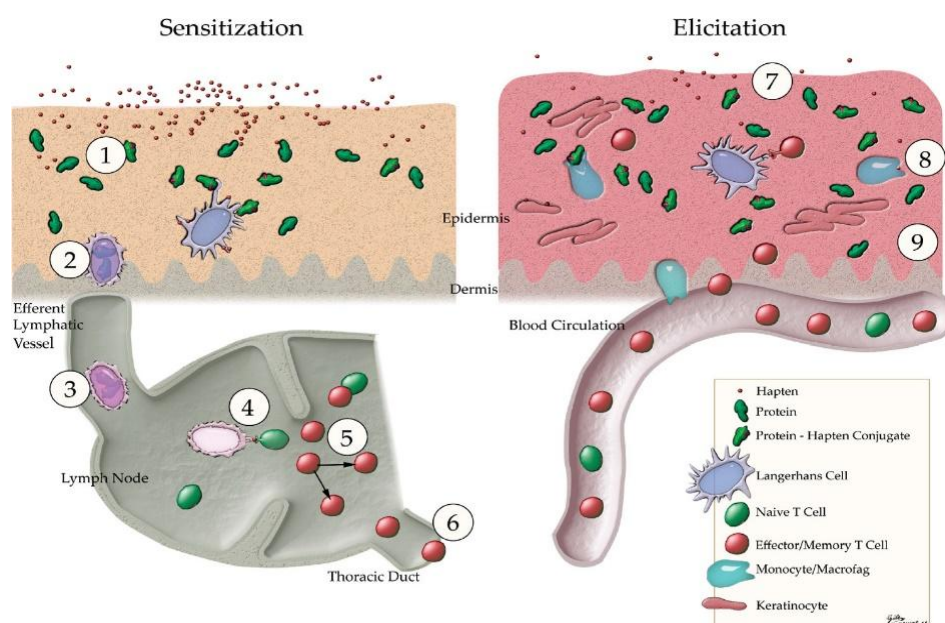


Figure 1.11 Mechanism of ACD, showing the sensitization phase (1-6) and elicitation phase (7-9). (1) Binding of haptens to proteins (2) Internalization of hapten-modified proteins. (3) Activation of Langerhans cells (LCs) by allergen-protein processing of hapten-protein complexes. (4) Presentation of antigens by LCs to naive specific T-cells. (5) Proliferation of antigen specific T-cells and formation of memory T-cells. (6) Hapten-specific memory T-cells leave the lymph node and enter the circulation. (7) Re-exposure to the hapten. (8) Release of cytokines and chemokines attracting cells to the skin from the circulation. (9) Inflammatory response within 24–48 h leading to symptoms of ACD⁸³.

1.5.1.1 The Sensitization Phase

To develop ACD, the patient must become sensitized to a compound, during the sensitization phase. The first step is exposure of the patient to an allergen, and the penetration of the allergen into the skin. For an allergen to do this, the molecule must be small (molecular weight less than 1000) and have appropriate lipophilicity ($\log P \sim 2$). Once the allergens have penetrated through the epidermis, they are too small to induce an immunological reaction on their own, and so they react with and bind to macromolecules, which are currently understood to be proteins [Figure 1.11 (1)]. In doing this, the allergen becomes haptenized (forming an allergen-protein conjugate)¹³. Haptenation occurs when an electrophilic molecule in the allergen covalently binds to a side chain of a nucleophilic amino acid in a protein, typically a cystine, lysine, histidine, arginine or tyrosine residue⁸⁴. This allows the allergen to become visible to the immune system and initiate further immunological events that result in a hypersensitivity reaction. This covalent binding between the allergen and protein can happen through various different chemical mechanisms, including Michael addition reactions, Schiff base formation, acylation, S_N2 reactions and S_NAr reactions⁸⁴. Cinnamaldehyde can act as both a Michael acceptor sensitizer and a Schiff base forming sensitizer, isocyanates act as acylating agents, Alkyl halides act as S_N2 electrophiles, and 2,4-dinitro-1-chlorobenzene (DNCB) acts as a S_NAr electrophile. In depth analysis on the modification of proteins by DNCB, a potent skin sensitizer, showed that approximately 5% of the proteome was haptenated, with many structural proteins and stress-response proteins acting as targets for haptenation⁸⁵. Also present in the epidermis are antigen presenting cells called Langerhans cells (LC). The allergen-protein conjugate is taken up by LCs and processed [Figure 1.11 (2)], with the antigens displayed on their surface by a class of major histocompatibility complexes called MHC Class II molecules¹³ [Figure 1.11 (3)]. The LCs then enter the lymph vessels and migrate to the lymph nodes where they present the processed allergen-protein conjugate (the antigen) to naïve T cells [Figure 1.11 (4)], which leads to the proliferation of effector and memory T cells specific for the allergen-protein conjugate, [Figure 1.11 (5)] which then

enter the lymph and blood circulation [Figure 1.11 (6)]. CD1a is also present on LCs and can present lipid antigens to naïve T cells. While there are no published studies investigating hapten-lipid binding, activation of the CD1a pathway by skin sensitizers suggests an interaction between contact allergens and lipids⁸⁴. CD1a has been shown to present small contact allergens like farnesol, benzyl cinnamate and urushiol to T cells⁸⁶.

1.5.1.2 The Elicitation Phase

To induce an inflammatory response, the patient must be re-exposed to the allergen [Figure 1.11 (7)]. Upon re-exposure, chemokines and cytokines are synthesised and released, drawing the memory and effector T cells from the circulation to the skin, at the site where the allergen was applied [Figure 1.11 (8)]. The allergen, bound to protein and processed by LCs, activates the T cells which induce a vigorous inflammatory response localised to the epidermis [Figure 1.11 (9)]. Activation of these Naïve T-cells requires two signals, signal 1 is T cell receptor recognition by the allergen-protein conjugate presented by MHC complexes, and signal 2 is described as a 'warning' signal, provided by APCs via CD80 and CD86 activation to produce cytokines like IL-1 β and IL-6. T cells will not be active without co-stimulation with signal 2. The activation of naïve T cells leads to the production of pro-inflammatory cytokines like tumour necrosis factor (TNF) and interferon- γ (IFN- γ) results in the presentation of ACD symptoms like itchiness, a rash, blistering and swelling⁸⁷. Current treatments for ACD include steroids and anti-inflammatories used to inhibit these pro-inflammatory cytokines and reduce inflammation.

1.5.2 Symptoms of Allergic Contact Dermatitis

The symptoms of ACD can be difficult to distinguish from ICD and other inflammatory skin conditions but generally consist of a well-demarcated pruritic rash that can turn weepy, as well as blistering and erythema. These symptoms are usually accompanied by a severe itch⁸⁸. The presentation of symptoms can differ depending on the location, for example ACD rash on the hands and the eyelids can differ slightly, but both will still be accompanied by severe itch as well as some pain or stinging. ACD can have a significant effect on quality of life, with a change of career often necessary in cases of occupational dermatitis.

1.5.3 Treatment of Allergic Contact Dermatitis

The primary treatment for ACD involves identifying the allergen responsible and avoiding it to prevent future outbreaks. An ACD diagnosis is confirmed through patch testing using one or more series of the 30 most frequent contact allergens, diluted in petroleum or water, or often with the patient's own products if one is suspected of causing an allergy (Figure 1.12)⁸⁹. Presence of an eczema-like rash at a test site indicates that the patient is allergic to the compound that was applied at this site. This a well-researched and trusted diagnostic method that has been used for well over 50 years.

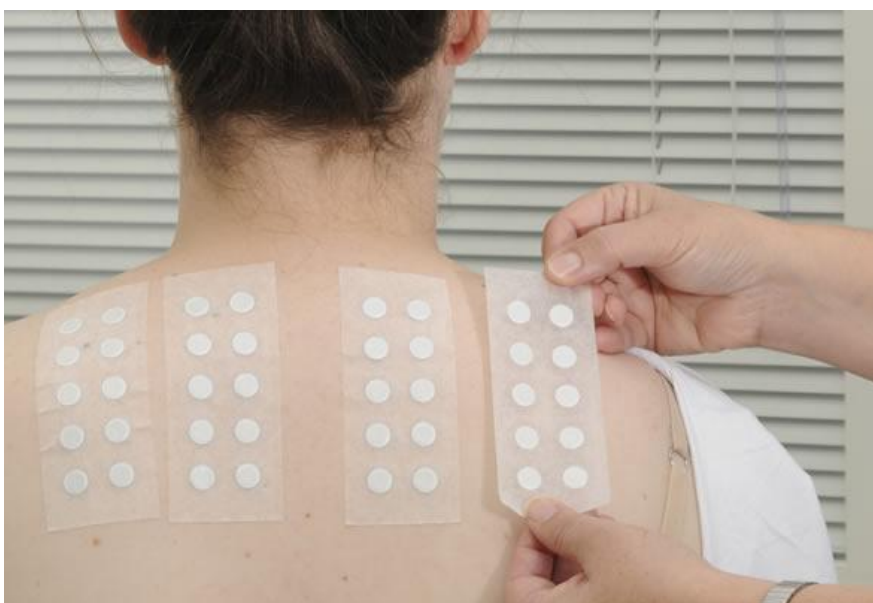


Figure 1.12 Patient undergoing an ACD patch test⁹⁰

Following on from avoidance, the next step in management of ACD is to treat the patient's symptoms. Sometimes avoidance is not enough to fully control ACD symptoms, with a study by Pryce et al (1989) showing that 70% of machinists continued to have symptoms of contact dermatitis for two years after diagnosis, regardless of whether they changed jobs to avoid the allergen⁹¹. Another study showed that 70% of patients with a diagnosed chromate allergy continued to show dermatitis for years after avoiding chromium⁹². Itching

is the most pressing symptom of ACD, as a chronic itch will lead to scratching of the lesions and increase the risk of an infection. Topical anti-pruritics or oral antihistamines can be used to relieve itching and sooth the rash. The rash itself can be treated with topical glucocorticoids which inhibit pro-inflammatory cytokines and downregulate the inflammatory response.

1.5.4 Metals in Allergic Contact Dermatitis

The rate of metal allergy is increasing rapidly worldwide due to the ubiquitous nature of metals such as nickel, cobalt and chromium in our everyday environment⁹³. Metal exposure can come from various routes including cutaneous exposure from contact with common everyday objects like jewellery and cosmetic products, and inhalation of metal dusts in high-risk occupations such as construction and metal workers⁹⁴. Additionally, exposure can come from the oral route, with many foods being high in metals such as nickel and palladium, as well as the release of sensitizing metals from dental implants. Nickel, chromium and cobalt are the most common metals that cause ACD, however ACD cases have also been reported with less common metals, such as an allergy to headphones that contained gold plated metal parts⁹⁵, and a cutaneous reaction to aluminium salts found in vaccines⁹⁶. Sensitization to metals occurs when electrophilic metals ionize and react with proteins, forming complexes that are processed by DCs and presented to T cells, inducing an inflammatory response⁹⁷.

Nickel is the most common contact allergen, with an 8-19% prevalence in the general population in Europe⁹⁸, with 1-3% of the population being allergic to cobalt or chromium⁹³.

1.5.4.1 Nickel

Nickel ACD is the most common cutaneous delayed-type hypersensitivity reaction worldwide. In 1994 the European Union introduced the Nickel Directive to regulate and minimize the levels used in the manufacture of jewellery and other metal objects and reduce the prevalence on nickel allergy⁹⁹. Since its introduction, the prevalence of nickel allergy has seen a significant reduction, from 19.8% in women aged 18-35 before implementation of the directive, to 11.4% following the implementation⁸¹. Nickel release is

unregulated in the United States, leading to a 20.1% prevalence of nickel sensitization¹⁰⁰. Nickel allergy has a high prevalence in children and adolescents with 10% of this population developing the allergy at some point in childhood¹⁰¹. This is due to the high rate of nickel exposure from common everyday appliances like jewellery, belt buckles and cutlery/cookery ware. Additionally, nickel allergy has a strong female dominance, with women being up to ten times more likely to develop nickel allergy than their male counterparts (8-15% prevalence for women vs 1-3% for males)¹⁰². Nickel allergy also appears to be more prevalent in overweight individuals. A study by Lusi et al. (2015) concluded the prevalence of nickel allergy in women with a BMI > 26 kg/m² to be 59.7%, in comparison to the 8-15% prevalence rate in the general population¹⁰³. Further research by Watanabe et al (2018) associated this increase in prevalence with a decrease in metabolic functions, such as an impairment in glucose homeostasis, higher insulin and higher HbA1C levels leading to an increase in inflammation and C-reactive protein levels, as well as hormonal dysregulation due to an impaired GH-IGF-1 axis function. Allergic patients showed lower baseline growth hormone (GH) and insulin like factor-1 (IGF-1) levels in comparison to their non-allergic counterparts, attributed to hyperinsulinism which suppresses GH production via direct action at the pituitary gland to inhibit GH synthesis and release¹⁰⁴.

Its widespread use means that there are many routes of nickel exposure including:

1. Occupational – construction workers, car mechanics, electricians, cleaners and nurses are just some of the occupations associated with nickel allergy due to use of work tools, electrical components, dental tools, dermatoscopes and cutting fluids¹⁰⁵
2. Consumer – via jewellery, metal on clothing like zips and buttons, toys, coins, utensils and belts^{106, 107}
3. Diet – nickel occurs naturally in drinking water and in various foods like chocolate, grains, nuts and canned food¹⁰⁸
4. Surgical implants – metallic orthopaedic and cardiovascular implants can release nickel ions into the systemic circulation¹⁰⁹

5. Dental materials – braces, fillings and retention wires are often made from metal alloys containing nickel, with the corrosive effect of the oral environment thought to increase nickel release. Due to oral tolerance, contact dermatitis from oral metals can present on other locations on the body, most frequently the hands¹¹⁰.

There have been many developments in the understanding of the mechanism behind nickel sensitization. The first obstacle in nickel sensitization is nickel's poor penetration through the skin, and in particular its very slow diffusion through the SC¹¹¹. Research has shown that nickel may penetrate through the SC through three routes; transcellular, intercellular or appendageal¹¹², however there is limited knowledge on the proportion of penetration through each route. Nickel is strongly chelated by histidine-rich filaggrin proteins, which are thought to slow SC penetration¹¹³, as well as a reaction with sweat to form divalent nickel ions that can penetrate through all three routes¹¹⁴. Time of flight secondary ion mass spectrometry (ToF-SIMS) has shown that nickel ions are mainly confined to the SC and that very little nickel penetrates through to the other layers of the epidermis, in comparison with cobalt and chromium ions which are present throughout the epidermis to a much higher extent¹¹⁵. TLR4 has been shown to have a crucial role in the sensitization process against nickel. Along with the main pathway for sensitization described in Figure 1.13, an additional secondary pro-inflammatory stimulus is required for efficient sensitization, which in turn upregulates extracellular matrix proteins that act as endogenous ligands for toll-like receptors. The activation of these receptors triggers the release of pro-inflammatory cytokines which promote the acquisition of skin sensitization. However, nickel has been shown to completely bypass the need for a secondary stimulus, due to its ability to bind directly to TLR4 with this mechanism instead acting as the secondary stimulus¹¹⁶. Nickel penetrates the SC and binds directly to TLR4 on DCs, in particular to two imidazole groups on the histidine residues His456 and His458¹¹⁷, initiating the IKK2-nuclear factor- κ B cascade leading to the production and release of cytokines and chemokines like IL-6 and IL-8 by dendritic cells and keratinocytes, delivering signal 2 to T cells¹¹⁸. His 456 and His458 are essential for TLR4 receptor dimerization. Nickel induces TLR4 receptor dimerization, and while

bacterial lipopolysaccharides (LPS) require myeloid differentiation protein 2 (MD2) to induce dimerization, a recent study concluded that nickel induces receptor dimerization independently of MD2¹¹⁹. Activated DCs migrate to the lymph nodes and nickel is presented to naïve T cells by major histocompatibility complex II (MHC II) molecules. In contrast to organic contact allergens which form stable covalent bonds with MHC-II molecules, nickel and other metal ions interact with nitrogen or oxygen in amino acid side chains on MHC II molecules to produce non-covalent coordination protein-metal complexes¹²⁰. Nickel is presented by the MHC II complex to the CD4+ T cells, leading to the production of skin-resident memory T cells which are activated upon re-exposure to nickel leading to the induction of an inflammatory response¹²¹. The involvement of TLR4 in nickel allergy remained undiscovered for a long period of time, due to the structural differences in the TLR4 molecule in animal models. Mouse TLR4 lacks the His456 and His458 residues critical for coordination with nickel ions¹²². Due to this, nickel cannot induce TLR4 dimerization in mice, and thus signal 2 is not produced. The absence of signal 2 means that naïve T cells are not fully activated, and sensitization is often ineffective in mice.

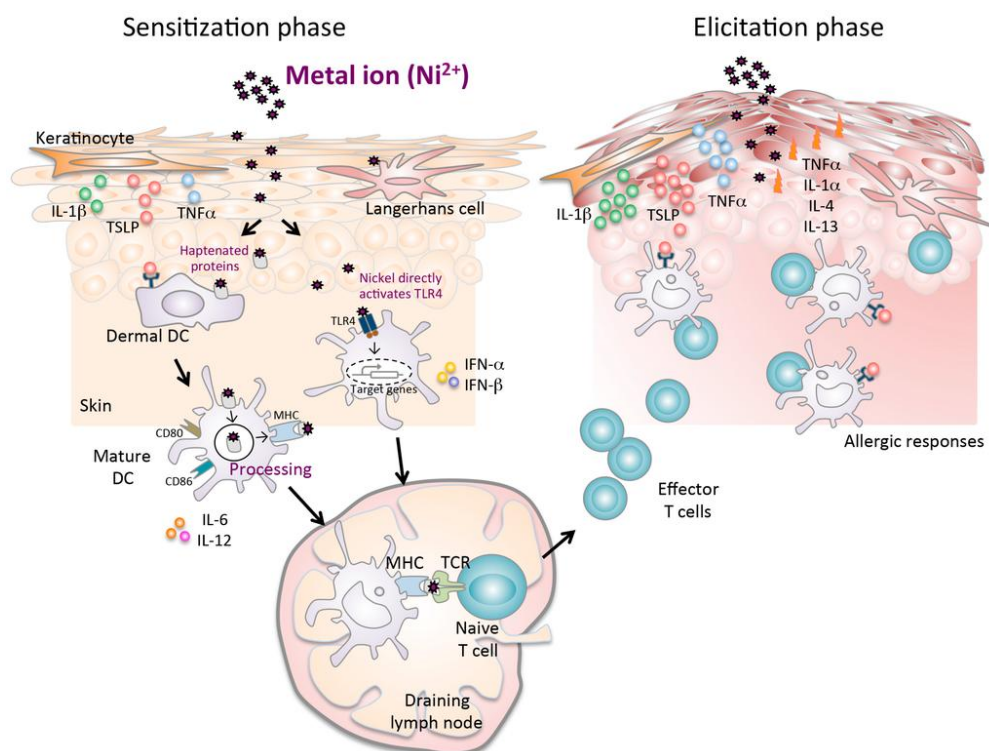


Figure 1.13 Mechanism of nickel allergy¹²³ In the sensitization phase, nickel penetrates into the skin and results in the production of pro-inflammatory cytokines, Thymic Stromal Lymphopoietin (TSLP) and chemokines, which activate DCs that migrate to the lymphatic system and process metal-protein haptens. Additionally, in humans nickel directly activates the toll-like receptor-4 (TLR4) pathway in DCs. DCs in the lymph node present the haptened peptide to naïve T cells, leading to the differentiation of hapten specific T cells, which migrate to the skin. Re-exposure to nickel in the elicitation phase leads to the activation of hapten specific T cells which produce pro-inflammatory cytokines, promoting the symptoms of ACD.

Other characteristic biomarkers of nickel allergy included an increase in metal-specific CD154⁺ and CD4⁺ memory T cells¹²⁴ and increase in interleukin-5 production and other cytokines released from type 1, 2, 9, 17 and 22 cells¹²⁵, an increase in Semaphorin 3A which activates mitogen-activated protein kinase (MAPK) and TNF¹²⁶, and changes in lipid profile of the skin including a decrease in DAGs and MAGs¹²⁷. While nickel has shown to induce secretion of many pro-inflammatory cytokines, nickel allergy has been shown to be mainly T_H1 mediated¹²⁸.

Nickel is classed as having weak sensitization potential¹²⁹, however there is a scarcity of information demonstrating its potency due to the unsuitability of in-vivo assays owing to the difference in histidine residues of TLR4 receptors in animal models. Guinea pig sensitization assays were initially the most common method to determine skin sensitization

potency, in which the metal is topically applied or injected intradermally at regular intervals for two to three weeks, followed by a rest period of one or two weeks, and finally the topical re-application of the metal to determine if skin sensitization has taken place. The assay originated from Magnusson and Kligman¹³⁰ who indicated a 10% positivity rate for nickel (II) sulfate (NiSO_4) skin sensitization, with optimized assays reporting positivity frequencies of up to 55%¹²⁹. This assay firmly places nickel as a category 1B sensitizer according to the potency categorization of skin sensitizers guidelines from the United Nations Globally Harmonized System¹³¹. 1B sensitizers are classified as weak to moderate sensitizers which elucidated a 15-60% positivity rate to <20% topical induction dose assessed by the guinea pig sensitization assay. The local lymph node assay (LLNA) is a more recent in vivo method for characterization of skin sensitizers. A study by Basketter et al (1999)¹³² used the LLNA to determine the skin sensitization potential of metal salts, with a nickel (II) chloride salt giving a positive dose response, however the result was not at a level high enough for classification as a skin sensitizer. However, a further non-validated LLNA study with a non-radioactive endpoint indicated a positive result, with an effective concentration 3 (EC3) value of 3.5% after interpretation of the Organisation for Economic Cooperation and Development (OECD) regulatory standard LLNA results¹³³. A 3.5% EC3 value identifies nickel as a 1B sensitizer, or a low to moderate sensitizer, which reinforces the results from the guinea pig sensitization assay and confirms nickel's status as a contact allergen.

1.5.4.2 Cobalt

Cobalt is a chemical element that presents as a hard silver-white metal and is found naturally in the Earth's crust in a chemically combined form¹³⁴. Cobalt compounds have been used for centuries to impart a rich blue colour to glass, glazes and ceramics, going as far back as Egyptian sculpture and Persian jewellery from the third millennium BC. These cobalt compounds include cobalt blue, cerulean and new blue. Cobalt is also commonly used to form alloys used in the production of jet engines, gas turbines, and some types of stainless steels. Cobalt allergy affects 2% of the general population¹³⁵ and common sources of contact allergy in recent times include¹³⁶:

1. Jewellery
2. Metal household items such as cutlery, coins, keys
3. As a dye in cosmetics, leather, clothing, shoes and hair dye
4. Medical devices such as orthopaedic implants, pacemakers, and stents
5. Dental alloys
6. Paints and ceramics

Cobalt is also a common ingredient of blue pigmented tattoo inks, which have been responsible for an increasing rate of contact dermatitis reactions¹³⁷. One study by Bagnato et al (1992) documented a case of a patient who developed a contact urticaria reaction around a tattoo, with patch testing revealing a positive test for cobalt chloride that was used in the blue inks of the patient's tattoo¹³⁸. Like other metal allergies, cobalt allergy is more prevalent in women than in men¹³⁹ with twice as many women as men developing sensitization to cobalt. Additionally, a study noted that prevalence of cobalt allergy is higher in patients of colour than in Caucasian patients, with 9.2% of black patients having sensitization to cobalt chloride in comparison to 6.6% of white patients. However, the authors note that while this increased rate of sensitization could be due to genetic differences related to race, it is more likely that this is due to differences in exposure to cobalt determined by race/ethnicity¹⁴⁰. Following the introduction of the EU Nickel Directive in 1994⁹⁹ the prevalence of cobalt allergy was expected to increase as it was thought that cobalt would replace nickel in consumer items, with many studies comparing the rates of allergy before and after implementation of the directive carried out in countries around Europe. However, the prevalence of cobalt allergy has fluctuated. In one study carried out in Sweden, there was a significant decrease in cobalt allergy following implementation of the nickel directive, with the prevalence in patients between the ages of 6 and 30 decreasing from 11.9% between 1995-1999 to 5.3% between 2012-2016, with similar declines observed for older populations¹⁴¹. However, in Denmark, a study reported that the prevalence of cobalt allergy increased significantly in women following implementation of the nickel directive, from 3.7% in 1992-1997 to 5.1% in 2004-2009, while

the prevalence rates in men did not undergo significant fluctuations in the same ten-year period¹⁴².

The sensitization mechanism involved in cobalt sensitization is similar to the mechanism involved in nickel sensitization described in Figure 1.13, in which cobalt ions bind to and are processed by DCs, and then form protein-metal coordination complexes with MHC-II which present the antigen to CD4+ T cells, resulting in the production of antigen specific memory T cells which are activated upon subsequent re-exposure and initiation of the elicitation phase¹⁴³. In addition, following the discovery of nickel's ability to bind directly to TLR4, it was discovered that cobalt too is a potent activator of DCs, identified by its ability to produce similar levels of interleukin-8 (IL-8) secretion to nickel¹⁴⁴. In addition, it was also concluded that, alongside nickel, cobalt induces TLR4 receptor dimerization independently of MD2¹¹⁹. However, in contrast to nickel which has very poor penetration through the SC, cobalt penetrates extensively through the SC and reaches the VE, with ToF-SIMS showing that after application of cobalt chloride to skin samples, cobalt is mainly located deep in the VE, in comparison to nickel which is found mainly in the SC¹¹⁵. A recent study reinforced the increased penetration capacity of cobalt salts, by concluding that a correlation can be drawn between cobalt skin exposure and cobalt levels in urine, indicating strong penetration through the SC and deeper layers of skin¹⁴⁵. In addition, cobalt is a more potent contact allergen in comparison to nickel. In the murine LLNA, cobalt sulfate was reported to have an EC3 value of 0.2%¹⁴⁶, which categorizes it as category 1A extreme sensitizer according to the United Nations Globally Harmonized systems classification guidelines, in comparison to nickel which sits in category 1B as a weak to moderate sensitizer.

1.5.4.3 Chromium

Chromium is a lustrous, silver-grey metal known for its strong chemical resistance, and extreme hardness. It is widely regarded as the strongest metal on earth, sitting between 8.5 and 9 on the Mohs hardness scale¹⁴⁷. Its resistance to corrosion promotes its use in nickel and iron alloys, and as an ingredient in stainless steels¹⁴⁷. Chromium is also commonly found in chromate salts as one of three chromium ions: chromium (II), chromium (III) or chromium (VI). Chromium (III) compounds such as chromium (III) chloride and chromium

(III) acetate are the most stable chromium salts, in comparison to their chromium (II) and (VI) counterparts. Trivalent chromium (Cr^{3+}) is a deep green colour and is often used as a colourant in inks, glasses, paints and ceramics, as well as for plating purposes. A chromium layer is typically applied to steel, copper, brass or plastic to give it a bright and shiny appearance. Trivalent chromium salts are the most commonly used chromium salts in plating¹⁴⁸. Hexavalent chromium (Cr^{6+}) compounds are powerful oxidising agents, and thus possess a considerable degree of toxicity and carcinogenicity¹⁴⁹. They are commonly used in industrial settings which can cause negative health consequences for those who live nearby. One study examined the effects of chromium exposure on villagers who lived near a chromium alloy plant, who had drunk water from a contaminated water well and suffered effects such as oral ulcers, diarrhoea, abdominal pain, vomiting and leukocytosis¹⁵⁰.

There are many sources of chromium exposure, the most historically important of which is cement¹⁵¹. Cement contains hexavalent chromium which has caused contact dermatitis in cement workers. EU and UK Regulations limiting the chromium content of cement have reduced the rate of cement induced dermatitis¹⁵², however prevalence has remained high amongst other countries. Analysis in Australia concluded that a minimum of 24 cases of chromium ACD could be attributed to cement exposure per year, and the prevalence rate has not changed over twenty years¹⁵³, in comparison to Europe which has seen a steady decline.

Leather is another significant source of chromium exposure, with 90% of leather produced globally containing trivalent chromium to provide softness, durability and flexibility, and also containing hexavalent chromium impurities. A European wide regulation restricting the content of hexavalent chromium in leather goods was implemented in 2014¹⁵⁴, however a recent study concluded that no immediate sign of improvement in chromium allergy had been seen in the first five years following implementation of the regulation¹⁵⁵. Recent analysis on the chromium levels in leather goods in Denmark found that there was an average chromium concentration of 41 mg/kg in leather goods¹⁵⁶ [equivalent to approximately 394 μM potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$)]. This level of chromium found in Danish leather goods is higher than the 3 mg/kg maximum limit of Cr(VI) content in leather

regulated by the European Union. Finally, mobile phones have become an increasingly relevant source of chromium allergy, with many components of mobile phones plated with chromium. The first case of mobile phone induced chromium allergy was reported in 2002¹⁵⁷, but a recent review of mobile phone dermatitis published in 2014 has shown that this is still a pertinent issue, with 10 out of 27 published patient cases reporting a positive patch test to potassium dichromate¹⁵⁸.

Like cobalt, chromium can extensively penetrate the SC¹¹⁵, however the exact extent of permeation depends on the oxidation state of chromium. Cr⁶⁺ passes more easily through the SC and VE than Cr³⁺, possibly due to the ability of Cr⁶⁺ to form stable complexes within the epidermis, or a higher rate of rejection of positive Cr³⁺ ions by the skin barrier¹⁵⁹. However, while Cr⁶⁺ is more toxic and can better penetrate the epidermis, it is incapable of reacting with proteins to form a hapten. It is first reduced to trivalent chromium within keratinocyte cells¹⁶⁰, which is widely accepted to be the chromium compound that forms a complex with proteins. As with the sensitization mechanisms for nickel and cobalt, the complex is processed by LCs and then forms a non-covalent protein-metal coordination complex with MHC-II which present the antigen to CD4+ T cells, resulting in the production of antigen specific memory T cells which are activated upon subsequent re-exposure and initiation of the elicitation phase (Figure 1.14)¹⁶¹. In addition, a secondary stimulus upregulates extracellular proteins that act as ligands for toll-like receptors, which upon activation trigger the release of pro-inflammatory cytokines¹⁶². After the discovery of nickel's direct involvement with TLR4, numerous other studies have taken place to investigate similar mechanisms induced by other allergenic metals, with one study confirming that neither Cr⁶⁺ or Cr³⁺ have the same level of potency for DC activation that both nickel and cobalt possess, suggesting that chromium could depend on microbial or tissue-derived cofactors to induce contact sensitization¹⁴⁴. Due to Cr⁶⁺'s high toxicity, even though it is unable to form complexes with proteins, its ability to cause cell death via ROS formation has prompted discussion on ROS involvement in ACD. The exact mechanism behind ROS involvement in Cr⁶⁺ hypersensitivity mechanisms has yet to be fully elucidated, but it has been shown that ROS production induced by hexavalent chromium can increase

interleukin-1 α (IL-1 α) and TNF production and has suggested that the administration of antioxidants could prevent chromium hypersensitivity through inhibition of ROS production¹⁶³. The most complete picture of Cr⁶⁺ sensitization yet has been given by Buters et al. (2017), who concluded that Cr⁶⁺ induced ROS can activate the NRLP3 inflammasome resulting in IL-1 β release, which could potentially activate antigen presenting DCs that process the reduced Cr³⁺ protein hapten and hence activate the production of chromium-specific T cells¹⁶⁴ (Figure 1.14).

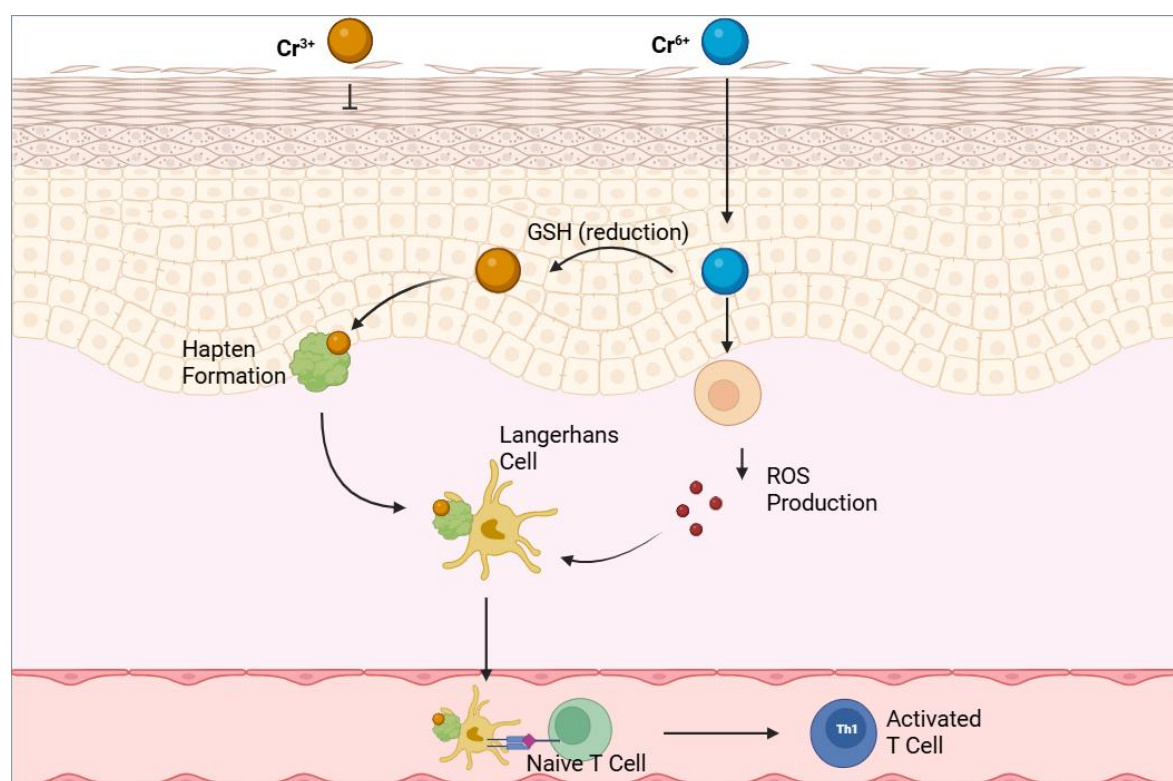


Figure 1.14 Mechanism of chromium sensitization. Cr⁶⁺ penetrates through the skin and follows two sensitisation pathways. It is reduced by antioxidants like glutathione (GSH) to Cr³⁺ which forms a hapten with a protein and activates Langerhans cells. Additionally, Cr⁶⁺ can induce reactive oxygen species (ROS) production in skin keratinocytes and ROS can then activate Langerhans cells.

This mechanism, so far, appears to be specific to Cr⁶⁺, as the authors noted that neither Cr³⁺ nor nickel (II) salts activated this pathway.

Chromium salts are very potent skin sensitizers, with many studies categorising potassium dichromate as an extreme sensitizer¹⁶⁵. In the guinea pig sensitization test reported by

Ikarashi et al¹⁶⁶, there was a 60% sensitization rate to 0.25% potassium dichromate, and a 100% sensitization rate at concentrations of 0.5% and 1% (84). This puts potassium dichromate firmly in the 1A extreme sensitizer category according to the United Nations Globally Harmonized systems classification guidelines¹³¹. Potassium dichromate produced an EC3 value of 0.08% in the LLNA¹⁶⁷, reinforcing its position as an extreme sensitizer, which encompasses all sensitizers with an EC3 value of less than 0.1%.

While protein-mediated mechanisms underlying metal-induced ACD have been extensively studied, the contribution of lipids to ACD pathophysiology remains poorly understood.

1.6 Lipid Changes Induced by Allergenic Metals

A recent study was carried out to examine lipid changes in ex vivo human skin samples treated with allergenic metals nickel (II), cobalt (II) and chromium (III) with each metal ion inducing changes in lipid composition¹²⁷. The most significant changes were seen in MAGs and DAGs in the SC and VE. Cobalt and chromium induced increases in the content of DAGs in the SC, with cobalt ions also inducing increases in MAGs in the VE. As well as the increases in DAGs in the SC, chromium also induced an increase in DAGs in the VE. In contrast to the effects of chromium and cobalt, nickel (II) ions resulted in a decrease of MAG and DAG levels in the VE.

A study using matrix-assisted laser desorption/ionization (MALDI) to investigate the penetration of nickel within porcine ear skin and its effect on lipids found that nickel was mainly retained in the SC and led to decreases in the levels of arginine and ceramide lipid species in the SC, as well as a downregulation in the levels of phospholipid and SM species in the VE and dermis layers of the skin¹⁶⁸. The lipid species affected are crucial for maintaining skin structure and function and their downregulation indicates that nickel compromises the skin barrier function. The lipid changes observed in lower layers of the skin, despite nickel's confinement to the SC indicated that response mechanisms are not dependent on direct contact with nickel ions. The decrease in PC and SM levels in the VE and dermis suggest that they could be degraded to bioactive lipids such as ceramides and FFAs to restore skin barrier function or trigger further immune responses.

Although skin lipids are known to play critical roles in maintaining barrier integrity and regulating immune responses, their involvement in ACD is not well defined. In particular, how allergens alter skin lipid composition, the mechanisms driving these changes, and the downstream impact of lipids on antigen presentation and immune activation remain poorly characterised. Addressing these gaps is essential to advance our understanding of the pathophysiology of ACD and provides the foundation for the investigations undertaken in this thesis.

1.7 Thesis Aims

The aims of thesis are:

1. To investigate the role of lipids in the pathophysiology of metal ACD by assessing whether allergenic metals can induce lipid peroxidation, using both a simplified single-lipid model and a keratinocyte cellular model.
2. To investigate the mechanisms of cell death triggered by metal allergen toxicity, with a focus on the potential role of lipid peroxidation.
3. To explore the role of lipid-antigen presentation molecules CD1a and CD1d in the immunological mechanisms underlying metal ACD, thereby evaluating their potential contribution to antigen recognition and immune activation.
4. To examine the distribution and impact of low-molecular weight organic allergens within the skin by employing imaging mass spectrometry techniques, with a

particular focus on their localization, interaction with skin lipids, and consequent effects on skin lipid composition.

Chapter 2: Induction of Lipid Peroxidation by Metal Allergens

2.1 Introduction

The first step in establishing the role of skin lipids in the pathophysiology of ACD was to investigate the ability of metal allergens to induce lipid peroxidation. Lipid peroxidation is the chemical reaction between lipids and ROS that are produced through enzymatic and non-enzymatic pathways during normal metabolism^{169, 170}. Polyunsaturated fatty acids (PUFAs) are particularly vulnerable to being attacked by oxygen-containing ROS including hydrogen peroxide (H_2O_2), superoxide, and hydroxyl radicals ($\cdot\text{OH}$) which can lead to cell membrane structural damage, further cellular dysfunction and widespread tissue damage¹⁷¹.

2.1.1 The Chemistry of Lipid Peroxidation

Non-enzymatic lipid peroxidation is a free radical chain reaction and consists of three stages: initiation, propagation and termination (Figure 2.1).

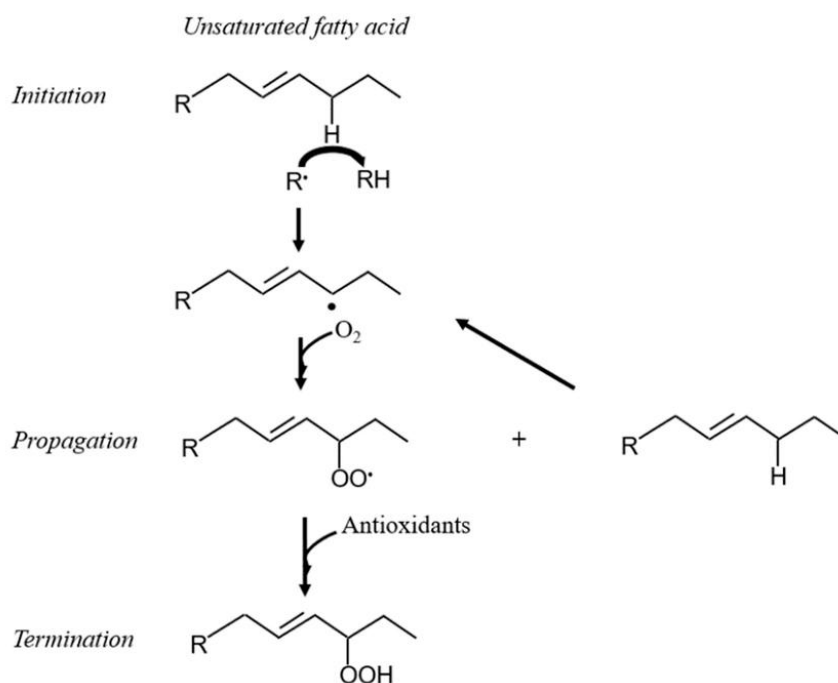


Figure 2.1 A Lipid Peroxidation Reaction. The stages in the lipid peroxidation reaction of an unsaturated fatty acid, consisting of the initiation, propagation and termination stages¹⁷².

Initiation is the formation of the first lipid-derived radical. PUFAs and other susceptible lipids are attacked by free radicals in biological systems generated by Fenton-type reactions

or metabolic reactions. Most commonly these attacking radicals are alkoxyl radicals or hydroxyl radicals. The radical abstracts a hydrogen atom from a PUFA, creating a carbon centred lipid radical and initiating the lipid peroxidation process. Propagation of the oxidative chain occurs when the lipid radical reacts with oxygen to form a lipid peroxy radical. The newly formed lipid peroxy radical abstracts a hydrogen from another lipid molecule generating a new lipid radical, which continues the chain reaction¹⁷³. Termination of the reaction occurs when two lipid radicals react with each other, forming a stable bond. The reaction can also terminate in the presence of a chain-breaking antioxidant such as vitamin E¹⁷⁴. The primary products of lipid peroxidation are lipid hydroperoxides, which can propagate further generation of ROS, or eventually lead to the generation of secondary products including highly reactive α , β -unsaturated aldehydes. 4-Hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA) and acrolein are the most reactive known lipid peroxidation products. These are electrophilic compounds that can interact with nucleophilic functional groups present in biomolecules, including the amino, thiol and hydroxyl groups of proteins, nucleic acids and lipids

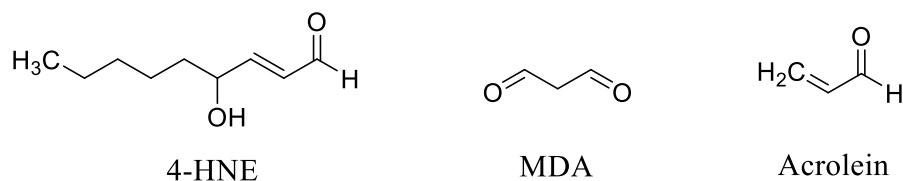


Figure 2.2 Selected Secondary Products of Lipid Peroxidation

These adducts are capable of inducing genotoxic and proteotoxic stress¹⁷⁵. The toxic lipid peroxidation products are thought to contribute to the pathophysiology of many human diseases.

2.1.2 Lipid Peroxidation in Human Health and Disease

Recent studies have demonstrated that lipid peroxidation derived aldehydes, 4-HNE and MDA, are implicated in many human pathological complications including cancer, diabetes, Alzheimer's disease and other neurological conditions, liver disease and autoimmune conditions like rheumatoid arthritis¹⁷⁶. The central nervous system is one of the major

targets of lipid peroxidation, due to its high oxygen consumption, high levels of PUFAs and high levels of redox transition metals in the brain, making it a prime target for free radical attack and induction of oxidative stress¹⁷⁷. An increase in lipid peroxidation markers in brain tissue have been observed in various neurodegenerative diseases including Parkinson's disease, Huntington's disease and Alzheimer's disease¹⁷⁸. Increased levels of free and protein-bound 4-HNE, MDA and acrolein have been identified in the brains of Alzheimer's disease patients¹⁷⁹, but also in patients with mild cognitive impairment, suggesting that lipid peroxidation is implicated in the pathophysiology of the earlier stages of Alzheimer's disease¹⁸⁰. 4-HNE modified proteins have also been identified at different stages of Alzheimer's disease, including modification of proteins involved in neuronal communication, energy metabolism, protein synthesis and neurite outgrowth. Enolase is a catalytic enzyme involved in glucose production, and is also important for the growth, survival and differentiation of developing and mature neurons. 4-HNE modified enolase was present in the brains of Alzheimer's disease patients in different stages of the disease¹⁸¹. It is suggested that this oxidative modification has numerous consequences including the disruption of neuronal energy metabolism and neuronal homeostasis, and that this may make neurons vulnerable to excitotoxicity and apoptosis, contributing to the pathophysiology of Alzheimer's disease. Collapsin response mediator protein 2 (CRMP2), a protein important for cytoskeletal organization, axonogenesis and neuronal polarity, also underwent 4-HNE modification in the brains of Alzheimer's disease patients¹⁸². This is suggested to result in the shortening of axons therefore negatively impacting neuronal communication. In addition, this modified protein can contribute to the development of neurofibrillary tangles and a loss of synapses, both hallmark characteristics of Alzheimer's disease.

With regard to skin inflammatory conditions, there is an established link between psoriasis and lipid peroxidation¹⁸³. Lipid peroxidation was up to two times higher in psoriatic patients than control patients, with elevated 4-HNE, 4-HNE-protein adducts, and MDA levels observed in skin biopsies, keratinocytes, and plasma of psoriatic patients^{184, 185}. 4-HNE-protein adducts can activate NFκB which increases the expression of pro-inflammatory

cytokines characteristic of psoriasis¹⁸⁶. In addition, 4-HNE stimulates apoptosis, accelerating the exfoliation of the epidermis¹⁸⁷. With the increased 4-HNE levels in psoriatic patients, it can be hypothesised that 4-HNE can contribute to the characteristic pathophysiology of psoriasis which includes the increased cell death of keratinocytes. Acrolein and acrolein-protein adduct levels are also increased in the blood of psoriatic patients¹⁸⁸, which has been shown to reduce the activity of anti-oxidant proteins, leading to increased oxidative stress and eventual cell death, characteristic of keratinocytes in psoriatic skin lesions.

The involvement of lipid peroxidation in psoriasis as well as AD¹⁸⁹ leads to the question of the role of lipid peroxidation in ACD and whether it can contribute to the pathophysiology of ACD. Recent reviews of the skin sensitization adverse outcome pathway emphasize that oxidative stress-induced lipid peroxidation generates reactive aldehydes such as MDA and 4-HNE, which can form covalent protein adducts resembling those produced by classical sensitizers¹⁹⁰. These adducts may act as endogenous danger signals, thereby amplifying or modulating sensitization outcomes. It has been previously established that allergens bind to peptides during the initial sensitisation phase of ACD¹⁹¹, and our hypothesis is that the due to the abundance of lipids present in the skin these may play a role in the pathophysiology of ACD. In this chapter we examine the ability of the metal allergens nickel, cobalt and chromium to induce lipid peroxidation. This has never previously been examined in the context of contact allergy and in a keratinocyte cell model.

2.1.3 The C11-BODIPY(581/591) Lipid Peroxidation Assay

C11-BODIPY is a fluorescent ratiometric probe used to measure lipid peroxidation and antioxidant efficacy in cellular systems. One of its earliest uses was published in 1999 in a study investigating lipid peroxidation in myocardial cells and rat-1 fibroblasts¹⁹². It is a lipophilic probe that incorporates into cellular membranes and undergoes a shift in fluorescence emission upon oxidation by lipid peroxides. C11-BODIPY detects peroxy radicals generated from lipid hydroperoxides, but does not respond to non-lipophilic hydroperoxides, providing a specific readout of lipid peroxidation in cells. The interaction

with peroxy radicals leads to oxidation of the C11-BODIPY probe at the phenylbutadiene residue, leading to a shift in its fluorescence. The maximum fluorescence of the intact probe is seen at 590 nm, but upon oxidation at the phenylbutadiene residue, conjugation is reduced and thus the maximum fluorescence shifts to 520 nm (Figure 2.3).

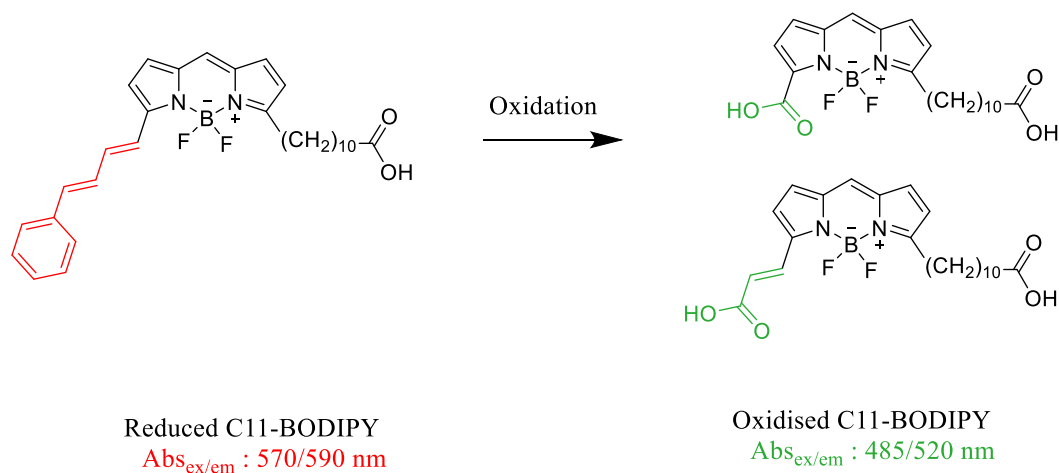


Figure 2.3 The mechanism of C11-BODIPY(581/591) oxidation by lipid peroxy radicals. The oxidation results in a shift in fluorescence emission from 590 nm (red) to 520 nm (green)¹⁹³

The advantages of using the C11-BODIPY(581/591) assay include its high sensitivity to oxidation by lipid peroxides due to its highly lipophilic nature, its adaptability to various different systems and cell lines, and its ability to be detected in various different platforms including confocal microscopy, flow cytometry and via a microplate fluorescence reader. Based on these advantages, this assay was chosen to investigate lipid peroxidation induction by metal allergens.

2.1.4 Aims and Objectives

The aim of this chapter is to evaluate the ability of metal allergens to induce lipid peroxidation. The objectives to fulfil this aim are:

1. To investigate the ability of metal allergens to induce lipid peroxidation in a single lipid model
2. To investigate the ability of metal allergens to induce lipid peroxidation in a HaCaT cellular model

3. To investigate the extent of ROS production by metal allergens in a HaCaT cellular model

2.2 Experimental Details

2.2.1 HaCaT Cells

The HaCaT cell line is a human immortalised keratinocyte cell line, derived from the epidermal cells of a 62-year-old Caucasian male (Figure 2.4). HaCaT cells are an indispensable model in dermatological research, facilitating a deeper understanding of skin biology and pathology through their resemblance to basal keratinocytes and their ability to undergo cell growth and differentiation. HaCaT cells were used for all viability, lipid peroxidation and ROS assays in this chapter.

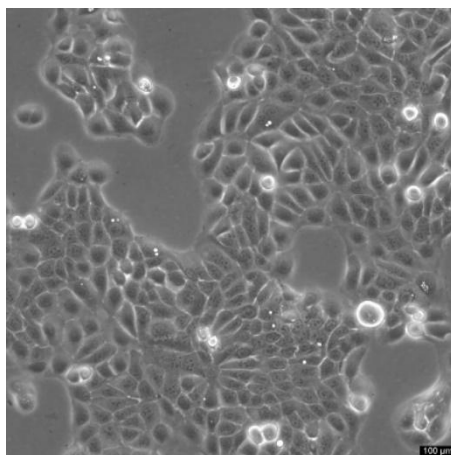


Figure 2.4 HaCaT Cell Culture¹⁹⁴

2.2.1.1 Thawing of HaCaT cells

When not in culture, cells were stored in liquid nitrogen vapour phase at a temperature of -178 °C preserved in 1 mL of a solution contain 5% DMSO and 95% Dulbecco's modified eagles' medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Cells were thawed in a 37 °C water bath and resuspended in a sterile 50 mL Falcon tube with 10 mL of DMEM containing 10% FBS in a sterile laminar flow unit. Cells were centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and pellet resuspended in DMEM containing 10% FBS and transferred to a T25 tissue culture flask. Cells were incubated at 5% CO₂ and 37 °C until the desired confluency was reached.

2.2.1.2 Sub-Culture of HaCaT Cells

HaCaT cells were cultured in DMEM high glucose with Glutamax™ (Gibco, 10566-016) supplemented with 10% foetal bovine serum (FBS) (Gibco, 12657029, Brazil). When the cells reached 70% confluency, they were sub-cultured. Media was removed and the cells were washed with 3 mL of room temperature Trypsin-EDTA solution (Sigma, T3924) to prevent interference of the media with trypsinisation. A further 3 mL of Trypsin-EDTA solution was added and the cells were incubated for approximately 5 minutes at 37 °C. Thereafter, 10 mL of culture media was added to neutralise the trypsin, and the cells were transferred to a 50 mL Falcon tube and centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 10 – 12 mL of fresh culture media. The cells were transferred to a new T75 flask at a split ratio of 1:3. Cells were sub-cultured at minimum on two independent occasions prior to experimental use following thawing from cryopreservation. The cells were used for a maximum of ten passages before a new batch of cells were thawed from cryopreservation.

2.2.1.3 Cryopreservation of HaCaT Cells

After trypsinisation and centrifugation, cells were resuspended in the freezing solution consisting of 5% DMSO and 95% DMEM supplemented with 10% FBS. Thereafter 1 mL of cell suspension was aliquoted into sterile cryotubes and placed on ice for 30 minutes, at -20 °C for 1 hour, at -80 °C overnight, and then transferred to liquid nitrogen for long term storage at -178 °C.

2.2.1.4 Counting of Cells

The cells were counted using a Bright-Line™ haemocytometer (Figure 2.5). A 10 µL aliquot of freshly suspended cells was mixed with 10 µL of Trypan Blue cell viability dye. 10 µL of the resulting cell suspension was loaded onto the haemocytometer and a clean cover slip was applied.

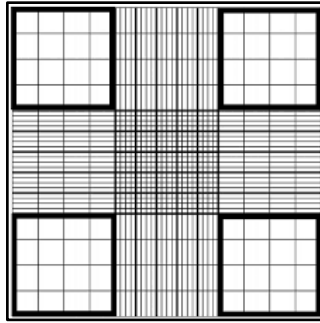


Figure 2.5 A representative haemocytometer grid. Cells in the outer four grids, outlined in black, are counted

The quantity of cells in each of the four quadrants was counted, excluding any cells stained with Trypan Blue. The following equation was used to calculate the quantity of cells per mL of suspension.

$$\frac{\text{Number of cells in four quadrants}}{4} \times 2 \times 10,000 = \text{number of cells in 1 mL}$$

2.2.2 alamarBlue Cell Viability Assay

HaCaT cells were seeded at a density of 1×10^4 cells per well in 100 μL culture media in a flat bottom sterile 96 well plate and allowed to incubate overnight. The cells were then treated with metal allergens in a deionised water vehicle for the intended treatment duration. The metals used were nickel (II) sulfate hexahydrate (CAS: 10101-97-0, Fluorochem, F494340), cobalt (II) chloride (CAS: 7646-79-9, ThermoScientific, B22031) and potassium dichromate (CAS: 7778-50-9, Acros Organics, 424110500) CumOOH (CAS: 81-15-9, Merck, 247502), was dissolved in DMSO, with total DMSO content in the well not exceeding 0.5%. An untreated control was also included. All treatments and controls were added to the wells in a 100 μL volume. Thereafter, the treatments were aspirated from the wells and the wells were washed with sterile PBS. A 10% solution of alamarBlue (ThermoFisher, DAL1100) was prepared using DMEM as a diluent. 100 μL of the diluted alamarBlue was added into each well. This was the procedure followed for alamarBlue assays in Figure 2.14 and all subsequent alamarBlue experiments. However, for the first alamarBlue assay (Figure 2.13), the metal treatments were not aspirated prior to adding the dye; instead, 10 μL of undiluted alamarBlue dye was added directly into each well. The plate was incubated in the dark at 5% CO_2 and 37 $^\circ\text{C}$ for the required treatment times; 2 –

4 hours or 24 hours, depending on the experiment. Fluorescence of the dye was measured using a Fluostar Omega microplate reader at an excitation wavelength of 540 nm excitation and 590 nm emission. Results were standardised to the untreated control and expressed as percentage viability.

2.2.3 C11-BODIPY 581/591 Lipid Peroxidation Assay

2.2.3.1 Single Lipid Model

A 100 μ M concentration of linoleic acid (Sigma-Aldrich, L1376) in 3 mL DMSO was incubated with 1 mL of the metal allergens dissolved in deionised water at patch test concentrations; 5% w/v (190 mM) nickel (II) sulfate, 1% w/v (80 mM) cobalt (II) chloride and 0.5% w/v (20 mM potassium dichromate), and stirred for two hours at 37 °C. The C11-BODIPY dye (Invitrogen, D3861) was reconstituted in DMSO to a final concentration of 10 mM, and 2.5 μ L was added to the reaction mixture. Controls included

- Linoleic acid + C11-BODIPY dye
- Metal Allergen + C11-BODIPY dye
- C11-BODIPY dye alone

Fluorescence of the reaction mixture was measured in a fluorescence cuvette using a Shimadzu RF-600 spectrofluorophotometer using the following parameters (table 2.1)

Excitation wavelength	480 nm
Emission wavelength	500-700 nm
Data Interval	2.0 nm
Scan Speed	6000 nm/min

Table 2.1 Spectrofluorophotometer parameters for C11-BODIPY(581/59) lipid peroxidation assay

2.2.3.2 C11-BODIPY Microplate Assay for Detection of Lipid Peroxidation *in vitro*

HaCaT cells were seeded at a density of 2×10^4 cells per well in 100 μ L in a flat bottom 96 well plate and allowed to incubate overnight. Prior to treatment the cells were pre-stained

with 100 μ L of 10 μ M C11-BODIPY dye and incubated at 5% CO₂ and 37 °C for 30 minutes. Thereafter, the cells were washed twice with sterile PBS. The metals, dissolved in deionised water, were then added to the cells in HBSS buffer. The metal concentrations included:

- NiSO₄ at a concentration range of 20 mM – 0.001 mM
- CoCl₂ at a concentration range of 10 mM – 0.001 mM
- K₂Cr₂O₇ at a concentration range of 5 mM – 0.001 mM

Cumene Hydroperoxide (CumOOH) was used as a positive control at a final concentration of 100 μ M for the assay and was prepared in a DMSO vehicle and added to the cells in HBSS buffer. The volume of DMSO in the well did not exceed 0.5% of total volume. The plate was incubated in the dark at 5% CO₂ and 37 °C for 2 hours. Fluorescence was measured using a BMG plate reader using the bottom optic. Unoxidized red fluorescence was measured at an excitation wavelength of 570 nm and an emission wavelength of 610 nm. The emission wavelength was altered from the 590 nm wavelength used in the single lipid model experiments, due to the observation of the λ_{max} at 610 nm in the C11-BODIPY fluorescence spectra. Oxidised green fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results were expressed as a fold change ratio, calculated using the following equation.

$$\text{Fold Change Ratio} = \frac{\text{Unoxidised fluorescence @ 610 nm}}{\text{Oxidised fluorescence @ 520 nm}}$$

For longer time point assays, e.g. 18-hour incubation, cells were treated with allergenic metals in DMEM supplemented with 1% FBS and incubated at 5% CO₂ and 37 °C. After incubation the cells were washed with PBS. C11-BODIPY dye (10 μ M) was added to the cells and the cells were incubated at 5% CO₂ and 37 °C for 30 minutes. Thereafter, the cells were washed with PBS x2, the wells were refilled with PBS and the fluorescence was measured using a BMG microplate reader. This modified methodology was adapted due to the instability of the C11-BODIPY dye for prolonged periods of time at 37 °C.

2.2.3.3 C11-BODIPY Confocal Microscopy Assay for Detection of Lipid Peroxidation *in vitro*

HaCaT cells were seeded at 4×10^4 cells per well in 200 μ L culture media in ibidi 8-well microscopy plates (ibidi, 80826) and incubated overnight at 5% CO₂ and 37 °C. The supernatant was removed, and the cells were stained with C11-BODIPY dye (10 μ M) and the cells were incubated at 5% CO₂ and 37 °C for 30 minutes. The cells were then washed twice with PBS and treated with NiSO₄ (20 mM), CoCl₂ (10mM), and K₂Cr₂O₇ (0.5 mM – 5 mM) and CumOOH (100 μ M) in HBSS buffer and incubated at 5% CO₂ and 37 °C for 2 hours. The cells were imaged using a fluorescence microscope and the images analysed using LAS X Office software.

2.2.4 H₂DCFDA Reactive Oxygen Species Assay

HaCaT cells were seeded at 1×10^4 cells in 100 μ L culture media per well of a 96 well plate and incubated overnight at 5% CO₂ and 37 °C. H₂DCFDA solution at a final concentration of 20 μ M was added to the cells and incubated at 5% CO₂ and 37 °C for 30 minutes. After incubation the supernatant was removed, and the cells were washed twice with PBS. The cells were treated with final concentrations of NiSO₄ (5 - 10 mM), CoCl₂ (1 - 10 mM), and K₂Cr₂O₇ (0.5– 5 mM) in a 100 μ L volume and incubated 5% CO₂ and 37 °C for two hours. CumOOH and *tert*-butyl hydroperoxide (*t*-BuOOH) (100 μ M) were used as positive controls as they are both known ROS inducers¹⁹⁵. After treatment, the fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 530 nm using a BMG microplate reader.

2.2.5 Statistical Analysis

The data for all experiments in this chapter was assessed using GraphPad Prism 10 for Windows (GraphPad software, USA). An ordinary one-way ANOVA test was used to analyse the data, with Tukey's multiple comparisons post-hoc test used to compare means. The mean $\pm$ SEM is displayed on the histograms of the results, and a *p*-value of less than 0.05 was considered to be statistically significant.

2.3 Results and Discussion

2.3.1 Lipid Peroxidation induced by Metal Allergens in a Single-Lipid Model

Salts of each of the metals, nickel (II) sulfate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), cobalt (II) chloride anhydrous (CoCl_2) and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) were incubated with linoleic acid, a C-18 PUFA, (99% purity) in DMSO, in the presence of the C11-BODIPY probe, after which the fluorescence was measured and quantified to assess the extent of linoleic acid peroxidation.

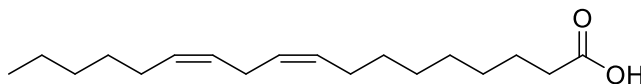


Figure 2.6 The structure of linoleic acid

The concentrations of metals selected were based on the standard concentrations described in the European Society of Contact Dermatitis baseline patch test guidelines¹⁹⁶. Nickel (II) sulfate (NiSO_4) is patch tested at a concentration of 5.0% (w/w) in petrolatum (190 mM), cobalt (II) chloride (CoCl_2) is patch tested at a concentration of 1.0% (w/w) in petrolatum (80 mM), and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) is patch tested at a concentration of 0.5% (w/w) in petrolatum (20 mM) in European contact allergy patch tests. These concentrations were chosen to investigate the ability of metal allergens to induce lipid peroxidation in a single lipid model, as they are representative concentrations that patients would be exposed to during patch testing.

2.3.1.1 Effect of Nickel (II) Sulfate on Peroxidation of Linoleic Acid

The C11-BODIPY(581/591) assay was conducted with NiSO_4 , the most common contact allergen, in the presence of linoleic acid alongside appropriate controls. The controls included linoleic acid and the C11-BODIPY probe, NiSO_4 and the C11-BODIPY probe, and the C11-BODIPY probe alone to get a baseline fluorescence reading of the probe in its ground state. The vehicle used in this experiment was DMSO, which is itself an oxidising agent¹⁹⁷. Including the C11-BODIPY alone control, which is dissolved in DMSO will establish whether DMSO is influencing the oxidation of the C11-BODIPY probe. The fluorescence of the

oxidised probe at 520 nm, and the unoxidized/reduced probe at 590 nm was read (Figure 2.7).

An indication of lipid peroxidation was the increase in fluorescence intensity of the oxidised probe at 520 nm, and a reduction in the fluorescence intensity of the reduced probe at 590 nm. When NiSO_4 was mixed with linoleic acid, and the C11-BODIPY dye was then added, the intensity of fluorescence at 520 nm was significantly increased in comparison to the fluorescence intensity in the NiSO_4 and linoleic acid control, and the C11-BODIPY standard [Figure 2.7(A)]. This indicated that the C11-BODIPY dye was being oxidised. The lower fluorescence at 520 nm in the linoleic acid only and NiSO_4 only controls indicated that oxidation of the C11-BODIPY dye required both NiSO_4 and linoleic acid, as fluorescence intensity was highest in the sample containing both chemicals. The fluorescence intensity of the unoxidized probe at 590 nm was also analysed. There was a reduction in fluorescence intensity of the probe at 590 nm in the sample with both NiSO_4 and linoleic acid in comparison to the linoleic acid control and the C11-BODIPY standard, but it was not statistically significant [Figure 2.7(B)]. This suggests that while lipid peroxidation may have occurred in the presence of NiSO_4 , it did not appear to be to a statistically significant extent. NiSO_4 also induced a non-significant reduction in fluorescence intensity of the unoxidized dye on its own in the absence of linoleic acid indicating some direct activity of NiSO_4 on C11-BODIPY dye. However, this non-significant reduction in fluorescence intensity coupled with the lack of increase in the intensity of the oxidised C11-BODIPY dye indicated that this direct activity of NiSO_4 on C11-BODIPY dye was insignificant, particularly in comparison to NiSO_4 in the presence of linoleic acid.

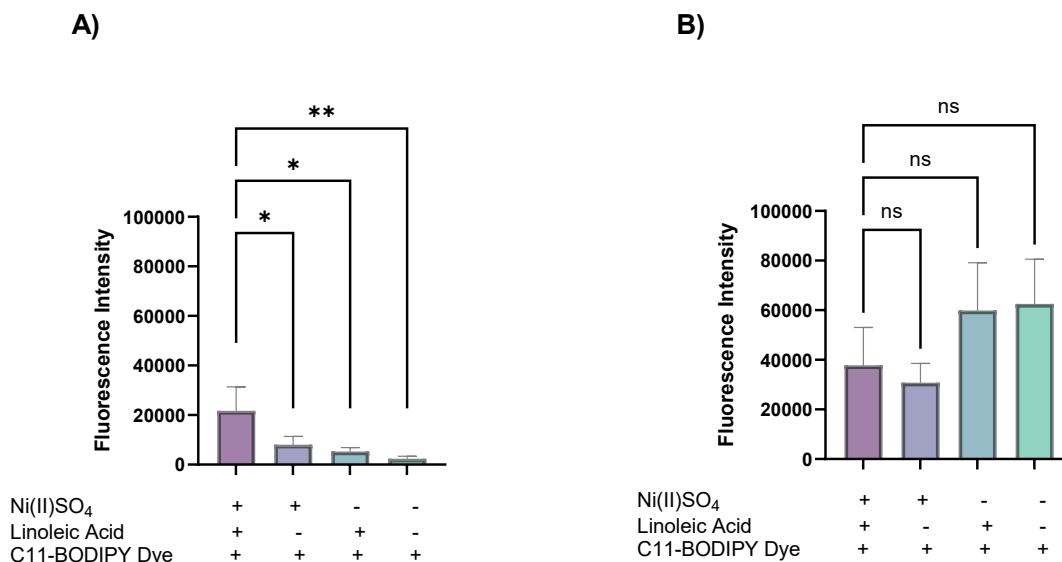


Figure 2.7 The C11-BODIPY(581/591) Assay with Nickel (II) Sulfate and Linoleic acid. The C11-BODIPY(581/591) assay with 5%/190 mM Nickel (II) Sulfate, and 0.1 M linoleic acid in a DMSO vehicle measuring the fluorescence intensity of (a) the oxidised dye at 520 nm and (b) the unoxidized dye at 590 nm. Nickel (II) sulfate was incubated with linoleic acid in the presence of C11-BODIPY probe for 2 hours. * $p < 0.05$, ** $p < 0.01$, calculated using one-way ANOVA test with Tukey's Multiple Comparisons Post-Hoc test, $n = 3$

Further confirmation that lipid peroxidation was occurring to some degree can be seen in the fluorescence spectra for the NiSO₄ and linoleic acid experiment and the C11-BODIPY standard (Figure 2.8). The spectrum for the C11-BODIPY standard displayed a characteristic curve, with maximum emission of fluorescence at 600-610 nm, with no fluorescence peak present at 520 nm, signifying that the C11-BODIPY dye in its ground state was completely unoxidized (Figure 2.8, red). Upon addition of NiSO₄ and linoleic acid, the maximum fluorescence emission peak at 600-610 nm was reduced, and the presence of an emission peak at 520 nm was observed, along with a smaller fluorescence emission peak at 550 nm which is characteristic for the oxidised C11-BODIPY dye and indicated that oxidation is progressing (Figure 2.8, black). Overall, the results indicated that lipid peroxidation had occurred.

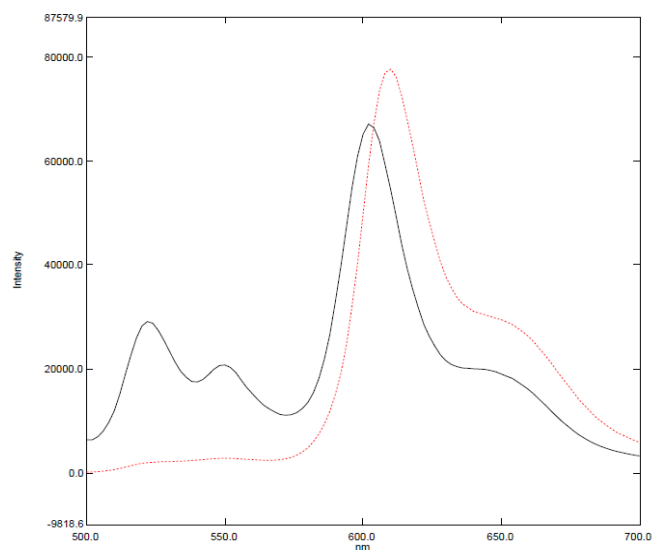


Figure 2.8 The Fluorescence Spectra of the C11-BODIPY(581/591) assay with Nickel (II) Sulfate. *Fluorescence Spectra of C11-BODIPY (red) and NiSO₄ + Linoleic Acid + C11-BODIPY (black). 5%/190 mM Nickel (II) Sulfate was incubated with 0.1 M linoleic acid in a DMSO vehicle in the presence of C11-BODIPY probe for 2 hours after which the fluorescence spectrum was obtained.*

2.3.1.2 Effect of Cobalt (II) Chloride on Peroxidation of Linoleic Acid

The assay was repeated with CoCl₂ which is a more potent allergen than NiSO₄. A series of controls consisting of combinations of CoCl₂, linoleic acid and the C11-BODIPY dye were used. The fluorescence of the oxidised dye at 520 nm and the unoxidized/reduced dye at 590 nm were measured (Figure 2.9). In the presence of CoCl₂ and linoleic acid, a statistically significant increase in the fluorescence intensity of the C11-BODIPY dye at 520 nm was observed in comparison to all other samples which showed little fluorescence emission at 520 nm [Figure 2.9(A)]. This indicated that in the presence of CoCl₂ and linoleic acid, the C11-BODIPY dye had undergone significant oxidation, whereas in the controls and standard, the C11-BODIPY probe had undergone very little to no oxidation. This suggested that CoCl₂ had induced peroxidation of linoleic acid. This was further confirmed using the fluorescence intensity of the C11-BODIPY probe at 590 nm [Figure 2.9(B)]. In the presence of both CoCl₂ and linoleic acid, the fluorescence intensity of the unoxidized probe underwent a statistically significant reduction, in comparison to both controls and the C11-BODIPY standard which showed minimal reduction in fluorescence intensity. The increase in fluorescence intensity at 520 nm, in addition to the reduction in fluorescence intensity at 590 nm in the presence of both CoCl₂ and linoleic acid indicated that lipid peroxidation was occurring. Additionally, the lack of changes in fluorescence intensity in the controls

confirms that oxidation of the C11-BODIPY depended on the presence of both CoCl_2 and linoleic acid.

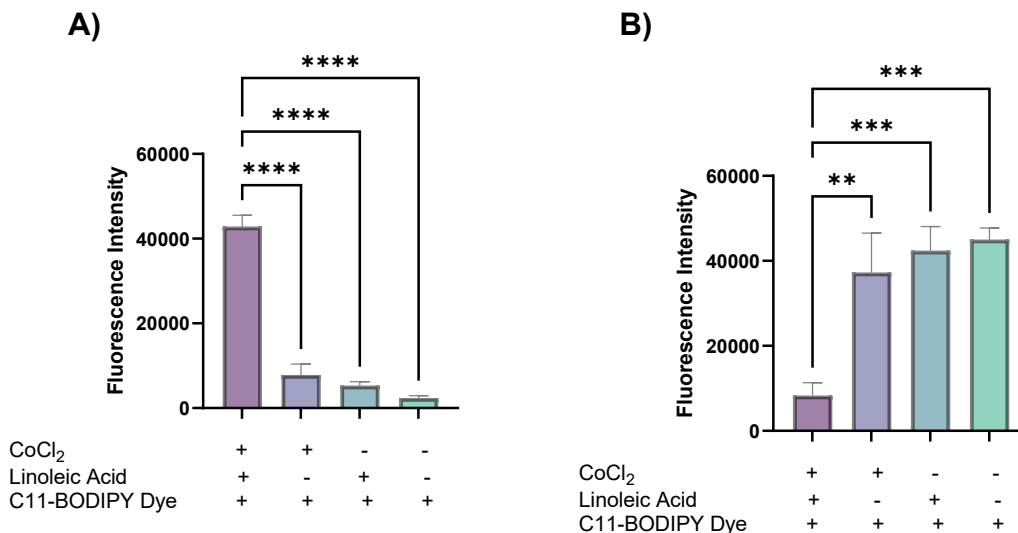


Figure 2.9 The C11-BODIPY(581/591) Assay with CoCl_2 . The C11-BODIPY(581/591) assay with 1%/80 mM CoCl_2 , and 0.1 M linoleic acid measuring the fluorescence intensity of (a) the oxidised dye at 520 nm and (b) the unoxidized dye at 590 nm in the presence of CoCl_2 . CoCl_2 was incubated with 0.1 M linoleic acid in a DMSO vehicle in the presence of C11-BODIPY probe for 2 hours, after which the fluorescence is measured ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0005$ calculated using one-way ANOVA test with Tukey's Multiple Comparisons Post-Hoc test, $n=3$

Lipid peroxidation can be confirmed by looking at the fluorescence spectra of the C11-BODIPY standard, and the C11-BODIPY in the presence of CoCl_2 and linoleic acid (Figure 2.10). In the presence of CoCl_2 and linoleic acid, the maximum fluorescence emission shifted almost completely from 600 nm in the C11-BODIPY standard (red, Figure 2.10) to 520 nm indicating that the C11-BODIPY dye had almost been completely oxidised (black, Figure 2.10). The presence of a very minor fluorescence emission peak at 600 nm indicated that a small amount of the C11-BODIPY dye had remained unoxidized. In comparison to NiSO_4 in 2.3.1.1 above, it appeared that a much higher degree of lipid peroxidation had taken place in the presence of CoCl_2 . This is consistent with the known allergenic potency of cobalt. This is classified as a strong allergen and has been documented to have a much higher potency as a contact allergen than nickel¹³². In addition, it appeared that CoCl_2 was

able to induce more lipid peroxidation at a concentration lower than that of NiSO_4 (1% and 5% w/w respectively).

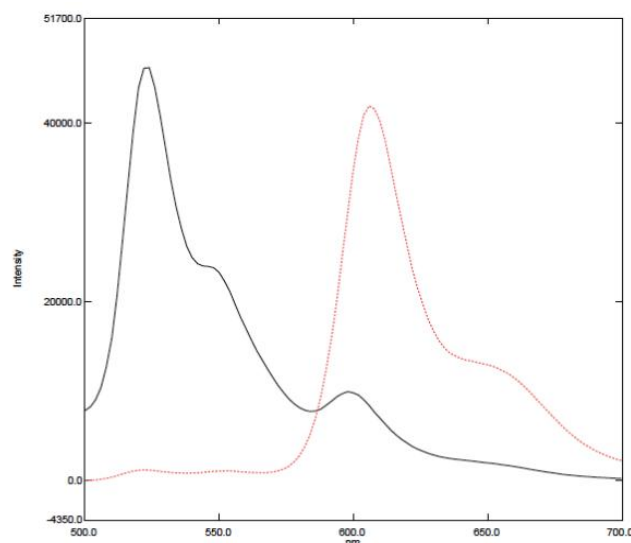


Figure 2.10 The Fluorescence Spectra of the C11-BODIPY(581/591) assay with cobalt (II) chloride. *Fluorescence Spectra of C11-BODIPY (red) and CoCl_2 + Linoleic Acid + C11-BODIPY (black). 1%/80 mM CoCl_2 was incubated with 0.1 M linoleic acid in a DMSO vehicle in the presence of C11-BODIPY probe for 2 hours after which the fluorescence spectrum was obtained*

2.3.1.3 Effect of Potassium Dichromate on Peroxidation of Linoleic Acid

The assay was lastly performed with potassium dichromate, the most potent metal allergen of the three metals studied. As with the preceding two experiments, a series of controls were run alongside the main experiment consisting of $\text{K}_2\text{Cr}_2\text{O}_7$, linoleic acid and the C11-BODIPY probe. The three controls, as before, included C11-BODIPY standard, linoleic acid and C11-BODIPY, and $\text{K}_2\text{Cr}_2\text{O}_7$ and C11-BODIPY. The fluorescence of the oxidised dye at 520 nm and the unoxidized/reduced dye at 590 nm was measured (Figure 2.11). In the presence of $\text{K}_2\text{Cr}_2\text{O}_7$ and linoleic acid, a significant increase in fluorescence intensity of the oxidised C11-BODIPY dye at 520 nm was observed in comparison to $\text{K}_2\text{Cr}_2\text{O}_7$ and the linoleic acid alone, which showed minimal fluorescence intensity in comparison to the C11-BODIPY standard [Figure 2.11(A)]. This indicates that lipid peroxidation was taking place in the presence of $\text{K}_2\text{Cr}_2\text{O}_7$. There appeared to be a small increase in fluorescence intensity at 520 nm in the $\text{K}_2\text{Cr}_2\text{O}_7$ only sample in comparison to the C11-BODIPY standard which raises

questions surrounding the specificity of the C11-BODIPY probe to oxidation by lipid radicals, however the increase in fluorescence intensity of the C11-BODIPY dye in the presence of linoleic acid and $K_2Cr_2O_7$ was significantly higher than that of the $K_2Cr_2O_7$ control, indicating that lipid peroxidation was occurring. In the presence of $K_2Cr_2O_7$ and linoleic acid, the C11-BODIPY dye exhibited minimal fluorescence emission at 590 nm, which indicated that the C11-BODIPY dye underwent complete oxidation in the presence of $K_2Cr_2O_7$ and linoleic acid (Figure 2.11B). In contrast, the fluorescence intensity of both controls and the C11-BODIPY dye remained high, indicating that little oxidation of the C11-BODIPY dye had occurred. There was a larger reduction in fluorescence intensity of the $K_2Cr_2O_7$ control in comparison to the C11-BODIPY standard and linoleic control, further indicating that $K_2Cr_2O_7$ had oxidised the probe. Furthermore, the difference in fluorescence intensity in comparison with the C11-BODIPY probe was shown to be statistically significant, furthering the suggestion that the C11-BODIPY probe shows minor susceptibility to oxidation by strongly oxidising transition metal ions. Overall, the significant increase in fluorescence intensity at 520 nm, as well as the significant decrease in fluorescence intensity at 595 nm, strongly suggested that $K_2Cr_2O_7$ had induced lipid peroxidation of linoleic acid.

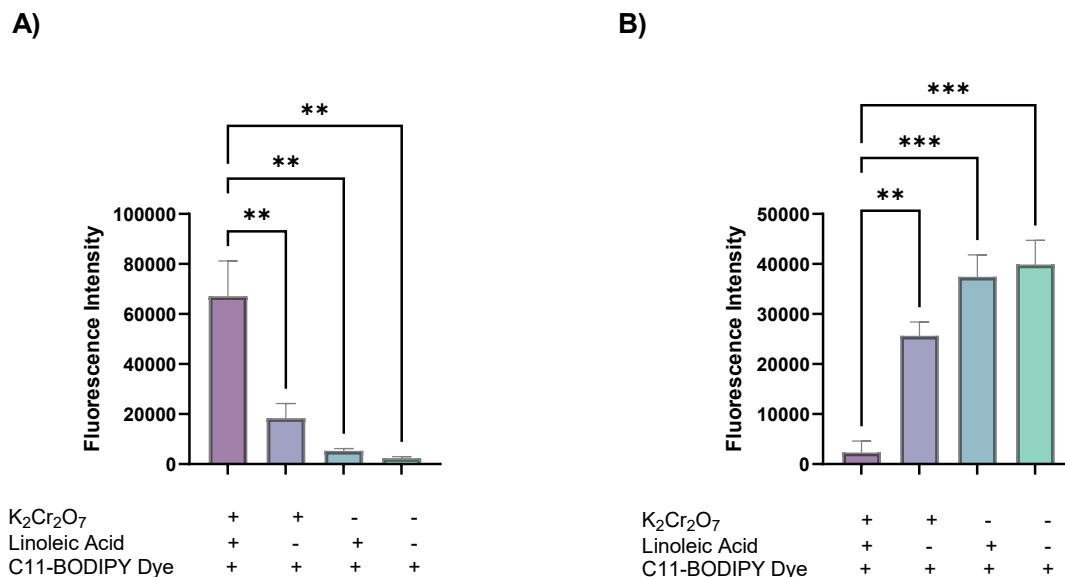


Figure 2.11 The C11-BODIPY(581/591) assay with Potassium Dichromate. The C11-BODIPY(581/591) assay with 0.5%/20 mM Potassium Dichromate and 0.1M linoleic acid, measuring the fluorescence intensity of (a) the oxidised dye at 520 nm and (b) the unoxidized dye at 590 nm in the presence of potassium dichromate, ** $p < 0.01$, *** $p < 0.005$, calculated using one-way ANOVA test with Tukey's Multiple Comparisons Post-Hoc test, $n=3$

Lipid peroxidation was confirmed by analysing the fluorescence spectra of the C11-BODIPY dye, and the C11-BODIPY dye in the presence of K₂Cr₂O₇ and linoleic acid (Figure 2.12). The spectrum for the unoxidized C11-BODIPY displays a characteristic maximum fluorescence emission peak at 590 nm, and no fluorescence emission peak at 520 nm (red, Figure 2.12). The spectrum for the C11-BODIPY dye in the presence of K₂Cr₂O₇ and linoleic acid indicated the complete absence of a fluorescence emission peak at 590 nm, and the complete shift of the maximum fluorescence emission down to 520 nm (black, Figure 2.12). This complete shift in fluorescence and the absence of fluorescence emission at 590 nm, coupled with the absence of a smaller shoulder peak at 550 nm, confirmed that the C11-BODIPY probe had been completely oxidised and that K₂Cr₂O₇ had strongly induced lipid peroxidation. The complete oxidation of the C11-BODIPY probe, in comparison to the results seen with NiSO₄ and CoCl₂, indicates that K₂Cr₂O₇ has the highest lipid peroxidation potency in this model. This result is even more significant considering that the concentration of K₂Cr₂O₇ used was lower than the concentrations of CoCl₂ and NiSO₄ used (0.5%, 1% and 5% w/w respectively).

This is consistent with the relative contact allergen potency of the metals, with chromium being classed as an extreme allergen, in comparison with cobalt's strong allergen status, and nickel's mild-moderate allergen status, in addition to the high oxidising potential of $K_2Cr_2O_7$ ¹³².

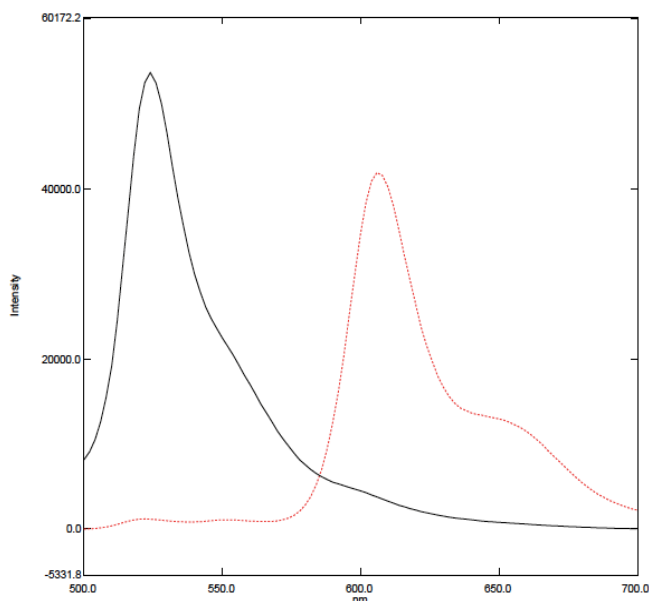


Figure 2.12 The Fluorescence Spectra of the C11-BODIPY(581/591) Assay with Potassium Dichromate. Fluorescence Spectra of C11-BODIPY (red) and $K_2Cr_2O_7$ + Linoleic Acid + C11-BODIPY (black). 0.5%/20 mM $K_2Cr_2O_7$ was incubated with 0.1 M linoleic acid in a DMSO vehicle in the presence of C11-BODIPY probe for 2 hours after which the fluorescence spectrum was obtained

2.3.2 Lipid Peroxidation Induced by Allergenic Metals in a Cellular Model

Following on from the observation of lipid peroxidation induction by allergenic metals in a single lipid model, we sought to apply the assay *in vitro* to investigate lipid peroxidation induction by metals in a more biologically relevant model. A HaCaT cell line was used for this model. HaCaT is an immortalised human keratinocyte cell line, frequently used in dermatological research¹⁹⁸.

2.3.2.1 Assessing the Effect of Metals on Cell Viability

Before the C11-BODIPY(581/591) assay was carried out, it was important to firstly assess the cytotoxicity of the metal salts on HaCaT cells. To do this, the alamarBlue cell viability assay was carried out. alamarBlue dye contains resazurin, an oxidation-reduction (REDOX)

indicator. Resazurin is a non-fluorescent blue dye that can be reduced by NADH and NADPH, in the presence of their respective dehydrogenase enzymes, in the cytoplasm of viable cells. Reduction of resazurin produces the pink, fluorescent compound resorufin. The intensity of the fluorescence of resorufin is directly correlated to the number of viable cells in the sample¹⁹⁹. A range of concentrations of each metal was tested. The highest concentration used was that of the standard patch test concentrations; 20 mM $K_2Cr_2O_7$, 80 mM $CoCl_2$, and $NiSO_4$ 190 mM. Concentrations 10 times lower and 100 times lower than the standard patch test concentrations were also tested. The assay was run over a four-hour incubation period, 2 hours to match the incubation period for the single lipid model C11-BODIPY(581/591) assay, plus an additional two hours when the alamarBlue dye was added to the cells (Figure 2.13).

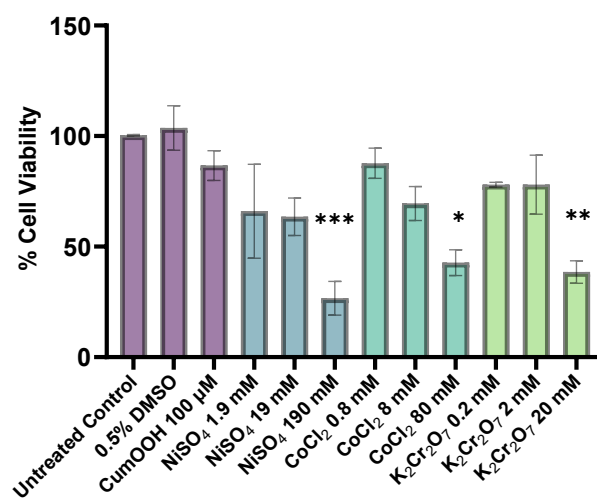


Figure 2.13 Cytotoxicity of Metals on HaCaT Cells HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells were treated with the metals at the indicated concentration and incubated for 2 hours. After this time the alamarBlue assay was carried out to assess cell viability, by adding alamarBlue directly onto the treated cells and incubating for a further 2 hours. In total the cells were in contact with metal salts for 4 hours. Results for each concentration are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. $N=3$ * $p<0.05$, ** $p<0.01$, *** $p<0.001$ in comparison to untreated control.

The patch test concentrations of all three metals were significantly toxic to the cells after 4 hours of treatment. This aligned with previously published work which found that patch test-relevant concentrations of metal salts caused significant toxicity on freshly excised

skin. NiSO_4 was tested at the 5% w/v patch test recommended concentration, equivalent to 190 mM, and was concluded to induce significant cell death via apoptosis, confirmed via the TUNEL assay, as well as reducing the metabolic activity of the skin²⁰⁰. $\text{K}_2\text{Cr}_2\text{O}_7$ at 2 mM and 0.2 mM concentrations did not have a significant effect on cell viability. This was also observed with the 8 mM and 0.8 mM concentrations of CoCl_2 and the 19 mM and 1.9 mM concentrations of NiSO_4 . In addition, a concentration-dependent effect was seen for all three metals, with cell viability increasing as the concentration of metal salt reduces. The cytotoxic patch test concentrations were removed and a range of concentrations between the non-cytotoxic concentrations were chosen to move forward with. The alamarBlue viability assay was carried out with these concentrations (Figure 2.14).

Lower concentrations of metal allergens were determined to be less toxic than the prior concentrations tested. $\text{K}_2\text{Cr}_2\text{O}_7$ had no effect on HaCaT cell viability at concentrations of 0.5 mM and 1 mM, with a slight reduction at 2 mM, but was significantly toxic at 5 mM. CoCl_2 did not have a significant effect on viability at the concentrations tested; 10 mM, 5 mM and 1 mM. However, there appeared to be a non-significant reduction in viability in the cells treated with 5 mM and 10 mM concentrations. NiSO_4 , at concentrations of 5 mM, 10 mM and 20 mM, also did not have a significant effect on cell viability after a two-hour incubation period, but there appeared to be a non-significant, concentration-dependent reduction in viability, with viability reducing to approximately 80% in cells treated with 20 mM NiSO_4 . The cytotoxicity of the metals in HaCaT cells has previously been studied and supports our results, however the assays were performed in different conditions, namely in DMEM culture media free of phosphate, and the metals appeared to be more cytotoxic at a shorter timepoint than was seen here²⁰¹. For example, CoCl_2 (1 mM) reduced the viability of HaCaT cells to 1% after four hours in the previous study, but in the present study after a two-hour treatment CoCl_2 had no effect on HaCaT cell viability. These differences can be attributed to differences in the experimental conditions.

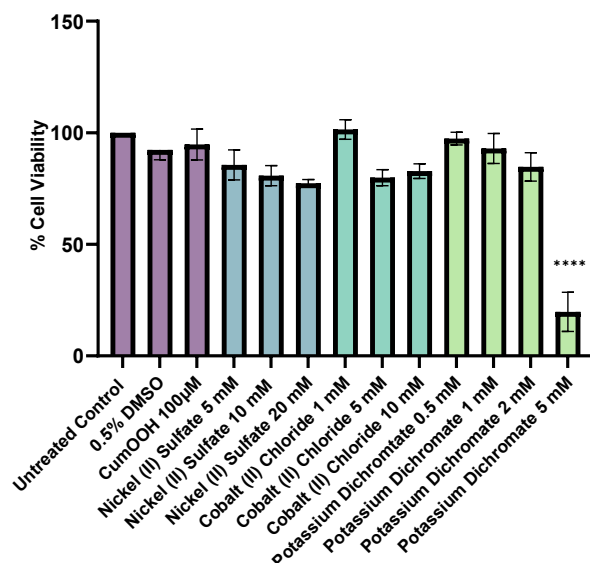


Figure 2.14 Cytotoxicity of Refined Concentrations of Allergenic Metals on HaCaT Cells. . HaCaT cells were seeded at a density of 2.0×10^4 cells per well of a 96 well plate. The cells were treated with NiSO_4 , CoCl_2 , and $\text{K}_2\text{Cr}_2\text{O}_7$ and incubated for 2 hours. After this time the metal salts were aspirated and the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. ** $p < 0.01$, **** $p < 0.0001$ in comparison to untreated control

Having assessed the effect of NiSO_4 , CoCl_2 , and $\text{K}_2\text{Cr}_2\text{O}_7$ on cell viability, the next step was to investigate their ability to induce lipid peroxidation in HaCaT cells using the BODIPY assay.

2.3.2.2 The C11-BODIPY(581/591) Assay in HaCaT cells

2.3.2.2.1 Optimisation of the Assay

The C-11 BODIPY(581/591) assay has been previously used in different cell lines such as HT-1080 cells²⁰², CHO cells²⁰³ and HeLa cells²⁰⁴. To investigate the ability of metals to induce lipid peroxidation in HaCaT cells, the assay methodology was first optimised using CumOOH as a positive control. CumOOH is a known lipid peroxidation inducer which, in the presence of transition metal ions like iron, produces cumoxyl radicals which react with PUFAs and initiate lipid peroxidation¹⁷³. The cumoxyl radical can also react with other CumOOH molecules to produce cumoperoxyl radicals which can also initiate lipid peroxidation¹⁷³. CumOOH has been used as a lipid peroxidation inducer since the 1980s, with a study by Weiss et al. in 1986 using it to induce lipid peroxidation in rat liver microsomes²⁰⁵. CumOOH

was first used as a positive control in the C11-BODIPY(581/591) assay in a study by Pap et al. in 1999 in an investigation into lipid peroxidation induction in Rat-1 fibroblasts¹⁹². To optimise the assay, HaCaT cells were seeded at 1.0×10^4 cells per well and 2.0×10^4 cells per well in a flat bottom 96-well plate to assess the optimal seeding density to allow detection of the C11-BODIPY dye via microplate reader. The cells were pre-stained with 10 μ M C11-BODIPY dye, as per manufacturers recommendation. After this the cells were treated with CumOOH at 50 μ M, 100 μ M, and 200 μ M. The fluorescence of the oxidised dye was measured at 485 nm excitation and 520 nm emission, and due to position of unoxidized peak in the spectra of linoleic acid peroxidation induced by $K_2Cr_2O_7$ in Figure 2.12, the fluorescence of the unoxidized fluorescence was measured at 570 nm excitation and 610 nm emission. (Figure 2.15).

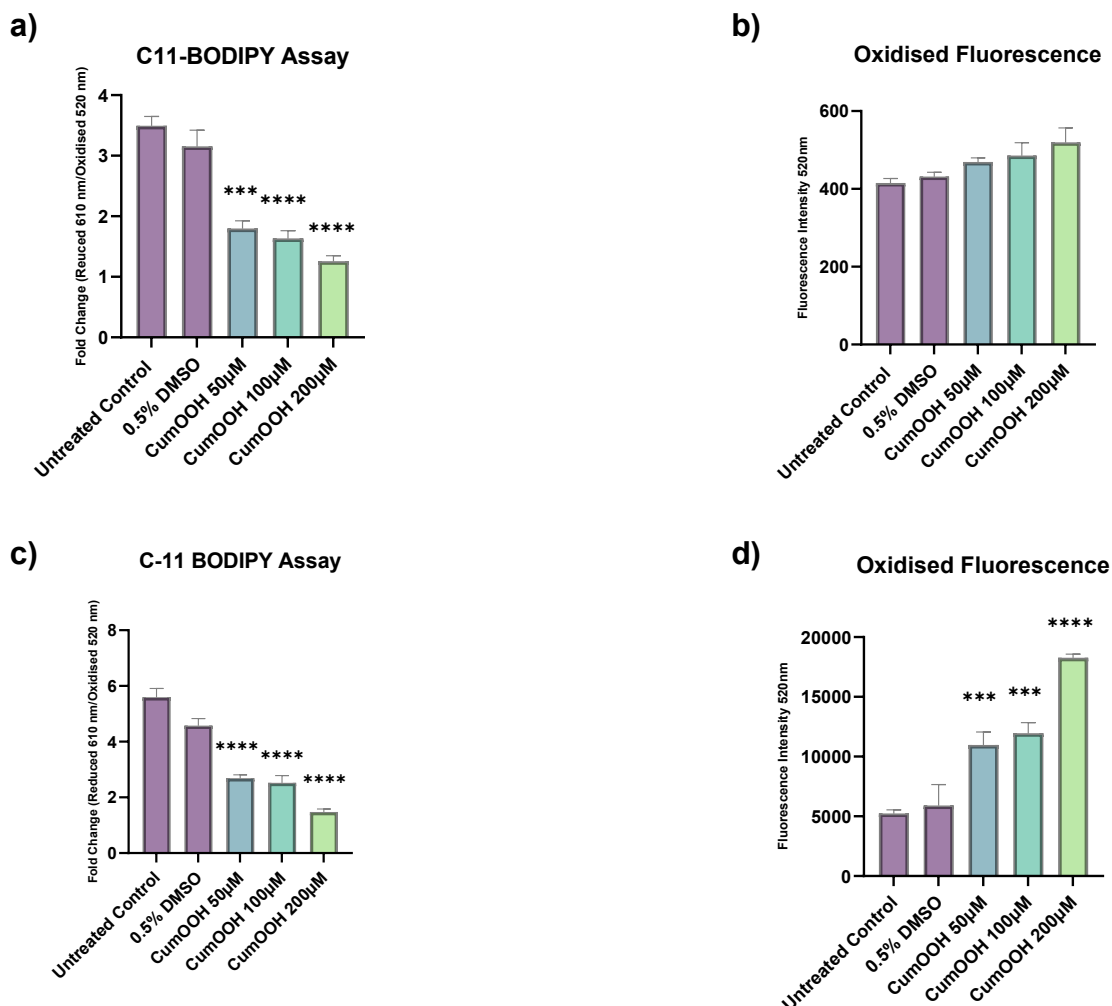


Figure 2.15 Optimisation of C11-BODIPY(581/591) Assay using CumOOH. HaCaT cells were seeded at a density of (a, b) 1.0×10^4 and (c, d) 2.0×10^4 cells per well and pre-stained with $10 \mu\text{M}$ C11-BODIPY dye for 30 minutes. Cells were then treated with CumOOH and incubated for 2 hours. Results are displayed in (a) and (c) as fold change ratio and in (b) and (d) as fluorescence intensity of oxidised (green) dye and are expressed as mean $\pm$ SEM. Results are analysed using one-way ANOVA with Tukey's post-hoc test for comparison of means. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ in comparison to untreated control.

To assess induction of lipid peroxidation induction, a ratio of the fluorescence of the unoxidized (red) dye at 610 nm emission and the fluorescence of the oxidised (green) dye at 520 nm emission was calculated. This is known as the fold change ratio. A decrease in the fold change ratio in comparison to the untreated control indicates that lipid peroxidation is occurring. There was a reduction in fold change ratio in both assay conditions, indicating that lipid peroxidation was occurring (Figure 2.15a, Figure 2.15c). Lipid peroxidation was evident at all concentrations of CumOOH tested, in a concentration-

dependent manner. However, the increase in fluorescence intensity of the oxidised (green) dye was more significant when the HaCaT cells were seeded at a higher density (Figure 2.15d). This indicated that lipid peroxidation was more significantly detected when the HaCaT cells are seeded at 2.0×10^4 cells per well, rather than at a density of 1.0×10^4 cells per well. Following on from these results, the HaCaT cells were seeded at a density of 2.0×10^4 cells per well for all future C11-BODIPY assay experiments investigating the ability of allergenic metals to induce lipid peroxidation.

2.3.2.2.2 Induction of Lipid Peroxidation in HaCaT Cells by Metals

Following optimisation of the C11-BODIPY assay using CumOOH, the assay was then performed with NiSO_4 at 20 mM, 10 mM and 5 mM, CoCl_2 at 10 mM, 5 mM and 1 mM and $\text{K}_2\text{Cr}_2\text{O}_7$ at 5 mM, 2 mM, 1 mM and 0.5 mM. These concentrations were selected as they were deemed to be non-cytotoxic from the earlier viability assays (Figure 2.14). The assay was carried out as per manufacturer's specifications^{206, 207}. The HaCaT cells were pre-stained with 10 μM C11-BODIPY dye for 30 minutes, after which the cells were washed with PBS and treated with the metals. As stated by the manufacturer's protocol, the metals were applied to the cells in HBSS buffer and incubated for two hours. Hank's Balanced Salt Solution (HBSS) is a buffered salt solution commonly used in cell culture to maintain the physiological environment for cells outside of a living organism. The use of HBSS buffer over standard cell media has several advantages. It can reduce background fluorescence caused by phenol red, a common component of culture media that can interfere with fluorescence imaging²⁰⁸. In addition, because the mechanism behind the C11-BODIPY assay involves its oxidation to cause the shift in fluorescence, the presence of antioxidants and serum can interfere with C11-BODIPY dye oxidation by scavenging ROS, leading to an underestimation of oxidative stress. Lastly, it has been noted that serum proteins in media can bind to fluorescent dyes such as C11-BODIPY. This would alter its lipophilicity and its ability to integrate into cell membranes and detect lipid peroxidation²⁰⁹. The results of the C11-BODIPY(581/591) assay with metals are displayed in Figure 2.16.

The results of the C11-BODIPY assay were expressed as a ratio between the reduced dye (red) and the oxidised dye (green), with a decrease in the ratio in comparison to the

untreated control indicating that lipid peroxidation has taken place. CumOOH 100 μ M was used as a positive control and induced a significant reduction in fold change. NiSO₄ treatment resulted in a reduction in the fold change ratio, however it was only statistically significant at the highest 20 mM concentration. This indicated that significant lipid peroxidation had taken place after treatment with 20 mM NiSO₄, but not at the lower concentrations of 10 mM or 5 mM. In comparison to the other metal treatments, it induced less lipid peroxidation. This was consistent with the results of the assay in the single lipid model, in which NiSO₄ treatment resulted in the lowest fluorescence shift of the C11-BODIPY dye from red to green.

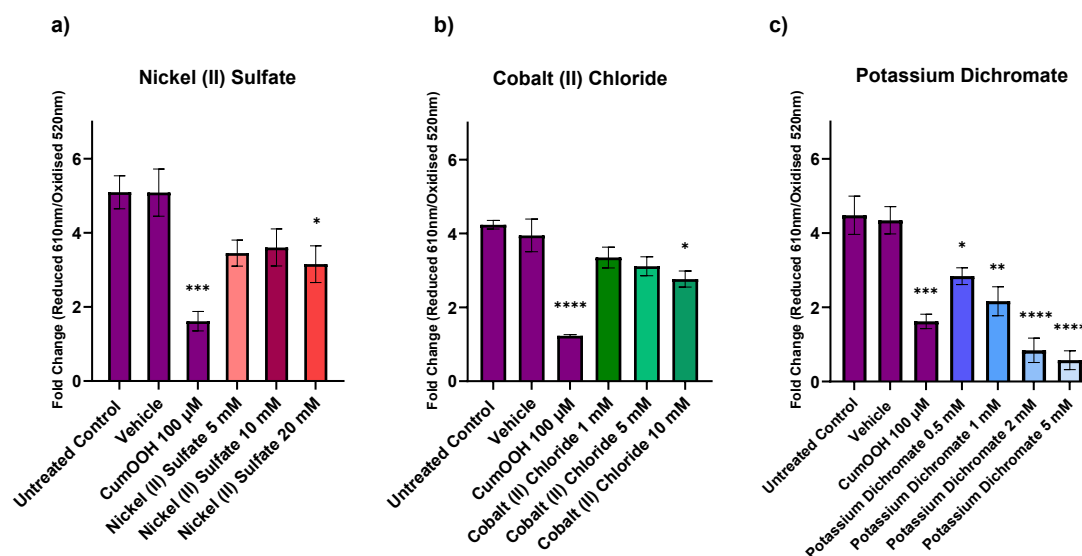


Figure 2.16 Induction of Lipid Peroxidation in HaCaT Cells by Allergenic Metals. HaCaT cells were seeded at a density of 2.0×10^4 cells per well and pre-stained with 10 μ M C11-BODIPY dye for 30 minutes. Cells were then treated with a) nickel (II) sulfate, b) cobalt (II) chloride and c) potassium dichromate in HBSS buffer and incubated for 2 hours. Vehicle = 0.5% DMSO. Results are displayed as fold change ratio, fluorescence of unoxidized (red) fluorescence/fluorescence of oxidised (green) fluorescence and are expressed as mean $\pm$ SEM. Results are analysed using one-way ANOVA with Dunnett's post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ in comparison to untreated control. $N = 3$.

CoCl₂ also only induced significant lipid peroxidation at the highest concentration tested, 10 mM. At lower concentrations, lipid peroxidation was not significant. A concentration dependent decrease in fold change ratio was also observed, with the ratio decreasing as the CoCl₂ concentration increased, indicating that the extent of lipid peroxidation induction is concentration dependent. Additionally, CoCl₂ did not induce lipid peroxidation to the

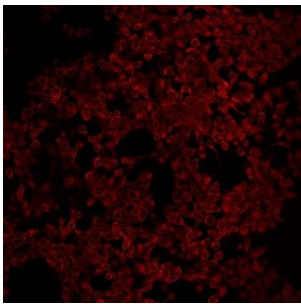
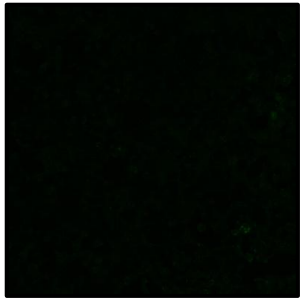
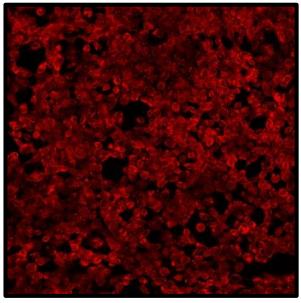
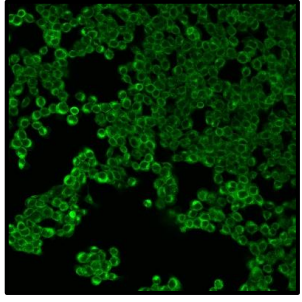
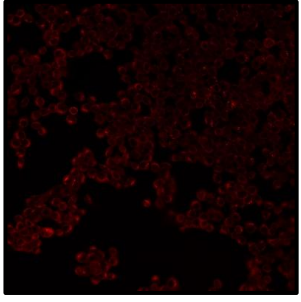
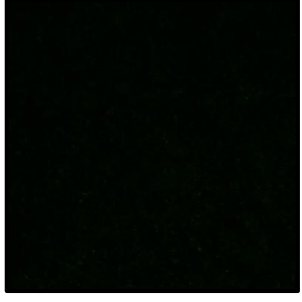
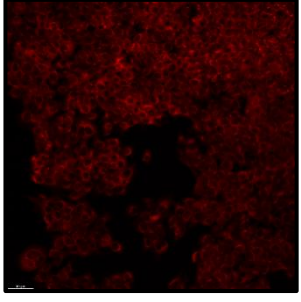
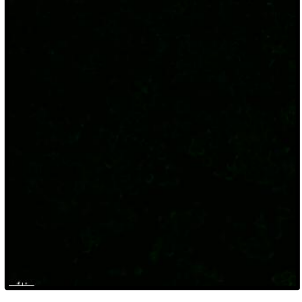
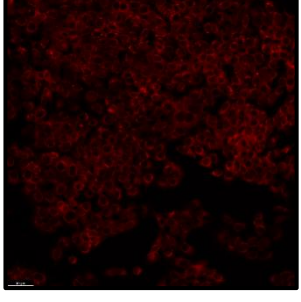
same extent as the CumOOH positive control. The ability of CoCl_2 to induce lipid peroxidation appeared to be stronger in the single lipid model, in which there appeared to be nearly a complete oxidation of the C11-BODIPY dye, in comparison to the HaCaT cell model, albeit the concentration of CoCl_2 used in the single lipid model was a higher concentration (80 mM) than the HaCaT cell model. Never-the-less the results suggested that antioxidant mechanisms in the cells can mitigate lipid peroxidation induction by CoCl_2 .

$\text{K}_2\text{Cr}_2\text{O}_7$ appeared to show the strongest lipid peroxidation ability out of the three allergenic metals. As with CoCl_2 , lipid peroxidation was concentration dependent, increasing as the concentration of $\text{K}_2\text{Cr}_2\text{O}_7$ increased. At the higher concentrations of 2 mM and 5 mM, lipid peroxidation induction appeared to be stronger in comparison to the CumOOH positive control. $\text{K}_2\text{Cr}_2\text{O}_7$ also induced lipid peroxidation at the lower concentrations of 0.5 mM and 1 mM. Its strength as a lipid peroxidation inducer in the HaCaT cells was mirrored in the single lipid assay. In this, $\text{K}_2\text{Cr}_2\text{O}_7$ treatment caused a complete oxidation of the C11-BODIPY dye. This strong lipid peroxidation effect is unsurprising, given $\text{K}_2\text{Cr}_2\text{O}_7$'s classification as an extremely strong oxidising agent²¹⁰, however this is the first observation of its ability to induce lipid peroxidation in HaCaT cells or other keratinocytes.

All three metals induced significant lipid peroxidation, although the extent of the effect appeared to differ between them. The relative levels of lipid peroxidation observed were consistent with trends from the single lipid model and with their reported order of potency as contact allergens. $\text{K}_2\text{Cr}_2\text{O}_7$, classified as the most potent sensitizer, showed the highest lipid peroxidation levels, while NiSO_4 , considered the weakest sensitizer among the three, showed the lowest. The mechanisms behind metal allergen induction of lipid peroxidation likely encompass multiple different pathways. One such possibility is the involvement of metal allergens in Fenton-like reactions. The well-known Fenton reaction involves the iron catalysed conversion of hydrogen peroxide (H_2O_2) into highly reactive hydroxyl radicals²¹¹. Iron is categorized as a redox-active metal, a group which also includes nickel, cobalt and chromium. These redox-active metals can undergo redox cycling reactions, thereby promoting oxidative stress through enhanced ROS generation. Chromium, particularly in its hexavalent form in $\text{K}_2\text{Cr}_2\text{O}_7$, can cause oxidative stress by generating ROS and depleting

cellular antioxidants²¹². Nickel and cobalt ions can induce ROS production through interference with DNA repair, and binding to thiol-like compounds like glutathione (GSH), limiting its antioxidant capabilities and resulting in increased ROS production²¹³. Metals have also been shown to effect cellular mechanisms important for redox balance within cells. Cobalt ions were shown to impair the activity of mitochondrial enzymes in the electron transport chain in macrophages, reducing oxidative phosphorylation and leading to an increase in oxidative stress²¹⁴. Chromium(VI) can inhibit several complexes in the electron transport chain, leading to its dysfunction and an increase in ROS production. Chromium(III) however was not shown to induce this mitochondrial dysfunction²¹⁵.

To visualise induction of lipid peroxidation by the metals, the cells were visualised using confocal microscopy. The cells were seeded on ibidi 8 well μ -slides for microscopy, after which the C11-BODIPY assay was carried out in HBSS as per the manufacturer's protocol. (Figure 2.17).

	Oxidised (Green) Fluorescence @ 520 nm	Unoxidized (Red) Fluorescence @ 610 nm
Untreated Control		
Vehicle		
CumOOH 100 μ M		
Nickel (II) Sulfate 20 mM		
Cobalt (II) Chloride 10 mM		

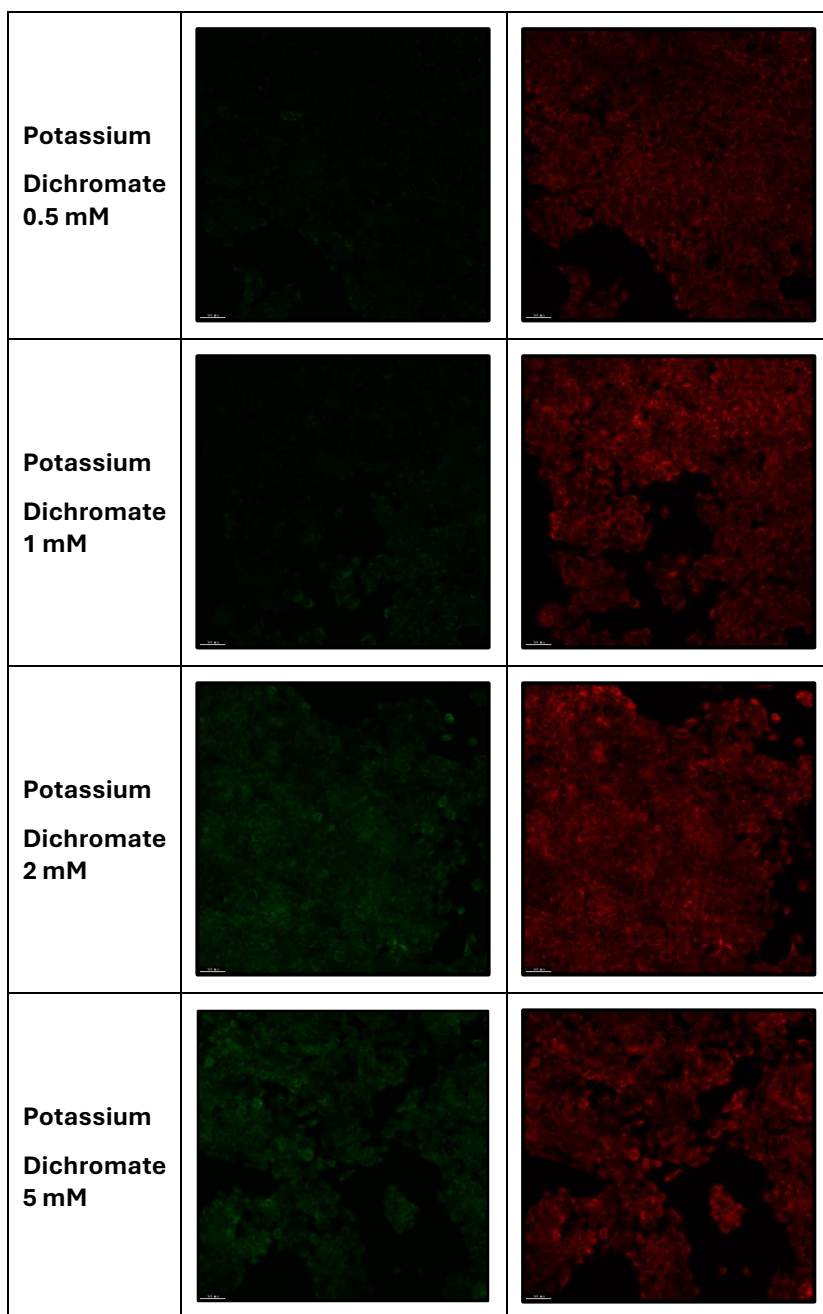


Figure 2.17 Confocal Microscopy Images of C11-BODIPY(581/591) Lipid Peroxidation Assay. HaCaT cells were seeded at a density of 4.0×10^5 cells per well and pre-stained with $10 \mu\text{M}$ C11-BODIPY dye for 30 minutes. Cells were then treated with NiSO_4 , CoCl_2 and $\text{K}_2\text{Cr}_2\text{O}_7$ in HBSS buffer and incubated for 2 hours. Vehicle = 0.5% DMSO. The cells are visualised using confocal microscopy. C11-BODIPY in its reduced, unoxidized state fluoresces red (610 nm), whereas oxidation of the probe during lipid peroxidation shifts the emission to green (520 nm). Thus, increased green fluorescence together with decreased red fluorescence relative to untreated controls indicates lipid peroxidation. $n = 2$; images shown are representative of one biological replicate

Metal concentrations that induced significant differences in the microplate assay (section 2.3.2.2.2) were used in the confocal microscopy assay. Green and red fluorescence were

imaged separately and analysed through image analysis software LAS X. The metal allergens were tested alongside an untreated control, vehicle control (0.5% DMSO) and CumOOH (100 μ M, positive control). When applied to the cells, the C11-BODIPY dye integrates into the cell membranes, which can be clearly seen in the images in Figure 2.17. The visualisation of a diminishing red fluorescence of the dye alongside a brightening green fluorescence indicated lipid peroxidation occurring. A strong increase in green fluorescence was observed upon treatment with the cells with the positive control CumOOH, accompanied by a lower intensity of red fluorescence. NiSO₄ (20 mM) was the only concentration of nickel that had a significant effect in the microplate assay; however, imaging of the cells did not appear to show an increase in green fluorescence in comparison to the untreated control. CoCl₂ (10 mM) also showed significant lipid peroxidation in the microplate assay, but this sample too did not appear to show an increase in green fluorescence upon imaging of the cells, in comparison to the untreated control. The lack of lipid peroxidation detected by confocal microscopy compared with the plate reader assay, could be due to the higher sensitivity of bulk fluorescence measurements achieved with a plate reader, which capture population-wide shifts in C11-BODIPY oxidation, whereas confocal imaging detects only localized changes within a subset of cells and is more limited by signal-to-noise and acquisition settings. All four concentrations of K₂Cr₂O₇ induced significant lipid peroxidation in the microplate assay, and so all four concentrations were included in the cell imaging assay. A concentration dependent effect on lipid peroxidation can be observed, with an apparent increase in green fluorescence intensity being observed as the concentration of K₂Cr₂O₇ increases. At 0.5 mM K₂Cr₂O₇, there did not appear to be an increase in green fluorescence in comparison to the untreated control, however as the concentration increases to 1 mM, the intensity of the green fluorescence appeared to increase. At the higher 2 mM and 5 mM concentrations of K₂Cr₂O₇ there was a visible increase in green fluorescence in comparison to the untreated control, indicating that there was lipid peroxidation taking place at these treatment conditions. This concentration dependent effect was nicely observed in the overlay image for the 5 mM concentration in Figure 2.18. In comparison to the untreated control, more green fluorescence could be seen in the

overlayed image and the intensity of the red fluorescence appeared to have decreased. Although $K_2Cr_2O_7$ treatment induced lipid peroxidation as evidenced by increased green fluorescence and decreased red fluorescence, the effect was less pronounced than that observed with the CumOOH positive control. Notably, CumOOH elicited stronger lipid peroxidation despite being applied at a much lower concentration, indicating that $K_2Cr_2O_7$ is a relatively less potent inducer.

The use of the C11-BODIPY assay in both a single lipid model and a HaCaT cell model, indicated that metal allergens can induce lipid peroxidation. However, further experiments could be carried out to complement these results and confirm lipid peroxidation induction by metals. These approaches could include further lipid peroxidation assays like the Liperfluo lipid peroxidation sensor. The Liperfluo assay is a fluorometric assay that emits intense fluorescence upon specific oxidation by lipid peroxides²¹⁶. The thiobarbituric acid reactive substances (TBARS) assay is a colorimetric assay that quantitatively measures MDA, a key product of lipid peroxidation. This assay, measuring a direct product of lipid peroxidation, could nicely complement the lipid peroxide data generated using the C11-BODIPY assay. Finally, a lipidomic approach using mass spectrometry techniques has previously been used to identify oxidised lipids in fragrance hydroperoxide treated HaCaT cells. This technique was able to identify modified phospholipids that were products of lipid peroxidation. This would provide the strongest complementary approach to the C11-BODIPY assay by identifying specific phospholipids that are peroxidised by metals.

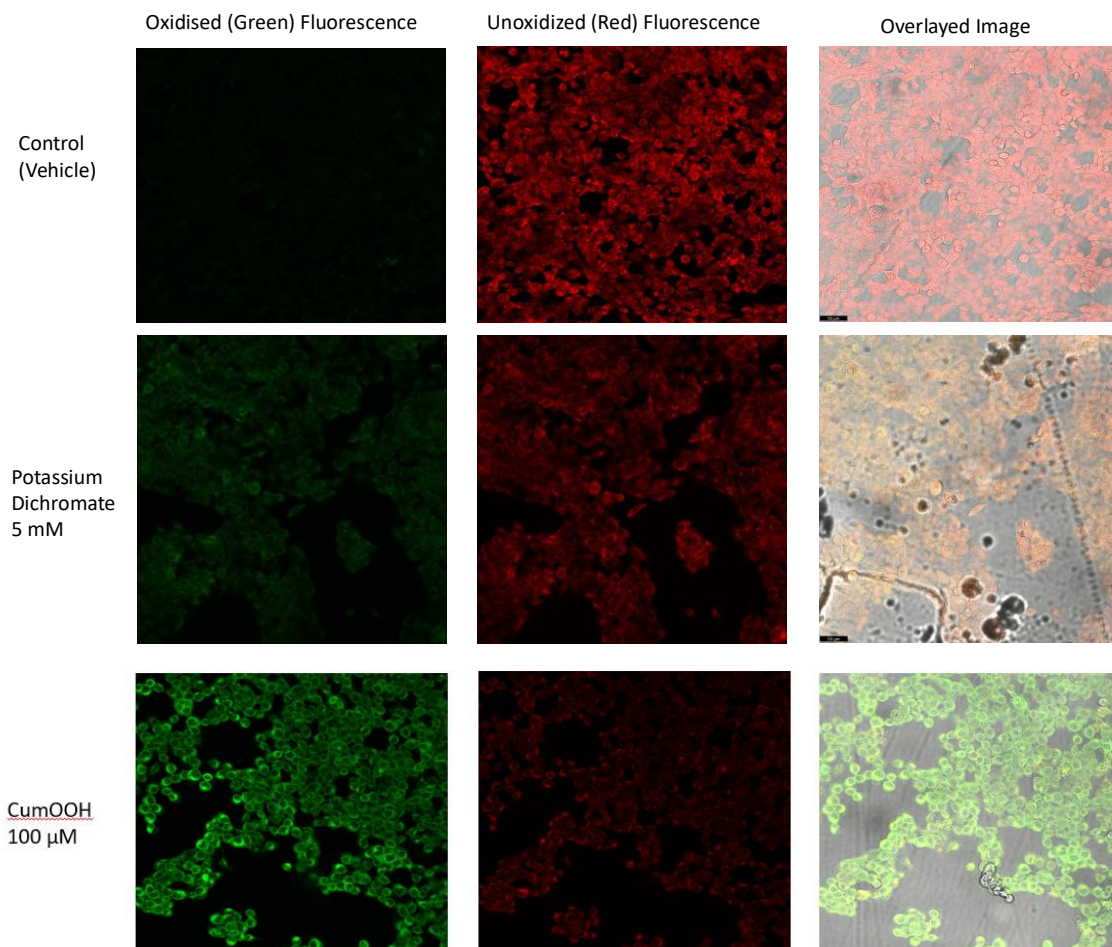


Figure 2.18 Confocal microscopy images showing the increase in green fluorescence and decrease in red fluorescence of C11-BODIPY(581/591) lipid peroxidation detector in cells treated with potassium dichromate (5 mM).

2.3.3 Investigating the effect of metals on ROS production using the H₂DCFDA ROS

Quantification Assay

To compliment the C11-BODIPY(581/591) lipid peroxidation assay, the 2, 7-dichlorodihydrofluorescein diacetate (H₂DCFDA) assay was performed to quantify the total ROS induction in HaCaT cells upon treatment with the metals. H₂DCFDA is a chemically reduced form of fluorescein used as an indicator for ROS in cells. Non-fluorescent H₂DCFDA is taken up by cells, where intracellular esterases cleave the acetate groups, yielding the non-fluorescent intermediate H₂DCF. Subsequent oxidation of H₂DCF by ROS generates the

fluorescent compound DCF which can be detected using a microplate reader (Figure 2.19)²¹⁷. DCFH itself is non-permeable to cell membranes, whereas H₂DCFDA is a diacetate ester form of H₂DCFH which increases its lipophilicity so it can readily cross the cell membrane and detect ROS²¹⁸. Once thought to be specific to H₂O₂ oxidation, it is now understood that H₂DCFH can also be oxidised by hydroxyl radicals, peroxynitrite, superoxide, nitric oxide and lipid peroxides along with H₂O₂²¹⁹.

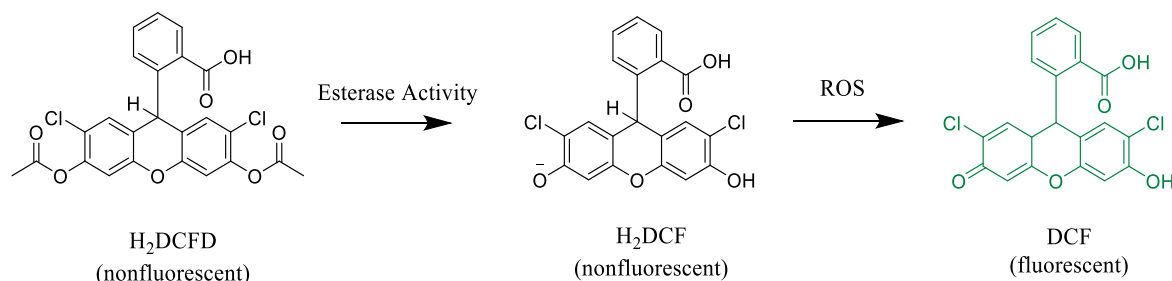


Figure 2.19 Mechanism of H₂DCFDA ROS Detecting Probe. Cell permeable H₂DCFDA undergoes deacetylation by intracellular esterases forming non-fluorescent H₂DCF, which is oxidised by ROS to form the fluorescent compound DCF

HaCaT cells were pre-stained with 20 µM H₂DCFDA, after which the cells were treated with NiSO₄, CoCl₂ or K₂Cr₂O₇ for 2 hours. Alongside the metal treated cells, untreated cells were run as a control, as well as CumOOH (100 µM) and *t*-BuOOH (100 µM) as positive controls as they are known ROS inducers²²⁰. The results of the assay were expressed as fluorescence intensity at 490 nm excitation and 530 nm emission, which can be directly correlated to a quantitative measure of ROS production. An increase in fluorescence intensity indicates an increase in ROS production (Figure 2.20).

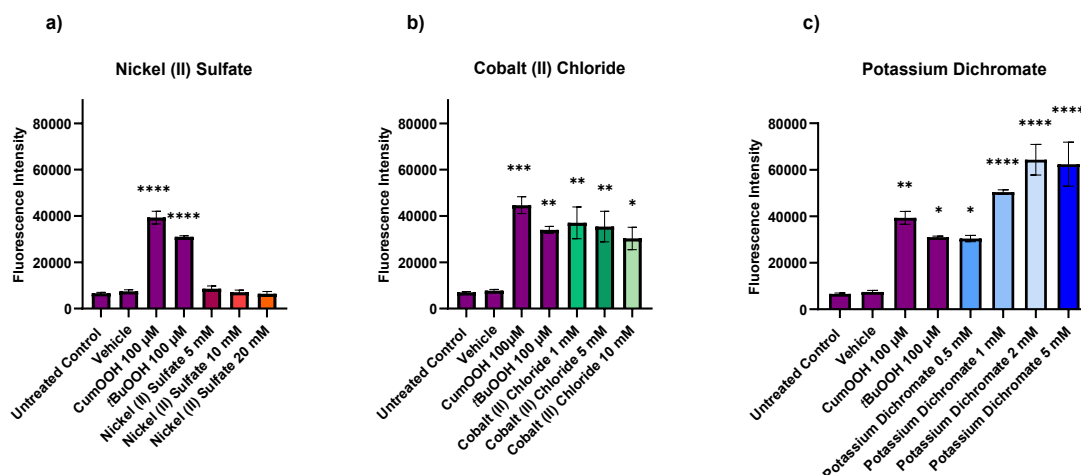


Figure 2.20 ROS Production in HaCaT Cells Treated with Allergenic Metals. HaCaT cells were seeded at a density of 2.0×10^4 cells per well in a 96 well plate. The cells were pre-treated with 20 μ M H_2DCFDA probe for 30 minutes followed by treatment with the metals at the indicated concentrations for two hours. Vehicle = 0.5% DMSO. Results for each concentration are expressed as mean $\pm$ SEM. Statistical analysis was carried out using one-way ANOVA with Tukey's post-hoc test for comparison on means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$, **** $p < 0.001$ in comparison to untreated control. $n = 3$

The positive controls CumOOH and *t*-BuOOH induced a significant increase in ROS production in comparison with the untreated controls. $NiSO_4$ did not induce significant ROS production in HaCaT cells across all concentrations tested. This correlates with the results of the C11-BODIPY(581/591) lipid peroxidation assay, which did not show significant increase in lipid peroxidation at 5 mM or 10 mM $NiSO_4$. It can be concluded that $NiSO_4$ induces minimal ROS and lipid peroxide production in HaCaT cells in these conditions. $CoCl_2$ induced a significant increase in ROS production at all concentrations tested, in comparison to the untreated control. The extent of ROS production appeared similar to that in the CumOOH and *t*-BuOOH controls, and Tukey statistical analysis concluded that there were not significant differences in ROS production between the positive controls and cobalt samples ($p > 0.1$). In the C11-BODIPY assay, there was only a significant increase in lipid peroxidation after treatment with 10mM $CoCl_2$, and not after treatment with lower concentrations of $CoCl_2$. The significant increase in ROS production seen in the H_2DCFDA across all concentrations indicates that while $CoCl_2$ is a strong ROS inducer in HaCaT cells, it is other ROS, for example hydroxyl radicals, nitric oxide or H_2O_2 that oxidises H_2DCF ,

leading to its conversion to the fluorescent DCF molecule. $K_2Cr_2O_7$ induced significant ROS production in HaCaT cells in comparison with the untreated control, across all concentrations tested. A concentration dependent effect was observed, with increasing $K_2Cr_2O_7$ concentration resulting in an increase in ROS production. Tukey analysis also concluded that ROS production by 2 mM and 5 mM concentrations of $K_2Cr_2O_7$ was significantly higher than ROS production induced by both positive controls. This result underlines $K_2Cr_2O_7$'s strength as an oxidising agent which was seen in HaCaT cells previously^{163, 221}. The results of the H₂DCFDA assay correlated with the results of the C11-BODIPY assay. The significant and concentration dependent induction of lipid peroxidation caused by $K_2Cr_2O_7$ treatment mirrors its ROS production ability seen in the H₂DCFDA assay, cementing its categorization as a strong ROS producer and inducer of lipid peroxidation in HaCaT cells.

2.3.4 Adaptation of Viability Assay to HBSS Conditions to Mimic Lipid Peroxidation Assay Conditions

Whereas the initial viability assays (Figure 2.14) were performed in DMEM culture media supplemented with 10% FBS, the lipid peroxidation assay was carried out in HBSS. In order to maintain consistency and support data interpretation the cell viability assay was repeated using HBSS. The assay was carried out similarly to the viability assays described in section 2.3.2.1, except that the metal allergens were added to the cells in HBSS buffer. Cell viability is maintained for 2 – 3 hours in HBSS solution²²² and the 2 hour incubation period also mirrors that of the C11-BODIPY lipid peroxidation assay.

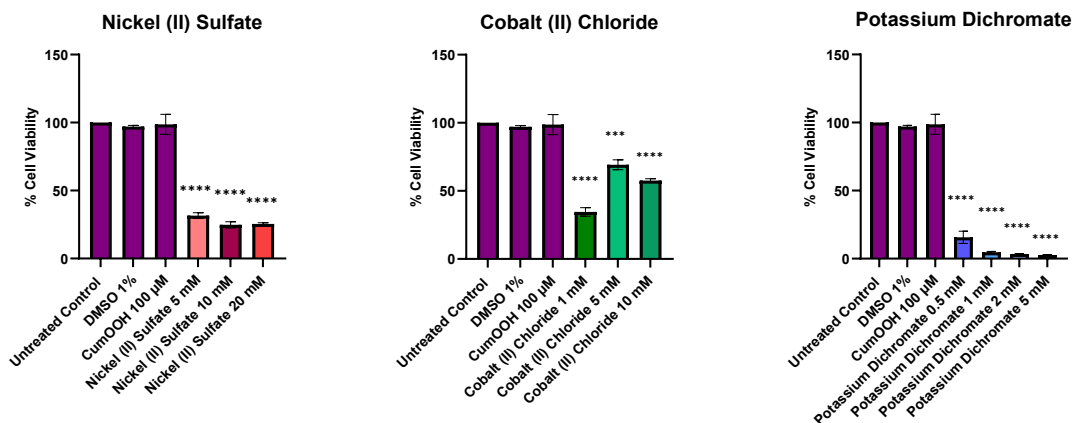


Figure 2.21 Cell Viability Assay Carried Out in HBSS Buffer. HaCaT cells were seeded at a density of 2.0×10^4 cells per well of a 96 well plate. The cells were treated with the metals at the concentrations indicated in HBSS buffer and incubated for 2 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. $n=3$. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. *** $p < 0.001$, **** $p < 0.0001$ in comparison to untreated control

Cell viability was significantly affected by all metals at all concentrations tested. NiSO_4 (5 mM), the lowest concentration tested, reduced cell viability to 30% in comparison with the untreated control, with viability reducing further to 25% to treatment with NiSO_4 at 10 mM and 20 mM. CoCl_2 reduced viability of HaCaT cells to a range of 70% to 30% in comparison to the untreated control. $\text{K}_2\text{Cr}_2\text{O}_7$ exhibited a significant, concentration dependent effect on HaCaT cell viability. This metal also appeared to be the most lethal to the cells, reducing cell viability to 15% after treatment with 0.5 mM. At the higher concentrations of $\text{K}_2\text{Cr}_2\text{O}_7$, cell viability was reduced to between 5% and 2.5% in comparison to the untreated controls. The difference in viability of the metals in HBSS and culture media, could be due to the complexation of metals with by amino acids and serum components that are present in media that reduce metal availability and toxicity. A study by Yang et al. (2013) showed that CHO cells in simple saline/glucose media accumulated 10-fold more nickel compared to cells in complete cell culture medium²²³. This effect is absent in HBSS. This significant impact on cell viability in HBSS, in conjunction with the significant lipid peroxidation, raises queries regarding their correlation and causation. One phenomenon could be causing the other, but it could also be a possibility that the cell viability and lipid peroxidation effects are independent of each other. The pattern between the C11-BODIPY assay, the H_2DCFDA assay

and the viability assay differs between each metal. $K_2Cr_2O_7$ induced significant lipid peroxidation and ROS production, but the viability of the cells was significantly affected. However, $NiSO_4$ showed minimal effect on lipid peroxidation or ROS production and yet still significantly affected the cell viability of the cells after a 2-hour treatment time using HBSS.

To investigate this further, both the cell viability and lipid peroxidation assays were run in culture media over an 18-hour treatment interval. Based on the initial cell viability assays (Figure 2.14), the concentrations tested were reduced for all three allergenic metals. The assays were performed in cell culture medium supplemented with 1% foetal bovine serum (FBS) to minimise serum interference with the C11-BODIPY dye, and similar to previous studies in which 1% FBS was used for viability and lipid peroxidation assays.²²⁴ The differences between the treatment conditions used in the original viability assays (Figure 2.13 and Figure 2.14) and the viability assays in Figure 2.21 and Figure 2.22 are outlined in Table 2.2.

Figure	Medium	Concentration Range of Metals	Incubation Time	Treatments Aspirated
2.13	DMEM with 10% FBS	NiSO ₄ 1.9 – 190 mM CoCl ₂ 0.8 – 80 mM K ₂ Cr ₂ O ₇ 0.2 – 20 mM	4 hours	No
2.14	DMEM with 10% FBS	NiSO ₄ 5 – 20 mM CoCl ₂ 1 – 10 mM K ₂ Cr ₂ O ₇ 0.5 – 5 mM	2 hours	Yes
2.21	HBSS	NiSO ₄ 5 – 20 mM CoCl ₂ 1 – 10 mM K ₂ Cr ₂ O ₇ 0.5 – 5 mM	2 hours	Yes
2.22	DMEM with 1% FBS	NiSO ₄ 1 – 1000 µM CoCl ₂ 1 – 1000 µM K ₂ Cr ₂ O ₇ 1 – 500 µM	18 hours	Yes

Table 2.2 Conditions used in alamarBlue Viability Assays in this chapter

The aim of these experiments was to determine if a concentration existed in which the metals induced lipid peroxidation but did not have a significant effect on HaCaT cell viability (Figure 2.22).

After an 18-hour treatment time in DMEM media supplemented with 1% FBS, NiSO₄ only had a significant effect on cell viability at the highest concentration (1000 µM), with lower concentrations deemed non-cytotoxic to the cells. Similarly, with CoCl₂ treatment, cell viability was only significantly affected at the highest concentration (1000 µM), however there also appeared to be a reduction in viability at a 500 µM concentration. K₂Cr₂O₇ had a significant effect on cell viability in a concentration dependent manner, with statistically significant cytotoxicity observed at concentrations ranging from 500 µM to 50 µM, while concentrations between 20 µM and 1 µM had no effect on cell viability.

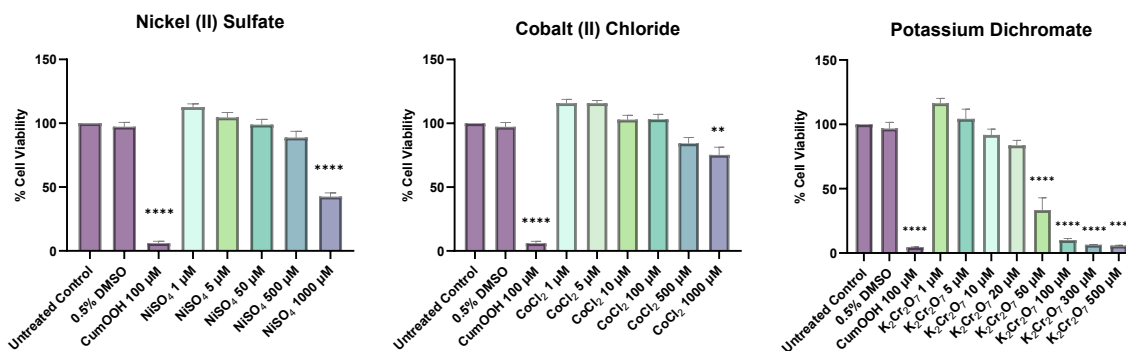


Figure 2.22 Cell viability of HaCaT cells treated with allergenic metals in DMEM supplemented with 1% FBS. HaCaT cells were seeded at a density of 2.0×10^4 cells per well of a 96 well plate. The cells were treated with the allergenic compounds and incubated for 18 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. $n=3$. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. $**p<0.01$, $****p<0.0001$ in comparison to untreated control.

Having established the cytotoxicity of the metals in these conditions, focus then turned to the C11-BODIPY assay to assess whether there was any evidence of lipid peroxidation taking place in the absence of cytotoxicity (Figure 2.23).

In the assay conditions tested, all metals induced a clear dose-response effect. NiSO₄ induced lipid peroxidation at 1000 µM which was cytotoxic to HaCaT cells. At 500 µM, NiSO₄ did not have a significant effect on cell viability, but lipid peroxidation was induced. CoCl₂ induced lipid peroxidation at 1000 µM and 500 µM. CoCl₂ was cytotoxic at 1000 µM, however at a 500 µM, while the viability of the cells was reduced to 84%, the effect was not statistically significant, suggesting the induction of lipid peroxidation at a non-cytotoxic concentration. K₂Cr₂O₇ induced lipid peroxidation at all concentrations that were deemed cytotoxic from the viability assay and also induced lipid peroxidation at the non-toxic concentration of 20 µM. K₂Cr₂O₇ induced the same magnitude of lipid peroxidation as the CumOOH positive control at concentrations between 50 and 500 µM. No lipid peroxidation was induced at the lowest concentrations of K₂Cr₂O₇.

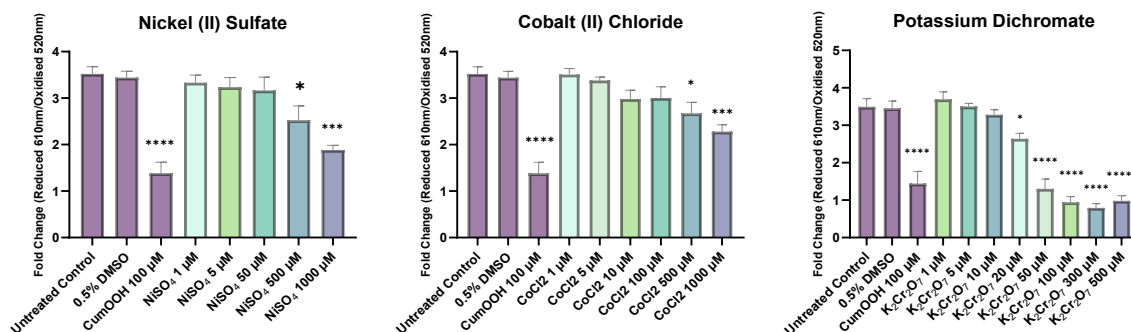


Figure 2.23 Lipid Peroxidation Induction by Allergenic Metals in DMEM supplemented with 1% FBS. HaCaT cells were seeded at a density of 2.0×10^4 cells per well and treated with (a) nickel (II) sulfate, (b) cobalt (II) chloride and (c) potassium dichromate in DMEM media supplemented with 1% FBS and incubated for 18 hours. Following treatment, the cells were then stained with $10 \mu\text{M}$ C11-BODIPY dye for 30 minutes. Results are displayed as fold change ratio, fluorescence of unoxidized (red) fluorescence/fluorescence of oxidised (green) fluorescence and are expressed as mean $\pm$ SEM. Results are analysed using one-way ANOVA with Tukey's post-hoc test for comparison of means. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ in comparison to untreated control.

With all three metals, there was evidence of lipid peroxidation at concentrations that were not toxic to HaCaT cells, indicating that cell death could be at least partially caused by, or a consequence of lipid peroxidation, rather than the other way around. However further investigation is needed to fully establish the relationship between lipid peroxidation and cell death for nickel, cobalt and chromium.

2.4 Conclusions of Chapter

The aim of this chapter was to investigate the ability of metal allergens to induce lipid peroxidation in a single lipid model and in keratinocytes. NiSO₄, CoCl₂ and K₂Cr₂O₇ all induced lipid peroxidation in a single lipid model using linoleic acid. All three allergenic metals also induced significant lipid peroxidation in HaCaT cells, treated in HBSS buffer, after a 2-hour treatment period, with lipid peroxidation induced by K₂Cr₂O₇ visualised using confocal microscopy. Additionally, CoCl₂ and K₂Cr₂O₇ induced significant ROS production in HaCaT cells, however NiSO₄ did not induce ROS production. When the conditions of the lipid peroxidation assay and viability assay were mimicked i.e. treatment in HBSS buffer over 2 hours, the allergenic metals had significant effects on HaCaT cell viability. Treatment of the cells with allergenic metals in DMEM culture media supplemented with 1% FBS over

a longer 18-hour treatment period identified concentrations in which lipid peroxidation was induced but cell viability was not significantly affected.

The findings in this chapter indicate that lipid peroxidation is not merely a secondary effect of cell death but may actively contribute to reduced cell viability following exposure to metal allergens. This suggests that oxidative lipid damage could represent an initiating event in metal-induced cytotoxicity, rather than simply a downstream consequence. To clarify the mechanistic basis of this relationship, it is important to determine whether regulated cell death pathways linked to lipid peroxidation are involved. The next chapter therefore looks to clarify the mechanism of cell death induced by metals, and investigates the potential role of ferroptosis, an iron-dependent form of cell death driven by lipid peroxidation, in mediating the cytotoxic effects of nickel, cobalt, and chromium in keratinocytes

Chapter 3: Investigation of Metal Allergen- Induced Cell Death Mechanisms in HaCaT Keratinocytes

3.1 Introduction

The previous chapter demonstrated that nickel, cobalt, and chromium induce lipid peroxidation in keratinocytes, and that this effect may contribute to reduced cell viability. These findings raised the possibility that a regulated cell death pathway associated with lipid peroxidation could be involved. In this chapter, we firstly assess whether metal induced cell death is apoptotic or necrotic, and whether ferroptosis, an iron-dependent and lipid peroxidation–driven form of regulated cell death, contributes to the cytotoxic effects of these metal allergens.

3.1.1 Mechanisms of Cell Death

Cell death is a biological process that results in the cessation of cell function and eventually cell death²²⁵. Its main purpose is to remove damaged or harmful cells to maintain tissue homeostasis. Cell death can also be triggered in response to injury or disease²²⁶. Cell death was first observed and understood in the 19th century, but it wasn't until the 1960s that several laboratories demonstrated that cell death was biologically controlled²²⁷. In 1972, the laboratory of pathologist John F. Kerr discovered programmed cell death and named it apoptosis²²⁸. Today, cell death is classified into two broad categories, programmed and necrosis²²⁹ (Figure 3.1). Necrosis, or non-programmed cell death, is caused by accidental, traumatic injury and leads to complete rupture of the cell membrane²³⁰. Programmed cell death includes apoptosis and autophagy as well as a recently discovered class of cell death termed programmed necrosis. Autophagy is involved in the regulation of almost all forms of cell death, and involves the encircling of cellular components by autophagic vesicles where they are broken down into small molecules²³¹. Autophagy has roles in protecting cells against various diseases. Programmed necrosis also leads to the compromise of the cell membrane and high levels of inflammation, and includes forms of cell death like necroptosis, pyroptosis and ferroptosis²³². Necroptosis, a programmed form of necrosis, is an inflammatory form of cell death with important functions in host defence against pathogens and tissue homeostasis²³³, while pyroptosis is a highly inflammatory form of programmed cell death, triggered by the detection of pathogens or endogenous danger

signals in the cytosol²³⁴. The ability of metal allergens to induce ferroptosis is the focus of this chapter.

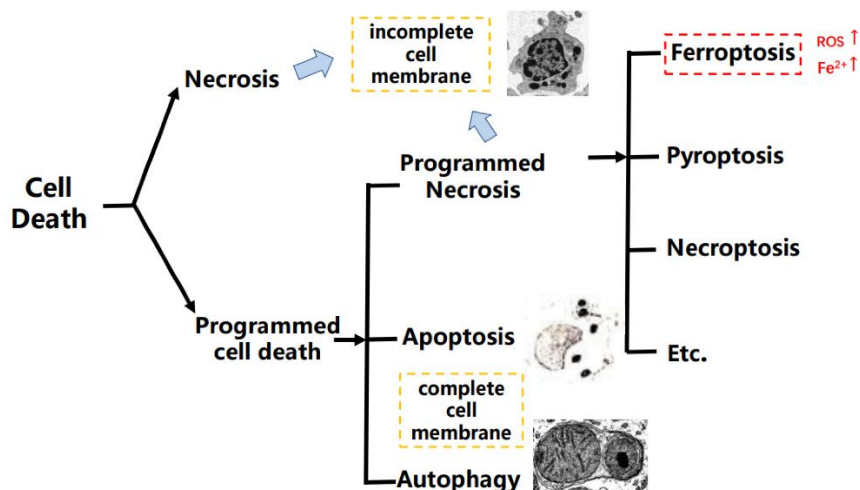


Figure 3.1 Classification of the Mechanisms of Cell Death²³⁵.

3.1.1.1 Cell Death Induced by Metal Allergens

NiSO₄ has been shown previously to induce apoptosis via activation of the mitochondria-dependent and endoplasmic reticulum (ER) stress pathways in human umbilical vein endothelial cells (HUVECs) at concentrations ranging from 62.5 to 1000 μ M²³⁶. Nickel chloride (NiCl₂) was shown to induce mitochondria mediated apoptosis in hepatic cells²³⁷. NiCl₂ has also been shown to induce autophagy in kidney and TCMK-1 cells, which promoted ferroptosis induced by NiCl₂²³⁸.

CoCl₂ has been shown to induce multiple forms of cell death. Both apoptosis and necrosis were induced by CoCl₂ at a range of 5–100 mg/L in cancer cell lines such as A549, PC-3 and IMR-32 after a 12–14-hour incubation time. Additionally, it was found that CoCl₂ induced more cell death in malignant cells in comparison to non-cancerous cells²³⁹. CoCl₂ also induced apoptosis at concentrations up to 300 μ M through mitochondria and death receptor mediated pathways in mouse embryonic stem cells after 48 hours of treatment²⁴⁰. Furthermore, CoCl₂ (300 μ M) induced ROS generation and necroptosis in HT-29 human colon cancer cells after 24 hours treatment²⁴¹.

$K_2Cr_2O_7$ has been shown to induce ROS, leading to DNA strand breaks and mitochondrial injury, as well as increasing lipid peroxidation in lung cell lines, characteristics of ferroptosis²⁴². $K_2Cr_2O_7$ also induces intrinsic and extrinsic pathways of apoptosis in renal proximal tubular cells (HK-2 cells) at 10 μ M. At higher concentrations, $K_2Cr_2O_7$ has been shown to increase a necrotic form of cell death. Concentrations above 30 μ M in HepG2 cells induced significant levels of oxidative stress and DNA damage consistent with necrosis²⁴³.

This chapter records investigations into the mechanism of cell death induced by metals in HaCaT cells, with particular interest in ferroptosis, since these metals were found to strongly induce lipid peroxidation.

3.1.2 Ferroptosis

Ferroptosis is a recently discovered iron and ROS-dependent form of regulated cell death. The term was first coined in 2012 by the lab of Dr Brent Stockwell, in which they concluded that ferroptosis is distinct from other types of regulated cell death such as apoptosis, necroptosis and autophagic cell death at morphological, biochemical, and genetic levels²⁴⁴. It is characterised morphologically by the presence of smaller than normal mitochondria with condensed mitochondrial membrane densities, reduction or vanishing of mitochondrial crista, and outer mitochondrial membrane rupture. The process is characterized by the accumulation of lipid peroxidation products and ROS derived from iron metabolism²⁴⁵ (Figure 3.2).

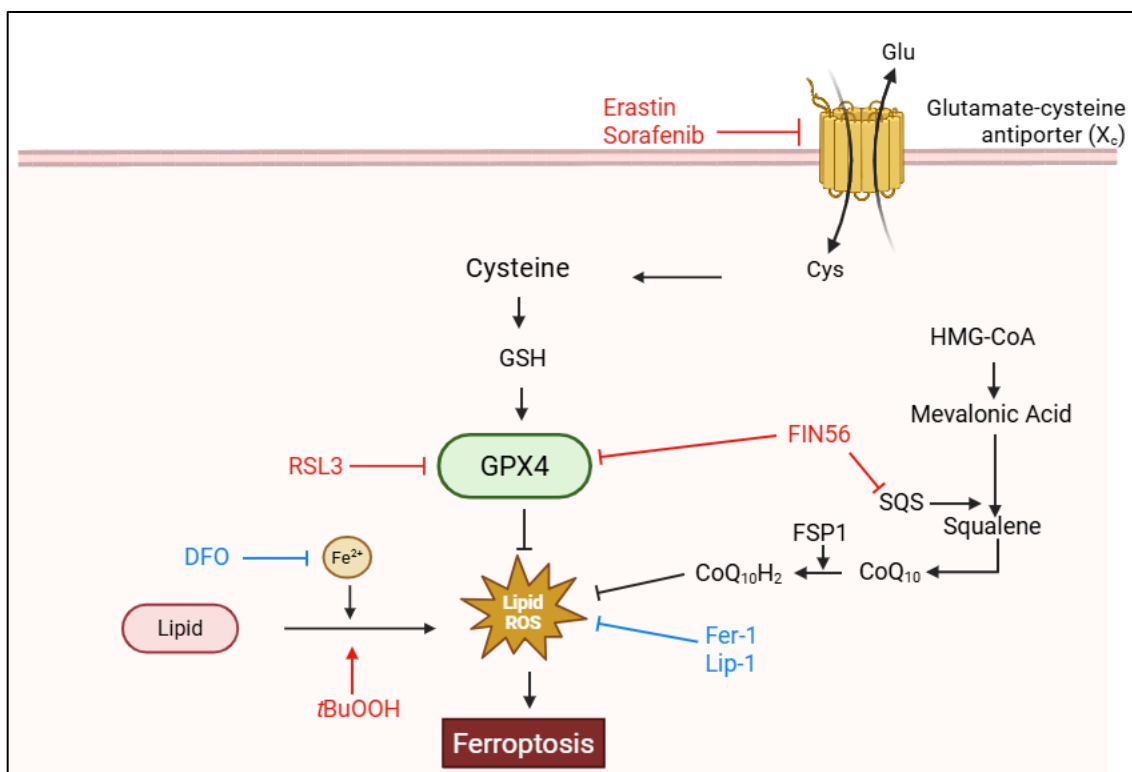


Figure 3.2 Mechanism of Ferroptosis Mechanism of ferroptosis induction in cells. Inducers of ferroptosis are coloured in red, and inhibitors of ferroptosis are coloured in blue. Abbreviations – DFO, deferoxamine; RSL3, RAS synthetic lethal 3; GSH, Glutathione; Fer-1, Ferrostatin-1; Lip-1, Lipoxystatin-1; GPX4, Glutathione Peroxidase 4; Cys, Cysteine; Glu, Glutamate; t-BuOOH, tert-Butylhydroperoxide; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; SQS, Squalene synthase, CoQ₁₀, coenzyme Q₁₀. Image generated using Biorender.

Glutathione peroxidase 4 (GPX4), heat shock protein beta-1 (HSPB1) and nuclear factor erythroid 2-related factor 2 (NFE2L2) function as negative regulators of ferroptosis by limiting ROS production and reducing cellular iron reuptake. Dysregulated ferroptosis has been implicated in multiple physiological and pathological processes such as cancer, tissue injury and T-cell immunity²⁴⁶.

3.1.3 Induction of Ferroptosis

Ferroptosis can be induced by multiple small molecules and drugs, which can be grouped into four categories based on their targets (Figure 3.3).

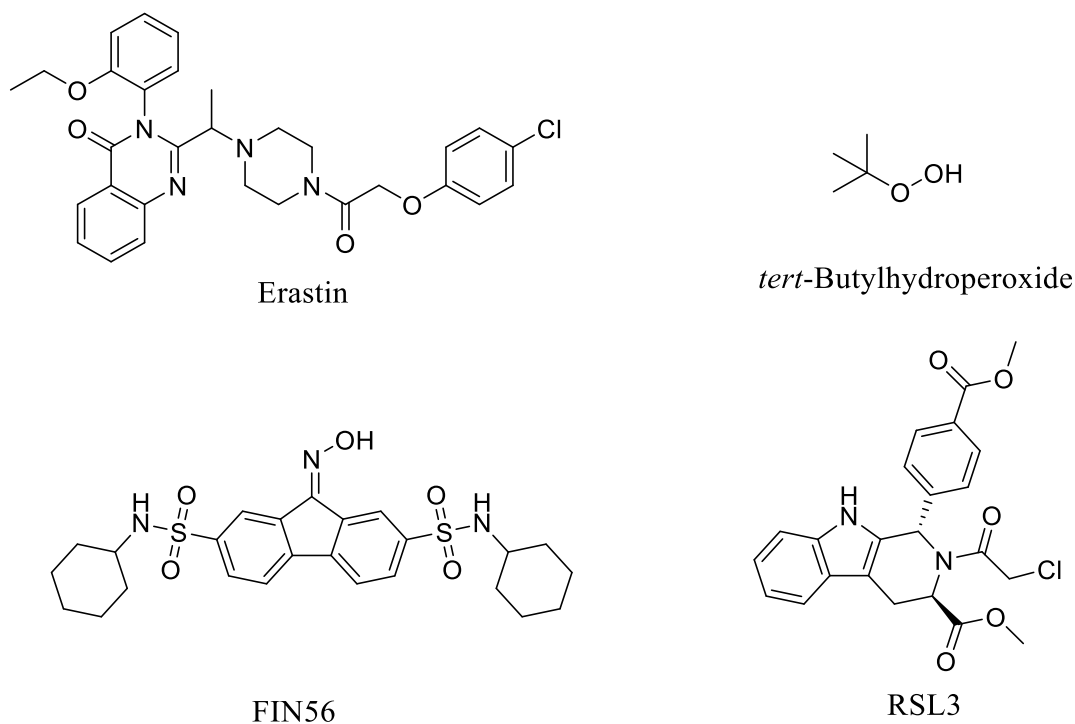


Figure 3.3 Structures of Ferroptosis Inducers

The first group of inducers target iron metabolism. Erastin is a ferroptosis inducer that can increase intracellular labile iron pools, increasing the amount of readily available iron sources within the cell, which is utilized by the cell to generate ROS via Fenton reactions. This leads to an increase in lipid peroxidation and ferroptosis²⁴⁷. Erastin also falls into a second category of ferroptosis inducers which target the glutathione (GSH)/GPX4 axis. Erastin inhibits the cysteine/glutamate antiporter (System Xc⁻) which inhibits the transport of cysteine into the cell²⁴⁸ (Figure 3.2). Cysteine is essential for the synthesis of GSH, an antioxidant. A reduction in cysteine within the cell leads to reduced GSH levels, which exacerbates oxidative stress and lipid peroxidation. Another ferroptosis inducer sorafenib also works in the same way²⁴⁹. RAS synthetic lethal 3 (RSL3) is another inducer that falls into this category. RSL3 acts on GPX4, an enzyme that protects cells against lipid peroxidation by detoxifying lipid peroxides²⁵⁰ (Figure 3.2). Inhibition of GPX4 leads to the accumulation of lipid peroxides in the cell leading to ferroptosis. The third category of ferroptosis inducers is those that affect lipid metabolism. *t*-BuOOH falls into this category, exerting its effect through the oxidation of cardiolipins and PUFAs, triggering ferroptosis²⁵¹ (Figure 3.2).

Finally, there are ferroptosis inducers that target ferroptosis suppressor protein 1/coenzyme Q10 (FSP1/CoQ) related pathways. FIN56 is one of these inducers, binding to squalene synthase, leading to the depletion of CoQ and increase in lipid peroxidation levels²⁵² (Figure 3.2).

3.1.4 Inhibition of Ferroptosis

Ferroptosis inhibitors mainly fall into two categories, those that reduce iron levels, and those that reduce the levels of lipid peroxides²⁴⁵.

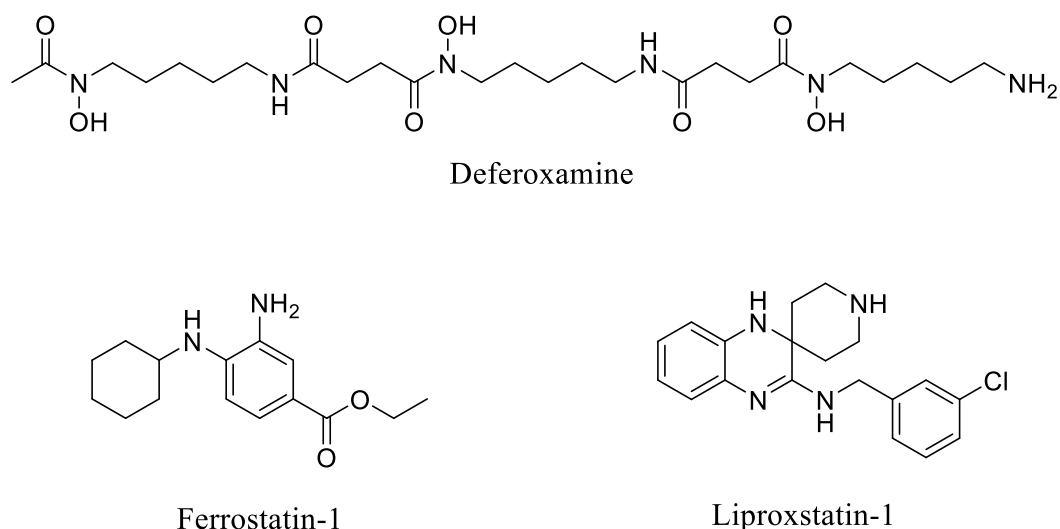


Figure 3.4 Structures of Ferroptosis Inhibitors.

Deferoxamine (DFO) is an iron chelator that can inhibit ferroptosis (Figure 3.4). DFO reduces the level of iron available in cells and upregulates intracellular levels of GPX4, which leads to reduced levels of lipid peroxides and inhibition of ferroptosis²⁵³. DFO has been reported to have a positive therapeutic effect on traumatic spinal cord injury²⁵⁴. Inhibitors that reduce levels of lipid peroxides include ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1) (Figure 3.4). Both Fer-1 and Lip-1 are radical scavenging agents²⁵⁵. Structure activity relationship (SAR) studies determined that the presence of the N-cyclohexyl moiety in their structure acts as a lipophilic anchor and is crucial to its mechanism of action²⁵⁶. Both Fer-1 and Lip-1 reduce overall intracellular oxidative stress and slow the accumulation of lipid hydroperoxides. It has also been reported that Fer-1 and Lip-1 can have an inhibitory effect on lipoxygenases²⁵⁷

3.1.5 Ferroptosis in Human Disease

Ferroptosis has been shown to contribute to the pathogenesis of several diseases such as cancer, ischemia, neurodegenerative diseases including Alzheimer's disease and Parkinson's disease, and dermatological diseases such as psoriasis²⁵⁸. Cancer cells accumulate high levels of iron compared to normal cells, leading to abnormalities in iron homeostasis²⁵⁹. Additionally, ferroptosis appears to function as an innate mechanism for tumour suppression, mediating the anticancer activity of several tumour suppressor genes like p53 and Kelch-like ECH-associated protein 1 (KEAP1) which partially suppress tumours by inducing ferroptosis in tumour cells²⁶⁰. Ferroptosis has also been used as target for cancer treatment, with drugs like 5-fluorouracil (5-FU) and paclitaxel linked with elevated ROS and depletion of GSH, leading to increased lipid peroxidation and ferroptosis^{261, 262}.

As mentioned previously in Chapter 2, lipid peroxidation has been identified as a hallmark characteristic in numerous neurodegenerative processes. In addition, however, iron accumulation in the brain regions affected by neurological diseases has also been reported^{263, 264}. Iron chelators have been trialled in Alzheimer's disease and Parkinson's disease, with deferiprone shown to alleviate neurological symptoms²⁶⁵ and have overall beneficial effects for Parkinson's patients in phase II studies²⁶⁶. This accumulation of iron is accompanied by increased oxidative stress which could lead to ferroptosis in neurodegenerative diseases²⁵⁸.

Ferroptosis has been implicated in the progression of various skin conditions including psoriasis, photosensitive dermatitis and melanoma²⁶⁷. In psoriasis, the keratinocytes within psoriatic lesions display molecular markers of ferroptosis, abnormalities in lipid metabolism and an increased activation of lipid oxidation pathways in comparison to healthy skin²⁶⁸. Additionally, the ferroptosis inhibitor Fer-1 can reduce inflammatory responses in keratinocytes associated with ferroptosis²⁵⁵. Ferroptosis has also been implicated in photosensitive dermatitis, as UV radiation exposure causes the accumulation of ROS and lipid peroxides in keratinocytes²⁶⁹. In addition, Fer-1 and Lip-1 can suppress UVB stimulation-induced keratinocyte ferroptotic cell death²⁷⁰. The established involvement of

ferroptosis in inflammatory skin conditions supports the hypothesis of its involvement in the pathophysiology of ACD, in particular due to the strong ability of allergenic metals to induce lipid peroxidation observed in the previous chapter.

3.2 Aims and Objectives

The aims of this chapter are to investigate the mechanism of HaCaT cell death induced by NiSO_4 , CoCl_2 and $\text{K}_2\text{Cr}_2\text{O}_7$, and its relationship to lipid peroxidation. As $\text{K}_2\text{Cr}_2\text{O}_7$ induced the most significant lipid peroxidation in the previous chapter, there was particular interest in its ability to induce ferroptosis in HaCaT cells.

The objectives to fulfil this aim include:

- Identifying whether metals induce apoptotic or necrotic cell death in keratinocytes using the Annexin-V PI assay, and by examining DNA fragmentation. The mechanism of cell death in keratinocytes in the context of contact allergy has not been studied before.
- Using ferroptosis inhibitors to investigate if metal allergens induce ferroptosis

3.3 Experimental Details

3.3.1 HaCaT Cells

HaCaT cells were selected for these experiments to maintain continuity with the previous experimental model, ensuring consistency in cell type across both sets of studies. The cells were cultured in the same way described in section 2.3.

3.3.2 Annexin V/PI Assay

The first step in identifying the method of HaCaT cell death induced by metal allergens was to establish whether the cell death was apoptotic or necrotic, or a combination of both. The Annexin V/PI assay was carried out using the FITC Annexin V Apoptosis Detection Kit with PI (640914, BioLegend). HaCaT cells were seeded at a density of 5×10^6 cells per well in 2 mL cell culture media in a 6 well plate and incubated overnight. Thereafter, the cells were treated with metals alongside untreated and vehicle controls. The assay was performed at two time points: 6 hours and 18 hours.

After the treatment time had elapsed, the cells were harvested for staining and flow cytometry. The cell culture media from each well was pipetted into labelled 15 mL Falcon tubes. Trypsin-EDTA solution was added to detach the cells from the wells, and the cells were added to their respective labelled Falcon tube. Each well was rinsed with PBS (1 mL), and the samples were centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in Annexin V binding buffer provided in the kit, at a concentration of 0.25×10^7 cells/mL. The samples were then transferred to labelled 1.5 mL Eppendorf tubes. The samples were centrifuged at 900 rpm for 5 minutes and the supernatant was discarded. FITC Annexin V (5 μ L) was added to each sample and the samples were incubated in the dark for 15 minutes at room temperature. Annexin V binding buffer (100 μ L) was added to each sample along with propidium iodide (PI) 1mg/mL (10 μ L) just prior to acquisition on flow cytometry. The data was acquired using a BD Accuri C6 flow cytometer. 10,000 events were acquired per sample. Compensation controls were prepared alongside the samples using compensation beads (OneComp eBeads™ Compensation Beads, 01-1111-42, Thermofisher Scientific) for the FITC compensation sample. For the PI compensation, a sample was prepared that contained a 1:1 ratio of dead cells (killed by heating of the cells to 65°C for 1 minute followed by immediate cooling in ice for 1 minute), and live cells. The fluorochromes were compensated on the flow cytometer prior to acquisition of the samples. The data was analysed using FlowJo LLC version 10.10.0 flow cytometry analysis software.

3.3.3 PI Cell Cycle Analysis for Detection of Apoptosis

HaCaT cells were seeded at a density of 5×10^6 cells in 2 mL culture media per well in a 6 well plate and incubated overnight. Thereafter, the cells were treated with metal allergens alongside untreated and vehicle controls and incubated for 24 hours. After the treatment time had elapsed the cells were harvested for staining and flow cytometry. The cell culture media from each well was pipetted into labelled 15 mL Falcon tubes. Trypsin-EDTA solution was added to detach the cells from the wells, and the cells were transferred to their respective labelled Falcon tube. Each well is rinsed with PBS (1 mL), and the samples were centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and the cells were

resuspended in Annexin V binding buffer provided in the kit, at a concentration of 0.25×10^7 cells/mL. The samples were then transferred to labelled 1.5 mL Eppendorf tubes. The samples were centrifuged at 900 rpm for 5 minutes and the supernatant was discarded. The cells were fixed by adding 1 mL of 70% v/v ice-cold ethanol dropwise to the samples, mixing frequently. The samples were left on ice for 30 minutes to ensure fixing of the cells. After fixing, the cells were washed with PBS and centrifuged at 900 rpm for 5 minutes. The cells were then resuspended in staining buffer, containing 100 µg/mL RNase (Life Technologies, R1253), 50 µg/mL PI solution (Biolegend, 421301) and PBS. The cells were incubated overnight at 4°C. The samples were then analysed using a BD Accuri C6 flow cytometer and the results analysed using FlowJo version 10.10.0 software.

3.3.4 Assay Using Ferroptosis Inhibitors to Assess Metal-Induced Ferroptosis

Two ferroptosis inhibitors, Fer-1 (Sigma-Aldrich, SML0583-5MG) and DFO (Sigma-Aldrich, D9533-1G), were used to investigate the induction of ferroptosis by allergenic metals. Fer-1 was prepared in DMSO and DFO was prepared in sterile deionised water. HaCaT cells were seeded at a density of 1×10^3 cells in 100 µL of culture media per well of a 96 well plate and incubated overnight at 37°C and 5% CO₂. The cells were then pretreated with either DFO in the concentration range of 50 µM - 250 µM, or Fer-1 in the range of 5 µM – 20 µM for 30 minutes at 37°C and 5% CO₂. The metals were then added directly to the well. The cells were treated with the metals at two time points, 6 hours and 18 hours. Untreated controls and vehicle controls were also included in the experiment. After the treatment time had elapsed, the treatments were aspirated from the wells and the wells were washed with sterile PBS. A 10% solution of alamarBlue was prepared using DMEM as a diluent. 100 µL of the diluted alamarBlue was added into each well and the plate was incubated in the dark at 5% CO₂ and 37 °C for 2 – 4 hours. Fluorescence of the dye was measured using a Fluostar Omega microplate reader at an excitation wavelength of 540 nm excitation and 590 nm emission. Results were standardised to the untreated control and expressed as % viability. No more than 0.5% DMSO was present in the samples. In addition, erastin and *t*-BuOOH (known ferroptosis inducers) were included as positive controls.

3.3.5 Concomitant Ferroptosis and Apoptosis Inhibition Assays

In these experiments, the ability of metals to induce apoptosis was investigated, and in addition experiments with both apoptosis inhibitors and ferroptosis inhibitors used concomitantly carried out. HaCaT cells were seeded at a density of 1×10^3 cells in 100 μL culture media per well of a 96 well plate and seeded overnight at 37°C and 5% CO_2 . The cells were then pre-treated for 30 minutes in the presence of 40 μM z-VAD-FMK (BD Biosciences, 550377) prepared in DMSO and DFO in various combinations and at various concentrations. These are outlined in table 3.1 below.

DFO 100 μM
DFO 250 μM
DFO 100 μM + z-VAD-FMK 40 μM
DFO 250 μM + z-VAD-FMK 40 μM

Table 3.1 Pre-treatments used in the Ferroptosis Inhibition Assays

After pre-treatment, metals were added to the well. The cells were treated with $\text{K}_2\text{Cr}_2\text{O}_7$ 100 μM or NiSO_4 1000 μM at two time points, 6 and 18 hours. After the treatment time had elapsed, the treatments were aspirated from the wells and the wells were washed with sterile PBS. A 10% solution of alamarBlue was prepared using 0% DMEM as a diluent. 100 μL of the diluted alamarBlue was added into each well and the plate was incubated in the dark at 5% CO_2 and 37°C for 2 – 4 hours. Fluorescence of the dye was measured using a Fluostar Omega microplate reader at an excitation wavelength of 540 nm excitation and 590 nm emission. Results are standardised to the untreated control and expressed as % viability. Controls included in these experiments were untreated controls, vehicle controls and *t*-BuOOH 100 μM prepared in sterile deionised water as a positive control. The vehicle controls contained the equivalent amounts of water and DMSO that were present in the metal samples. No more than 0.5% DMSO was present in the samples.

3.3.6 Statistical Analysis

The data for all experiments in this chapter was assessed using GraphPad Prism 10 for Windows (GraphPad software, USA). An ordinary one-way ANOVA, or two-way ANOVA, test

was used to analyse the data, with Tukey's multiple comparisons post-hoc test used to compare means. The mean $\pm$ SEM is displayed on the histograms of the results, and a p -value of less than 0.05 was considered to be statistically significant.

3.4 Results and Discussion

3.4.1 Mechanism of Metal Induced HaCaT Cell Death as Apoptotic or Necrotic

3.4.1.1 Annexin-V/PI Assay

The most popular assay described for discriminating apoptosis from necrosis is the Annexin-V/PI assay. In viable cells, PS is located on the inner leaflet of the plasma membrane. However, during early apoptosis, PS translocates to the outer leaflet and becomes exposed on the cell surface²⁷¹. Fluorescent conjugated Annexin-V has high binding affinity to PS and so is used to detect apoptotic cells using flow cytometry.

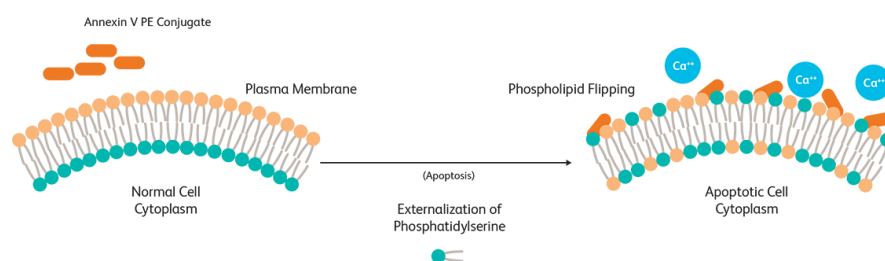


Figure 3.5 Phosphatidylserine exposure and recognition by Annexin-V²⁷²

In this assay, the cells are co-stained with PI, a cell impermeable nuclear dye, that can identify cells with loss of membrane permeability which is a hallmark of necrosis but also present in late apoptotic cells²⁷³. To differentiate between apoptotic and necrotic cells, the assay can be run at two different timepoints; an earlier 6-hour time point and a later 18-hour timepoint. These timepoints were chosen to enable identification of early apoptotic cells at an earlier timepoint of 6 hours which would then progress to late apoptotic cells which were identifiable at 18 hours. This was essential in differentiating whether the type of cell death is apoptotic or necrotic. Membrane changes leading to PS exposure occur rapidly in apoptotic cells and are visible at the early timepoint as Annexin V positive and PI negative (Q3). Upon progression of apoptosis, apoptotic cells will lose their membrane

integrity at a later timepoint and appear as both Annexin V positive and PI positive (Q2). In contrast, during necrosis PS exposure coincides with permeabilization of the outer cell membrane, and cells immediately become positive for both Annexin V and PI (Q2) and bypass the intermediate Annexin V positive/PI negative stage (Q3) (Figure 3.6).

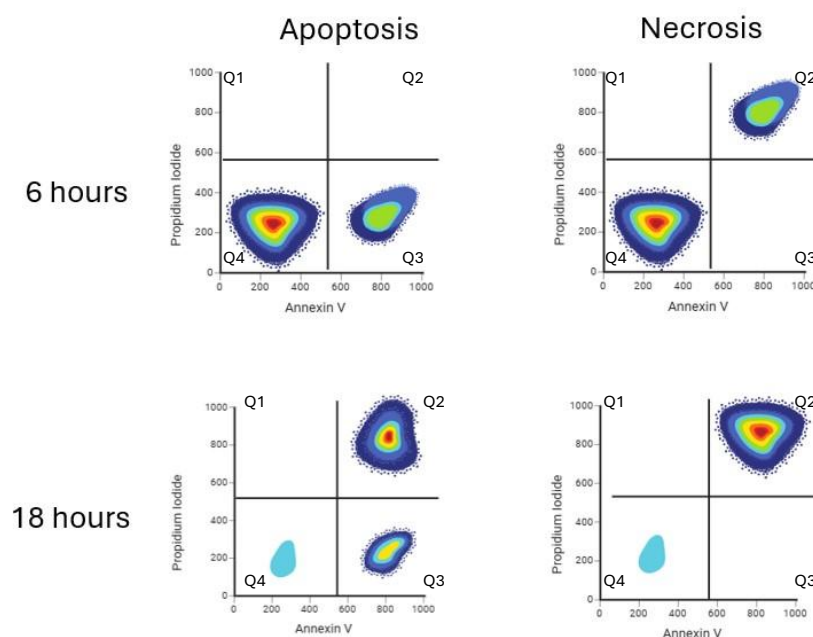


Figure 3.6 Annexin-V/PI assay to differentiate between apoptosis and necrosis

The Annexin-V PI assay was performed using the metals NiSO_4 , CoCl_2 and $\text{K}_2\text{Cr}_2\text{O}_7$, at a 6-hour time point and an 18-hour time point to investigate whether the cells pass through the Annexin-V positive PI negative intermediate stage characteristic of apoptosis, or progress directly to the Annexin-V positive PI positive stage characteristic of necrosis (Figure 3.7). The Annexin V/PI assay was performed with $\text{K}_2\text{Cr}_2\text{O}_7$ (20 μM – 300 μM). These concentrations were chosen as $\text{K}_2\text{Cr}_2\text{O}_7$ was shown to affect HaCaT cell viability at these concentrations in the alamarBlue assay (Figure 2.22). The Annexin V/PI assay showed that $\text{K}_2\text{Cr}_2\text{O}_7$ had a concentration dependent effect on HaCaT cell viability at both the 6 and 18-hour timepoints. This correlates with the alamarBlue viability assays discussed in section

2.22. In the cells treated with $K_2Cr_2O_7$ (300 μM) 0.73% of cells were in the early apoptotic stage, while 4.7% of cells were in the necrotic stage and 30% were in the late apoptotic/necrotic stage [Figure 3.7(a)]. At 18 hours, similarly to the 6-hour timepoint, there were minimal cells passing through the early apoptotic stage in any of the $K_2Cr_2O_7$ treated samples, with many cells in the necrotic stage (Q1) or the late apoptotic/necrotic stage (Q2) [Figure 3.7(b)].

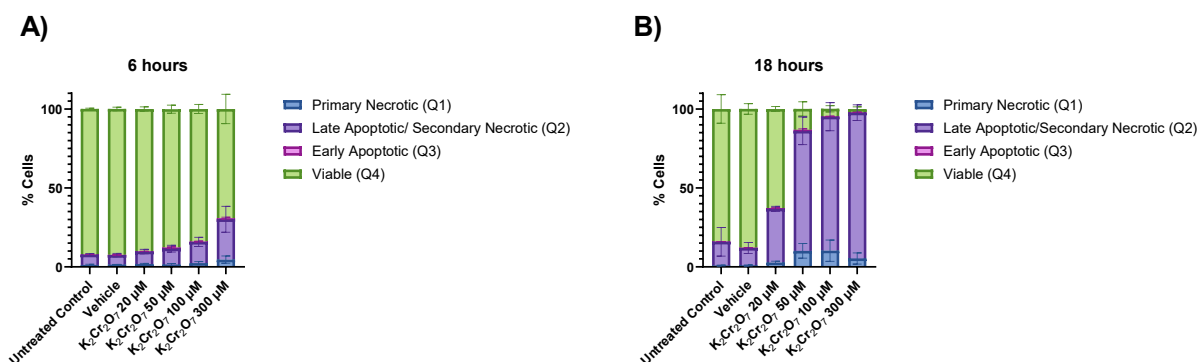


Figure 3.7 Annexin-V/PI Assay with $K_2Cr_2O_7$ for differentiation between Apoptosis and Necrosis. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with $K_2Cr_2O_7$ at concentrations indicated and incubated for A) 6 hours and B) 18 hours. Vehicle was an equivalent amount of sterile deionised water added to cell culture media. The cells were stained with 5 μL Annexin-V per sample and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. $n=3$.

The lack of cells in the early apoptotic stage at both timepoints indicated a necrotic form of cell death. The flow cytometry dot plots of one replicate of the $K_2Cr_2O_7$ treated samples at the 6-hour and 18-hour timepoints are displayed below. (Figure 3.8).

At 6 hours, across all concentrations, cells transitioned directly from Q4 (viable; Annexin V^-/PI^-) to Q2 (late apoptotic/necrotic; Annexin V^+/PI^+), with very few detected in early apoptosis (Q3, Annexin V^+/PI^-). By 18 hours, the proportion of non-viable cells increased further, with more cells in Q1 (necrotic; Annexin V^-/PI^+) compared with controls, and many appearing to progress into Q2. The scarcity of early apoptotic cells at both time points supports necrosis, rather than apoptosis, as the predominant mechanism of cell death.

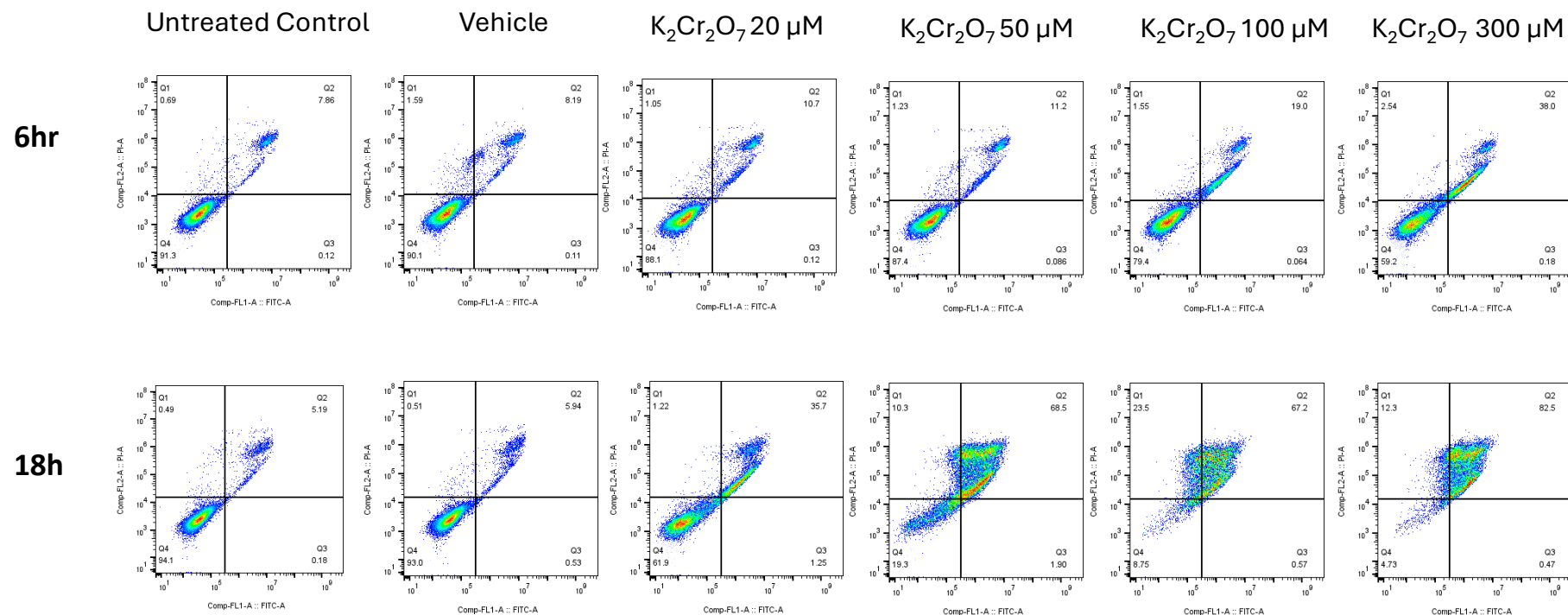


Figure 3.8 Annexin-V/PI Assay with $K_2Cr_2O_7$. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with $K_2Cr_2O_7$ at concentrations in the range of 20 μM – 300 μM and incubated for two timepoints – 6 hours and 18 hours. The cells were stained with 5 μL Annexin-V per sample and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. The images are representative of one replicate. $n=3$. The data was analysed using FlowJo version 10.10.0 software.

Cobalt (II) chloride treatment also induced a concentration dependent effect on cell viability, as assessed by the Annexin-V/PI assay (Figure 3.9). At 6 hours, viability was largely unaffected: 0.14% of cells in the vehicle control, 0.31% in CoCl₂ (500 µM), and 0.51% in CoCl₂ (1000 µM) were in early apoptosis, with no significant differences compared to controls. The majority of cells remained viable (Q4), with only minimal increases in necrotic cells (Q1). After 18 hours, CoCl₂ treatment markedly reduced viability. In the late apoptotic/necrotic stage (Q2), 40% of cells were detected at 500 µM and 47% at 1000 µM, compared with 12% in the vehicle control. Despite this loss of viability, the proportion of early apoptotic cells (Q3) remained very low across all conditions.

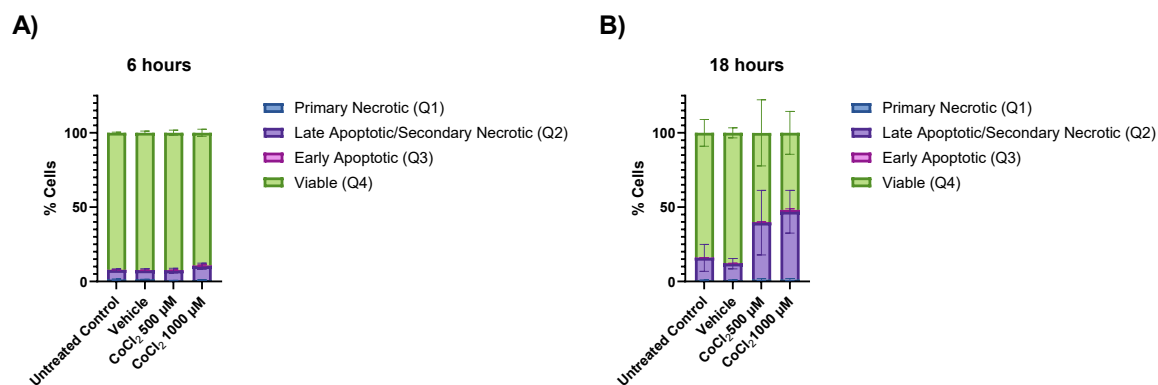


Figure 3.9 Annexin V/PI assay performed with CoCl₂ to differentiate between apoptotic and necrotic cell death. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with CoCl₂ at concentrations indicated and incubated for A) 6 hours and B) 18 hours. Vehicle was an equivalent amount of sterile deionised water added to cell culture media. The cells were trypsinized and stained with 5 µL Annexin-V per sample for 15 minutes in the dark and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. $n=3$.

The flow cytometry plots (Figure 3.10) illustrate this shift in cell populations. At 6 hours, most cells clustered in Q4, (viable cells; Annexin V⁻/PI⁻), with only a slight redistribution into Q1 (necrotic; Annexin V⁻/PI⁺). By 18 hours, however, a pronounced redistribution was observed, with large proportions of cells occupying Q1 (necrotic; Annexin V⁻/PI⁺) and Q2 (late apoptotic/necrotic; Annexin V⁺/PI⁺). The consistent absence of cells in Q3 (early apoptosis; Annexin V⁺/PI⁻) across concentrations and time points indicates that cells bypassed the early apoptotic phase and transitioned directly into necrotic stages. This

pattern supports necrosis, rather than apoptosis, as the predominant mechanism of CoCl_2 -induced cell death.

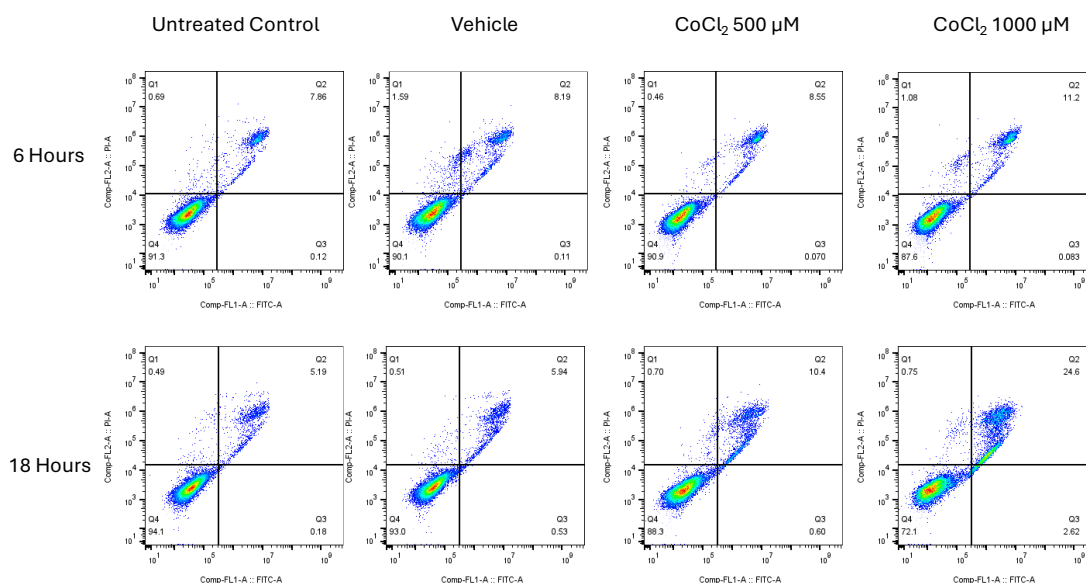


Figure 3.10 Annexin-V/PI Assay with CoCl_2 . HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with CoCl_2 at concentrations of 500 μM and 1000 μM and incubated for two timepoints – 6 hours and 18 hours. The cells were stained with 5 μL Annexin-V per sample and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. The assay was run in triplicate and data was analysed using FlowJo version 10.10.0 software.

The Annexin V/PI assay was performed with NiSO_4 (500 and 1000 μM), concentrations selected based on their effects on viability in the alamarBlue assay (Figure 2.22). At 6 hours, neither concentration significantly reduced viability compared with untreated or vehicle controls [Figure 3.11(A)]. The proportion of early apoptotic cells also remained low, with 0.055% in the vehicle control, 0.36% in NiSO_4 (500 μM), and 0.69% in NiSO_4 (1000 μM). Most cells were viable (Q4), and few were detected in the early apoptotic quadrant (Q3). At 18 hours, NiSO_4 caused a concentration-dependent reduction in viability, with only 16% of cells remaining viable at 1000 μM [Figure 3.11(B)]. This was accompanied by an increase in necrotic cells, with 13% of cells in Q1 compared to the vehicle control. Finally, there was no significant increase in the proportion of cells in the early apoptotic phase in the NiSO_4

(1000 μM) and NiSO_4 (500 μM) treated cells in comparison to the untreated or vehicle treated controls.

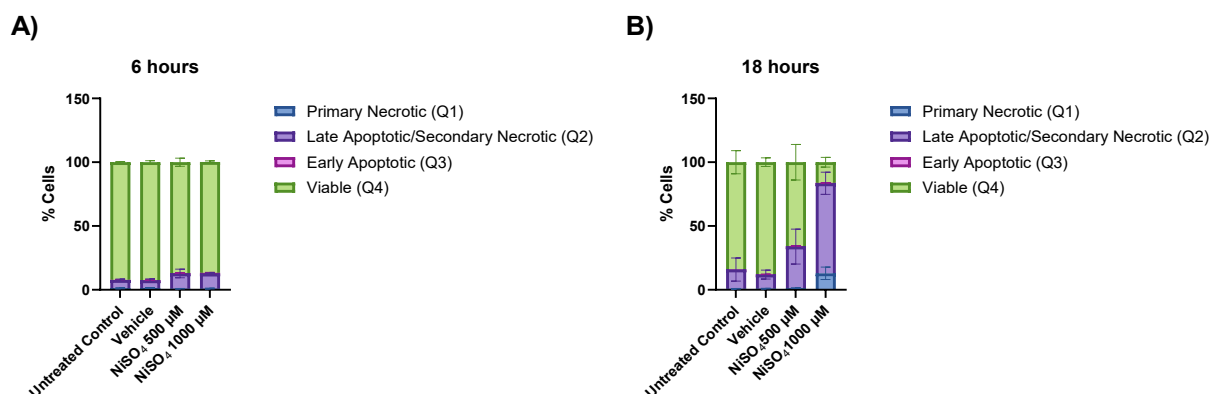


Figure 3.11 Annexin V/PI assay performed with NiSO_4 to differentiate between apoptotic and necrotic cell death. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with NiSO_4 at concentrations indicated and incubated for A) 6 hours and B) 18 hours. Vehicle was an equivalent amount of sterile deionised water added to cell culture media. The cells were trypsinized and stained with 5 μL Annexin-V per sample for 15 minutes in the dark and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. $n=3$.

Flow cytometry plots (Figure 3.12) showed that, similar to CoCl_2 and $\text{K}_2\text{Cr}_2\text{O}_7$, NiSO_4 -treated cells bypassed early apoptosis: at 6 hours, non-viable cells were primarily in Q2 (late apoptotic/necrotic; Annexin V^+/PI^+) rather than Q3 (early apoptosis), despite the short treatment time being insufficient to allow full progression into late apoptosis²⁷⁴. After 18 hours, particularly at 1000 μM , cells shifted further out of Q4 (viable; Annexin V^-/PI^-) into Q1 (necrotic; Annexin V^-/PI^+) and Q2 (late apoptotic/necrotic; Annexin V^+/PI^+), consistent with a necrotic mechanism of cell death in which cells do not pass through an early apoptotic stage but instead progress directly into necrosis or late apoptosis/necrosis.

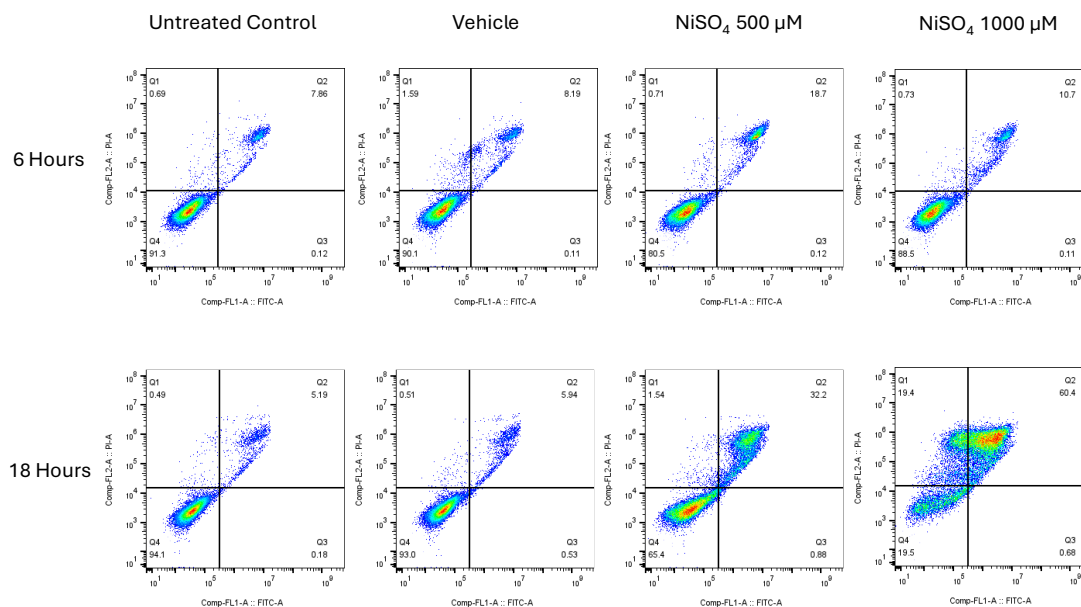


Figure 3.12 Annexin-V/PI Assay with NiSO₄. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with NiSO₄ at 1000 μM and 500 μM and incubated for two timepoints – 6 hours and 18 hours. The cells were stained with 5 μL Annexin-V per sample and stained with PI immediately prior to data acquisition using a BD Accuri C6 flow cytometer. The assay was run in triplicate and data was analysed using FlowJo version 10.10.0 software.

Annexin-V/PI assay showed the absence of cells in early apoptosis upon treatment with each of the three metals and indicated a necrotic mechanism of cell death rather than an apoptotic mechanism of cell death. However, the appearance of the cells on the dot plots for the Annexin-V/PI assay indicate that they results may be unreliable. This could be due to issues in compensation, as the FITC representing the Annexin V was compensated using beads, and the PI was compensated using cells. Compensating with both cells and beads is generally discouraged due to differences in how they behave and their differences in autofluorescence and light scatter properties. A study that compared cell-based compensation to bead based compensation showed that the fluorescence spillover measured on beads did not correctly represent the spillover of the fluorophore on cells, leading to improper compensation calculation²⁷⁵. Therefore, further experiments were needed to confirm the lack of apoptosis and to identify whether the metals could be inducing a ferroptotic mechanism of necrotic cell death.

3.4.1.2 PI Cell Cycle Analysis using PI Staining

In addition to its use in the Annexin-V/PI assay, PI is also commonly used for cell cycle analysis. PI binds to DNA, with the intensity of its fluorescence correlating to the amount of DNA in the cell. As DNA content varies throughout the cell lifecycle [Figure 3.13(a)], staining with PI can differentiate cells in different phases of the cell cycle²⁷⁶.

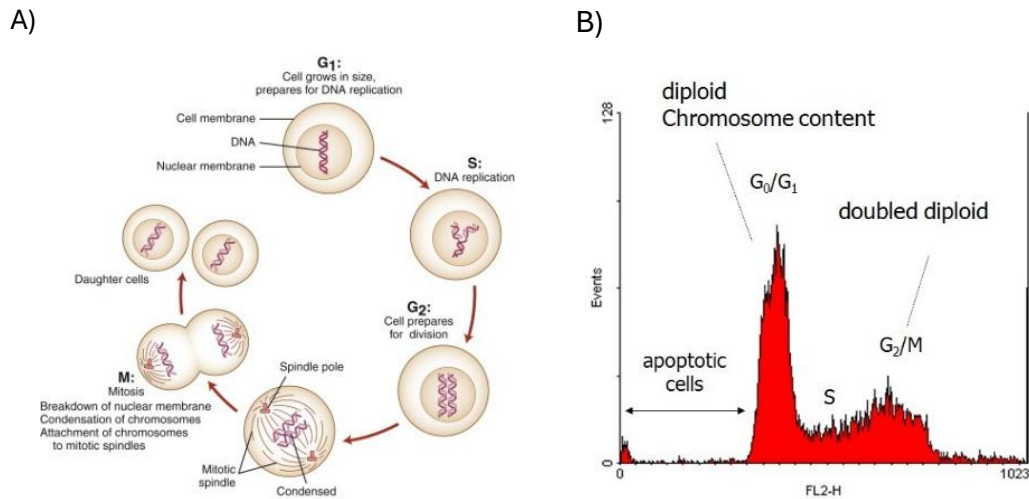


Figure 3.13 The Cell Cycle and Its Visualisation Using PI. A) The standard lifecycle of a cell and its DNA contents, beginning at the G₁ phase and progressing through the S phase, G₂ phase and M phase²⁷⁷ B) Visualisation of the cell cycle using PI flow cytometry analysis²⁷⁸

In a normal diploid cell, the DNA content is represented as 2N in the G₀/G₁ phase²⁷⁹. PI has a low fluorescence intensity in these cells as cells have the least amount of DNA at this stage of the cell cycle. However, cells in this stage account for the highest proportion of a typical growing population of mammalian cells and so appear as the tallest peak on the PI fluorescence histogram²⁸⁰. When the cell is in the S phase DNA synthesis is taking place and so the fluorescence intensity of PI in these cells is higher than in the G₀/G₁ phase. In the G₂ phase the DNA content in the cell is double that of the cell in G₀/G₁ phase, making these cells tetraploid cells²⁷⁹. These cells express the highest PI fluorescence.

Extensive DNA cleavage is a hallmark of apoptosis. When apoptotic cells are stained with PI, they display a broad hypodiploid (sub-G₁) peak which can be easily discriminated from the narrow G₀/G₁ peak of normal diploid DNA content. This additional peak is due to the fragmented DNA present in apoptotic cells that possesses lower PI fluorescence than the

cells in the G_0/G_1 phase²⁸¹. The presence of this sub- G_1 peak indicates the presence of apoptosis, whereas the absence of the sub- G_1 peak could be indicative of a necrotic mechanism of cell death. Necrotic cells do not show DNA fragmentation and so do not produce a sub- G_1 peak upon PI staining and flow cytometry analysis²⁸².

HaCaT cells treated with $K_2Cr_2O_7$ at a range of concentrations did not produce any sub- G_1 peak in comparison with the untreated control, which indicated that apoptosis was not taking place (representative histogram in Figure 3.14). Analysis of all three experiments in Figure 3.16 indicated a percentage of 4.1% and 4.7% of HaCaT cells in sub- G_1 cells upon treatment with $K_2Cr_2O_7$ at concentrations of 100 μM and 50 μM , possibly indicating a minimal non-significant presence of apoptotic cells at these concentrations, however there was no clear presence of a sub- G_1 peak. The highest proportion of cells were present in the G_0/G_1 phase as expected, and the G_2/M peak could also be clearly seen in the histograms. The absence of the sub- G_1 peak in HaCaT cell populations treated with $K_2Cr_2O_7$ indicated the absence of apoptosis and correlated with the lack of early apoptotic cells identified in the Annexin-V/PI assay.

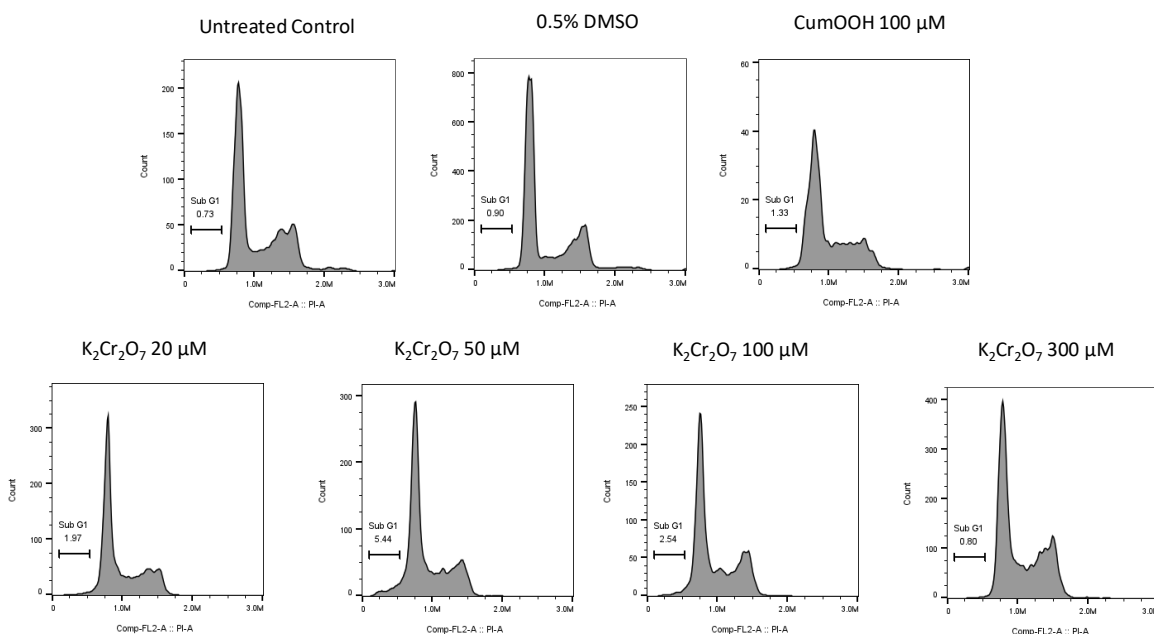


Figure 3.14 Cell Cycle Analysis of $K_2Cr_2O_7$ Treated HaCaT Cells. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with $K_2Cr_2O_7$ at concentrations in the range of $20 \mu M$ – $300 \mu M$ and incubated for 24 hours. The cells were stained with $50 \mu g/mL$ PI in the presence of $100 \mu g/mL$ RNase and incubated overnight at $4^\circ C$. The cells were acquired using a BD Accuri C6 flow cytometer. The data was analysed using FlowJo version 10.10.0 software. Data representative of one experiment. $N=3$

The assay was then run with $NiSO_4$ and $CoCl_2$ at varying concentrations (Figure 3.15). Similarly to the $K_2Cr_2O_7$ treated HaCaT cells, there was an absence of a sub- G_1 peak in the $NiSO_4$ and $CoCl_2$ treated cells. Analysis of all three experiments in Figure 3.16 showed that in the cells treated with $1000 \mu M$ or $500 \mu M$ $NiSO_4$ there were 4.7% and 2.6% of cells in the sub- G_1 area in comparison to 0.65% of cells in the untreated control. However, there was an absence of any sub- G_1 peak in these samples in Figure 3.15. In the $1000 \mu M$ $NiSO_4$ treated cells there appeared to be a decrease in the proportion of cells in the G_0/G_1 phase to 43% in comparison to 54% in the untreated control. There was a concomitant increase in the proportion of cells in the G_2/M phase to 37% in comparison to 26% in the untreated control [Figure 3.16(A)]. This increase in cells in the G_2/M phase can indicate DNA damage from oxidative stress which results in G_2/M checkpoint arrest²⁸³. In HaCaT cells treated with $CoCl_2$ at a concentration of $1000 \mu M$ or $500 \mu M$, 2.1% and 1.7% of cells were in the sub- G_1 phase in comparison with 0.65% in the untreated control. The absence of a sub- G_1 peak in HaCaT

cell populations treated with NiSO_4 or CoCl_2 indicated that these metals were not inducing apoptosis in these experimental conditions, suggesting that the mechanism of cell death induced was necrotic in nature.

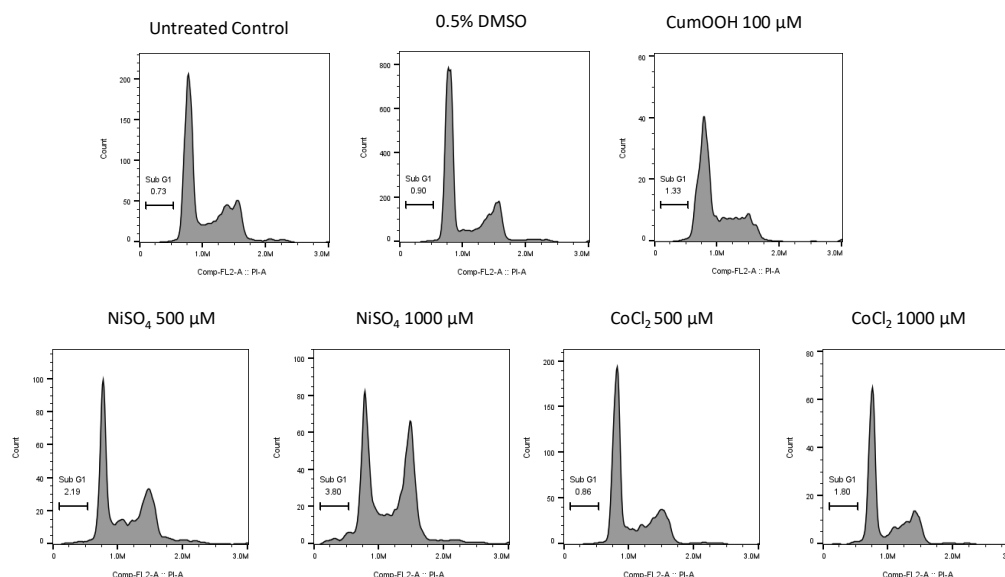


Figure 3.15 Cell Cycle Analysis of NiSO_4 Treated and CoCl_2 Treated HaCaT Cells HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with NiSO_4 or CoCl_2 at concentrations of 1000 μM and 500 μM and incubated for 24 hours. The cells were stained with 50 $\mu\text{g}/\text{mL}$ PI in the presence of 100 $\mu\text{g}/\text{mL}$ RNase and incubated overnight at 4°C. The cells were acquired using a BD Accuri C6 flow cytometer. The data was analysed using FlowJo version 10.10.0 software. N=3, vehicle control = 0.5% DMSO. Data is representative of one experiment.

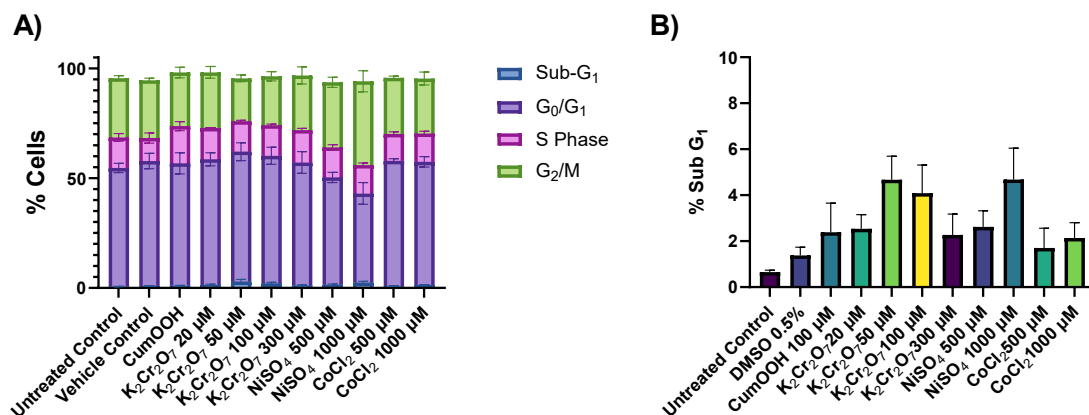


Figure 3.16 Cell Cycle Analysis Using PI Staining. HaCaT cells were seeded at a density of 5×10^6 cells per well in a 6-well plate and treated with CumOOH at a concentration of 100 μ M, NiSO₄ or CoCl₂ at concentrations of 1000 μ M and 500 μ M, and K₂Cr₂O₇ at concentrations in the range of 20 μ M – 300 μ M and incubated for 24 hours. The cells were stained with 50 μ g/mL PI in the presence of 100 μ g/mL RNase and incubated overnight at 4°C. The cells were acquired using a BD Accuri C6 flow cytometer. A) The proportion of cells in each phase of the cell cycle after treatment with metal allergens. B) Proportion of HaCaT cells in the sub-G₁ phase after treatment with metal allergens. Vehicle = 0.5% DMSO. The data was analysed using FlowJo version 10.10.0 software. Data was analysed using one-way ANOVA with Tukey's post hoc test for comparison of means. N=3

The percentage of cells present in the sub-G₁ peak in HaCaT cells treated with metal allergens were statistically analysed and compared to the untreated control. There appeared to be increases in the percentage of sub-G₁ cells across the metal treated samples. There appeared to be particular increases in the K₂Cr₂O₇ (100 μ M and 50 μ M) treated samples, as well as in the NiSO₄ 1000 μ M samples. While there appeared to be increases in sub-G₁ cell populations upon treatment with metal allergens, none of these changes were statistically significant. The visual absence of sub-G₁ peaks in the flow cytometry histograms coupled with the non-significant changes in the percentages of sub-G₁ cells present in HaCaT cell populations treated with metal allergens indicated that the mechanism of cell death induced by metal allergens in the conditions tested was not apoptotic in nature, and thus is likely to be a necrotic form of cell death, especially considering previous results in section 3.4.1.1.

Having observed that the mechanism of cell death induced by metal allergens in these conditions is more likely necrotic rather than apoptotic, investigations then turned to whether ferroptosis could be a form of necrotic cell death induced by metal allergens.

3.4.2 Use of Ferroptosis Inhibitors to Investigate Ferroptosis Induction in HaCaT cells by Metals

With the previous experiments having indicated that the metals were inducing a necrotic form of cell death, focus then turned to investigating whether the cells were dying via ferroptosis. The most currently used methods to investigate the induction of ferroptosis include the measurement of GPX4 levels using western blotting techniques, the measurement of iron levels using colorimetric or fluorometric assays, using known ferroptosis inhibitors and measuring the change in cell viability, and measuring lipid peroxidation production, as lipid peroxides are a well characterized hallmark of ferroptosis. As lipid peroxidation had already been quantified in the previous chapter, the model chosen for the investigation into ferroptosis induction by metals included the use of ferroptosis inhibitors and their effect on metal treated HaCaT cell viability. This model was chosen due to its extensive evidence base, simplicity, time-effectiveness and cost-effectiveness. Two ferroptosis inhibitors were used, Fer-1 and DFO. These two ferroptosis inhibitors work in different manners. DFO is an iron-chelator and Fer-1 is radical scavenging agent that reduces lipid peroxide content in the cell to prevent ferroptosis. Using two different ferroptosis inhibitors allowed for the investigation into ferroptosis inhibition by two different mechanisms. In addition, the known ferroptosis inducer erastin was used as a positive control for the assay. Erastin induces ferroptosis by inhibiting cysteine import into the cell therefore reducing the production of GSH, leading to increases in lipid peroxides in the cell.

In this assay, HaCaT cells were treated with metal allergens in the presence or absence of Fer-1 and DFO at two timepoints – 6 and 18 hours, after which the alamarBlue assay was used to assess HaCaT cell viability. Before running the assay with the metals, the effect of erastin on HaCaT cell viability was investigated (Figure 3.17). In this assay the cell density was lowered from 2×10^4 cells per well in a 96 well plate used in previous experiments to 1×10^4 cells per well. This change was made due to a study that concluded that the cell-cell contacts in highly confluent HaCaT cell cultures can protect against erastin induced ferroptosis²⁸⁴.

3.4.2.1 Effect of erastin and *t*-BuOOH on HaCaT cell viability

Prior to carrying out the ferroptosis inhibition assays, the effect of the erastin positive control, at a range of concentrations, on HaCaT cell viability was assessed (Figure 3.17). While erastin did influence HaCaT cell viability, it was only at the 20 μ M, 30 μ M and 50 μ M concentrations at which these reductions in viability were statistically significant. In addition, erastin treatment did not reduce the viability of HaCaT cells to the levels expected. One study showed that erastin (10 μ M) induced 60% cell death in a HaCaT cell culture²⁸⁴, and another showed that at the same concentration of erastin induced 40% cell death²⁸⁵. Treatment with erastin (10 μ M) in the conditions used in our experiments reduced viability to just 77% of the untreated control, equivalent to 23% cell death.

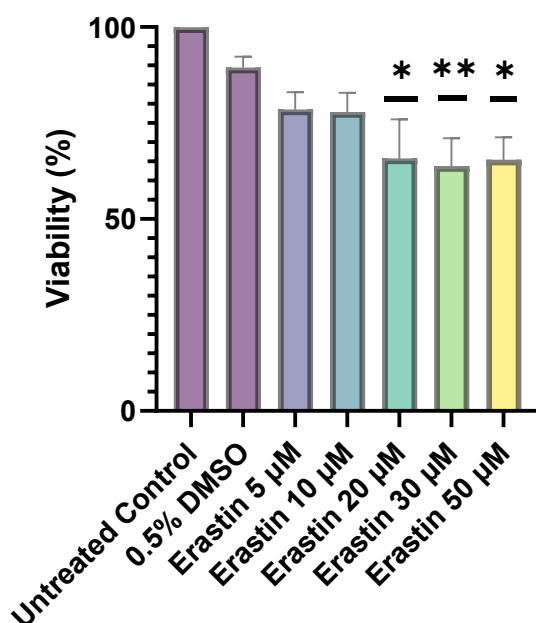


Figure 3.17 The effect of Erastin on HaCaT Cell Viability. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells are treated with Erastin and incubated for 24 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean

This weak effect on cell viability led to the trial of another known ferroptosis inducer as a positive control, *t*-BuOOH (Figure 3.18). Treatment with *t*-BuOOH at both 100 μ M and 200

μM concentrations led to a significant reduction in cell viability, equivalent to 92% cell death after 24 hours of treatment. This result was in line with previous literature about the toxicity of *t*-BuOOH on HaCaT cells, which reduced viability to approximately 20% at 100 μM .²⁸⁴

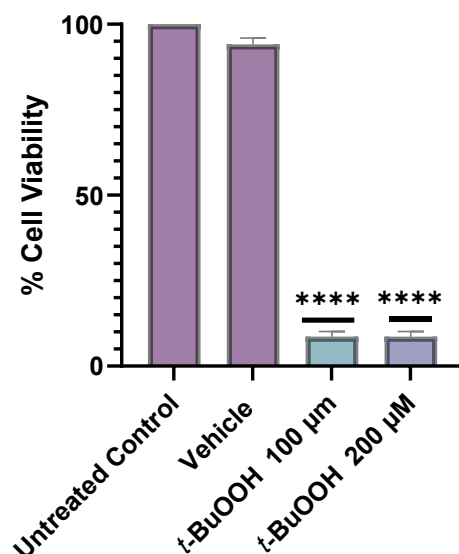


Figure 3.18 Effect of *t*-BuOOH on HaCaT Cell Viability. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells are treated with *t*-BuOOH and incubated for 24 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. Vehicle = 0.5% DMSO, $n=3$

Due to its increased effect on reducing cell viability, *t*-BuOOH was chosen as a positive control for the assays investigating ferroptosis induction by metal allergens.

3.4.2.2 Effect of Ferroptosis Inhibitors on HaCaT Cell Viability

The next step was to assess the effect of Fer-1 and DFO on the viability of HaCaT cells to ensure that they themselves were not negatively interfering with the assay (Figure 3.19). DFO did not reduce cell viability of HaCaT cells at any of the three concentrations tested. Fer-1 also did not have any significant effect on the viability of HaCaT cells at any of the concentrations tested. The viability assay concluded that neither DFO nor Fer-1 at any of the concentrations tested had any significant effect on viability in comparison to the untreated control sample. Thus, all the tested concentrations were taken forward for assays involving metal allergens and their ability to induce ferroptosis.

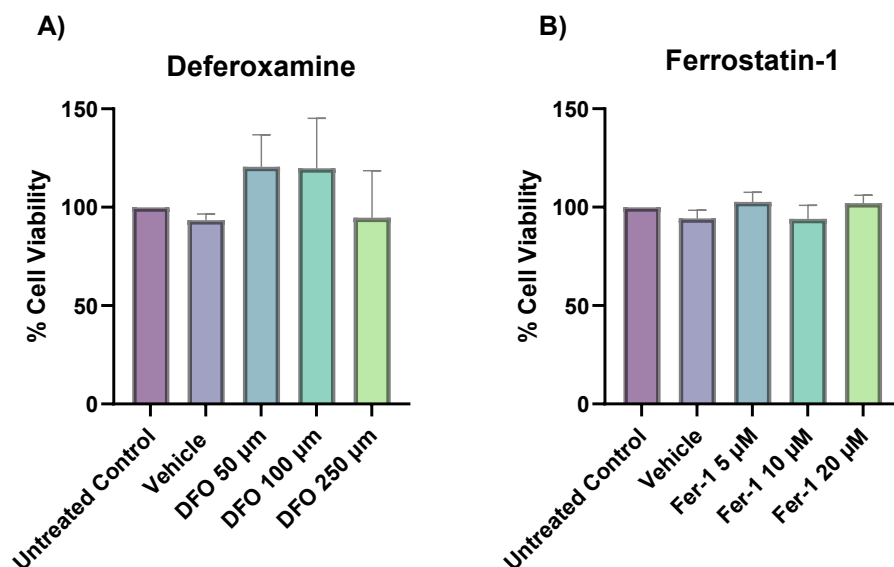


Figure 3.19 Effect of Ferroptosis Inhibitors on HaCaT Cell Viability. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96-well plate. The cells are treated with the A) DFO and B) Fer-1 at various concentrations and incubated for 24 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for each concentration are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of mean. Vehicle = 0.5% DMSO, $n=3$.

3.4.2.3 Optimisation of assay conditions

To optimise the assay and identify the most effective concentrations of the ferroptosis inhibitors DFO and Fer-1, the assay was first carried out with *t*-BuOOH at two different time points – 6 hours due to the rapid nature of ferroptosis as a cell death mechanism, and a 24-hour timepoint. The concentrations of inhibitors trialled were adopted from previously published studies on ferroptosis^{286, 287}. The cells were pre-treated with DFO at concentrations between 50 μ M and 250 μ M, and Fer-1 at concentrations between 5 μ M and 20 μ M (Figure 3.20). After a 6-hour treatment period, DFO effectively reduced *t*-BuOOH (100 μ M and 200 μ M) induced cell death at all concentrations tested from 50 μ M to 250 μ M [Figure 3.20(a)]. The viability of the cells was increased in the presence of DFO to the extent that there was no statistically significant difference in the viability of the untreated control and the cells treated with *t*-BuOOH in the presence of DFO. It appeared that DFO almost completely rescued the HaCaT cell population after 6 hours *t*-BuOOH treatment. After 24 hours *t*-BuOOH treatment, there also appeared to be an increase in cell viability in

the presence of DFO, particularly at 100 μ M or 250 μ M concentrations [Figure 3.20(b)]. However, the viability was not increased to the same extent seen at 6 hours of *t*-BuOOH treatment. When the cells were treated with 100 μ M *t*-BuOOH, there was a statistically significant increase in cell viability when cells were in the presence of 250 μ M DFO in comparison to cells without DFO. While an increase was seen in the presence of 100 μ M DFO, this was not deemed to be statistically significant. Overall, DFO significantly increased the viability of cells treated with *t*-BuOOH at 6 hours and appeared to cause an increase in the viability of cells treated with *t*-BuOOH for 24 hours at higher DFO concentrations, albeit the increases were not always statistically significant.

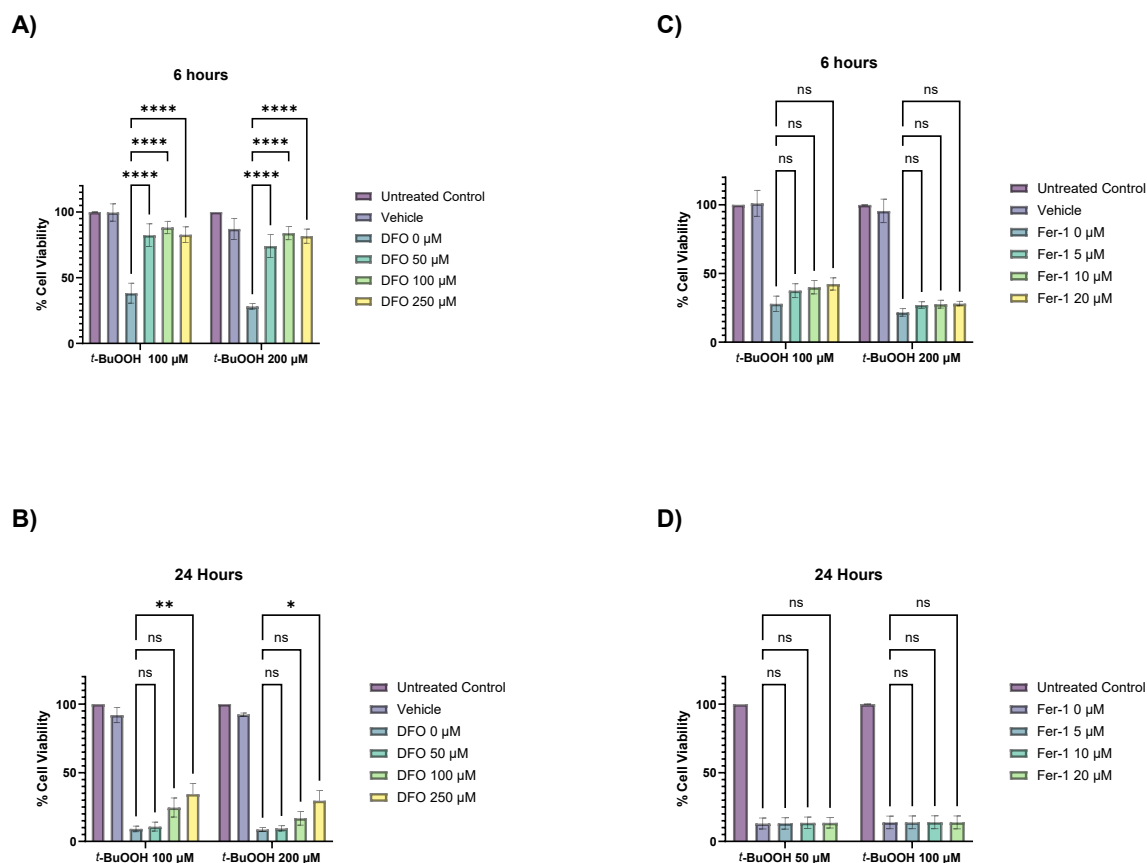


Figure 3.20 Optimisation of Ferroptosis Inhibitors using *t*-BuOOH. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells were pre-treated with either DFO or Fer-1 for one hour prior to treatment with *t*-BuOOH for either 6 hours or 24 hours. Untreated control and vehicle control are not treated with *t*-BuOOH. After this time the alamarBlue assay was carried out to assess cell viability. A) Effect of DFO on *t*-BuOOH induced cell death after 6 hours treatment. B) Effect of DFO on *t*-BuOOH induced cell death after 18 hours treatment. C) Effect of Fer-1 on *t*-BuOOH induced cell death after 6 hours treatment. D) Effect of Fer-1 on *t*-BuOOH induced cell death after 18 hours treatment. Results are expressed as mean $\pm$ SEM. N=3. Vehicle was made up of equivalent amounts of DMSO (0.5%) and sterile deionised water as the experimental samples. Data analysed by two-way ANOVA with Tukey's post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

The ferroptosis inhibitory effect of Fer-1 was not as notable as that seen with DFO. The viability of HaCaT cells treated with *t*-BuOOH in the presence of Fer-1 appeared to increase in comparison to cells treated in the absence of Fer-1. This was particularly noted in cells treated with 100 μ M *t*-BuOOH in the presence of 20 μ M Fer-1, however none of the apparent viability increases were statistically significant [Figure 3.20(c)]. Pre-treatment with Fer-1 did not have any effect on the viability of HaCaT cells treated with either 100 μ M *t*-BuOOH, or 50 μ M *t*-BuOOH, for 24 hours [Figure 3.20(d)]. These results indicated that Fer-

1 had some mildly ferroptosis inhibitory effects at 20 μM and after 6 hours *t*-BuOOH treatment but had no effect on cell viability after 24 hours *t*-BuOOH treatment. It was also observed that DFO had a greater effect at inhibiting *t*-BuOOH induced ferroptosis at both timepoints than Fer-1.

3.4.2.4 Effect of ferroptosis inhibitors on metal induced HaCaT cell death

$\text{K}_2\text{Cr}_2\text{O}_7$ was chosen as the focus of experiments investigating ferroptosis induction by metal allergens due to its high lipid peroxidation induction ability recorded in Chapter 2, therefore having the highest likelihood of ferroptosis induction. NiSO_4 was also tested due to its high frequency as a contact allergen²⁸⁸. HaCaT cells were first pre-treated with the ferroptosis inhibitor, either Fer-1 or DFO, for one hour, after which the metal allergen was added into the well with the ferroptosis inhibitor. The cells were then incubated for two timepoints – 6 or 24 hours (Figure 3.21). The assay was carried out using two different concentrations of $\text{K}_2\text{Cr}_2\text{O}_7$ (100 μM and 300 μM for 6-hour experiments, and 50 μM and 100 μM for 24-hour experiments). *t*-BuOOH (100 μM) was a positive control. At 6 hours, Fer-1 pre-treatment appeared to induce a slight increase in viability of *t*-BuOOH treated cells, particularly at 10 μM and 20 μM Fer-1, however it was not statistically significant. A similar effect was observed in the $\text{K}_2\text{Cr}_2\text{O}_7$ treated samples. Fer-1 pre-treatment did not have any significant effect on the viability of cells treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (300 μM or 100 μM). Some very slight increases in viability were seen. There was a 3% increase in cell viability in $\text{K}_2\text{Cr}_2\text{O}_7$ (100 μM) treated HaCaT cells pre-treated with Fer-1 (5 μM) in comparison to cells treated in the absence of Fer-1, and there was a 5% increase in cell viability in $\text{K}_2\text{Cr}_2\text{O}_7$ (300 μM) treated HaCaT cells pretreated with 5 μM Fer-1 in comparison to cells treated in the absence of Fer-1. At the 24-hour timepoint, Fer-1 pre-treatment had no effect on either the *t*-BuOOH treated samples or the $\text{K}_2\text{Cr}_2\text{O}_7$ treated samples. Fer-1 had a similar effect in Fig. 3.20 on cells treated with a known ferroptosis inhibitor.

Using DFO as a ferroptosis inhibitor, led to a significant increase in the viability of *t*-BuOOH treated cells at a 6-hours. No statistically significant increase in viability was seen in the samples treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (300 μM or 100 μM). A 4% increase in viability was observed in HaCaT cells treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (100 μM) in the presence of DFO (50 μM) in comparison

to cells treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (100 μM) in the absence of DFO. A 7% increase in viability was observed in HaCaT cells treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (300 μM) in the presence of DFO (50 μM) in comparison to cells treated with $\text{K}_2\text{Cr}_2\text{O}_7$ (300 μM) in the absence of DFO. At 24-hours of treatment there appeared to be a concentration dependent effect on viability in HaCaT cells treated with *t*-BuOOH in the presence of increasing concentrations of DFO in comparison to HaCaT cells treated with *t*-BuOOH in the absence of DFO. While these changes were not statistically significant, it was clear that DFO is having a protective effect, with DFO (250 μM) pre-treatment increasing viability to 26% from 7% viability in cells not pre-treated with DFO. Similarly to the 6-hour timepoint, DFO pre-treatment did not have any protective effect on $\text{K}_2\text{Cr}_2\text{O}_7$ (100 μM or 50 μM) induced cell death.

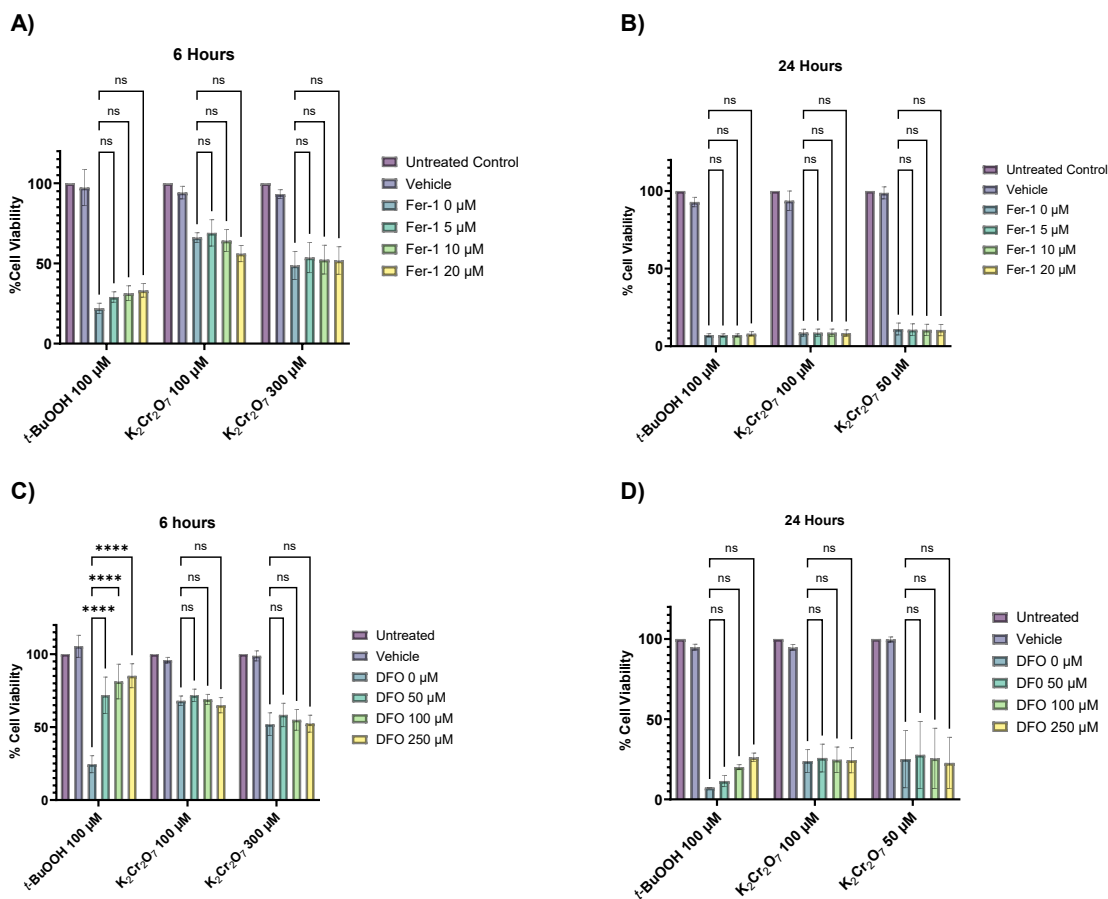


Figure 3.19 Effect of Ferroptosis Inhibitors on K₂Cr₂O₇ induced HaCaT Cell Death. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells were pre-treated with either DFO or Fer-1 for one hour prior to treatment with K₂Cr₂O₇ for either 6 hours or 24 hours. Untreated control and vehicle control are not treated with t-BuOOH or K₂Cr₂O₇. After this time the alamarBlue assay was carried out to assess cell viability. A) Effect of Fer-1 on K₂Cr₂O₇ induced cell viability after 6 hours treatment. B) Effect of Fer-1 on K₂Cr₂O₇ induced cell viability after 24 hours treatment. C) Effect of DFO on K₂Cr₂O₇ induced cell viability after 6 hours treatment. D) Effect of DFO on K₂Cr₂O₇ induced cell viability after 24 hours treatment. Vehicle is made up of equivalent amounts of DMSO (0.5%) and sterile deionised water. N=3. Results are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by two-way ANOVA with Tukey's post-hoc test for comparison of means. **** $p < 0.0001$, ns = $p > 0.05$.

The absence of a protective effect in cells pre-treated with Fer-1 or DFO suggests that K₂Cr₂O₇ does not induce ferroptosis. However, since Fer-1 also failed to protect HaCaT cells from t-BuOOH-induced death—a condition known to involve ferroptosis—it cannot be conclusively determined whether K₂Cr₂O₇ triggers ferroptosis in HaCaT cells.

The assay was then repeated with NiSO₄ to investigate whether NiSO₄ induces HaCaT cell death through ferroptosis (Figure 3.20).

NiSO₄ (1000 µM) had no significant effect on HaCaT cell viability at 6 hours, so the assay was performed using NiSO₄ (1000 µM) for 24 hours. Similarly to K₂Cr₂O₇, a *t*-BuOOH positive control was included. DFO had a concentration dependent effect on the viability of cells exposed to *t*-BuOOH (100 µM), with higher DFO (100 µM and 250 µM) having a significantly higher protective effect on *t*-BuOOH induced cell death [Figure 3.20 (a)]. DFO appeared to cause a slight increase in cell viability in NiSO₄ treated samples, and while it appeared to be concentration dependent, the increases in viability were not statistically significant [Figure 3.20(a)]. Pre-treatment with DFO (250 µM) increased NiSO₄ treated HaCaT cell viability to 42.7% in comparison to cells treated with NiSO₄ in the absence of DFO which had a viability of 35.7%. Similarly to K₂Cr₂O₇ (Figure 3.19), Fer-1 pre-treatment did not have any protective effect on NiSO₄ or *t*-BuOOH induced HaCaT cell death [Figure 3.20(b)]. The lack of effect of Fer-1 on *t*-BuOOH induced cell death meant that its effect on NiSO₄ induced cell death could not be concluded, and thus its ability to induce ferroptosis cannot be confirmed. The slight increase in viability seen with DFO 250 µM pretreatment ($p = 0.81$) indicated the potential for ferroptosis playing a role in NiSO₄ induced HaCaT cell death.

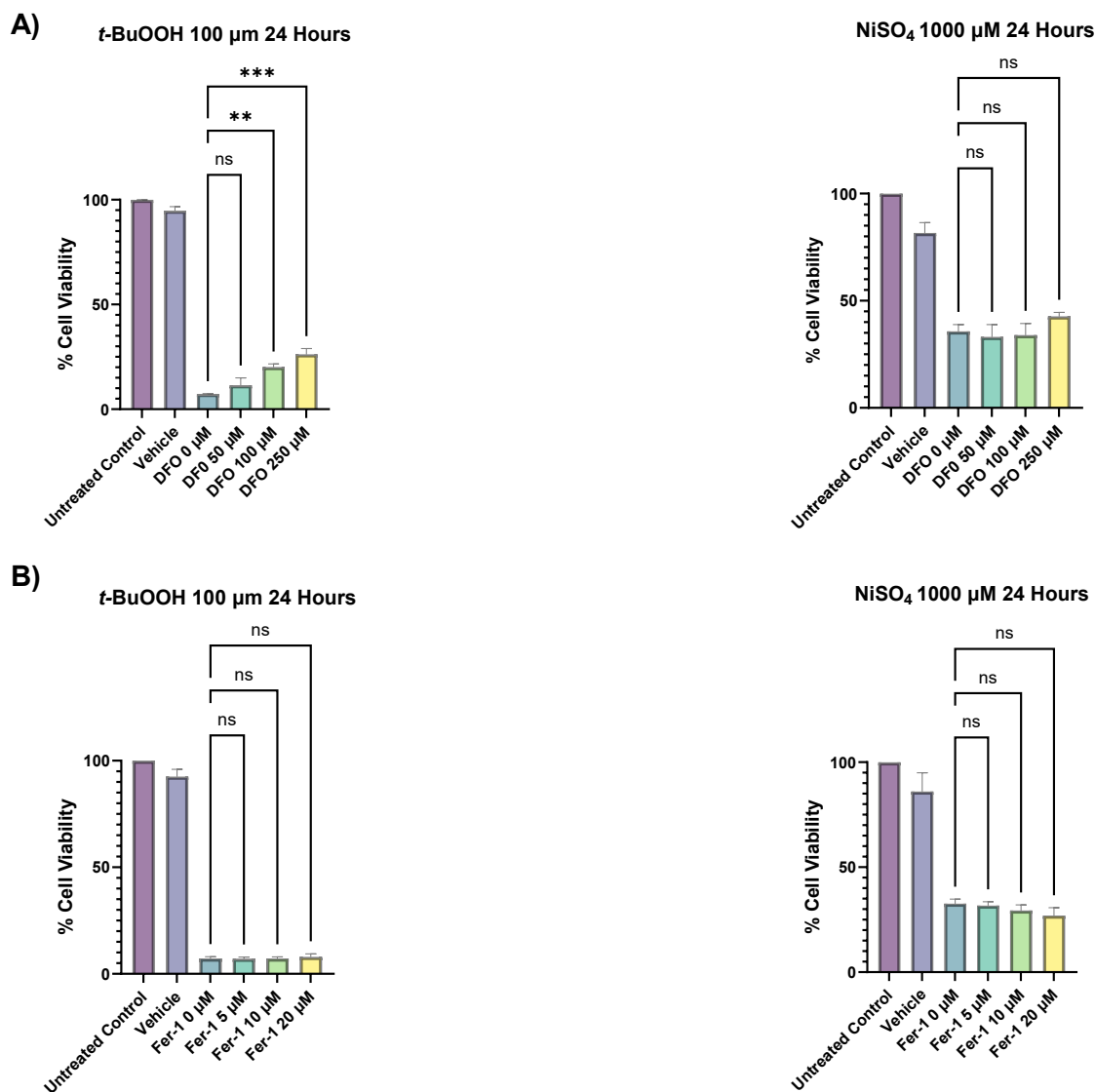


Figure 3.20 Effect of Ferroptosis Inhibitors on NiSO₄ Induced HaCaT Cell Death. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells were pre-treated with either DFO or Fer-1 at the concentrations indicated for one hour prior to treatment with NiSO₄ (1000 μM) or t-BuOOH (100 μM) for 24 hours. Untreated control and vehicle control are not treated with t-BuOOH or NiSO₄. Vehicle is comprised of an equivalent amount of DMSO (0.5%) and sterile deionised water. After this time the alamarBlue assay was carried out to assess cell viability. A) Effect of DFO on NiSO₄ induced cell death after 24 hours treatment. B) Effect of Fer-1 on NiSO₄ induced cell death after 24 hours treatment. Results are expressed as mean $\pm$ SEM. N=3. Data analysed by two-way ANOVA with Tukey's post-hoc test for comparison of means. ****p<0.0001

3.4.3 Combining Ferroptosis and Apoptosis Inhibitors to Further Investigate Metal-Induced Cell Death

To clarify the mechanism of metal induced cell death further, an apoptosis inhibitor was added into the assay and tested in combination with the ferroptosis inhibitors Fer-1 and

DFO, to assess its effect on metal allergen induced HaCaT cell death. z-VAD-FMK was chosen as the apoptosis inhibitor used for the assay. z-VAD-FMK is a cell permeable, irreversible pan-caspase inhibitor. It binds to the active site of caspases which blocks their ability to cleave target proteins and initiate the apoptotic cascade²⁸⁹. The first step was to assess the cytotoxicity of z-VAD-FMK on HaCaT cells (Figure 3.21). z-VAD-FMK was not observed to be cytotoxic to HaCaT cells at concentrations ranging from 20 μ M to 100 μ M as assessed through the alamarBlue cytotoxicity assay. This concentration range was chosen based on previously published literature documenting the use of z-VAD-FMK as an apoptosis inhibitor in keratinocytes. A range of 20 μ M – 50 μ M has been reported for inhibition of apoptosis. One study concluded that 20 μ M z-VAD-FMK was sufficient to reverse cell death induced by apoptosis inducers²⁹⁰, however a 40 μ M concentration of z-VAD-FMK was more common in literature. One study noted its use at 40 μ M in HaCaT cells prior to periplogenin (PPG) induced cytotoxicity²⁹¹, with another study pre-treating HaCaT cells with 40 μ M z-VAD-FMK prior to treatment with convallatoxin²⁹². Thus, 40 μ M was chosen as the concentration of z-VAD-FMK in the assays going forward.

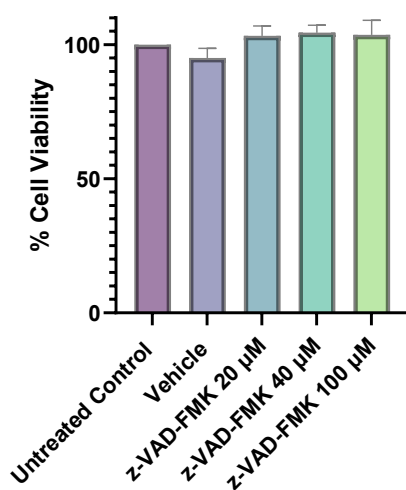


Figure 3.21 Cytotoxicity of z-VAD-FMK on HaCaT Cells. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96-well plate. The cells are treated with z-VAD-FMK at various concentrations and incubated for 18 hours. After this time the alamarBlue assay was carried out to assess cell viability. Vehicle = 0.5% DMSO. Results for each concentration are expressed as mean $\pm$ SEM. N=3. Data analysed by one-way ANOVA with Tukey's post-hoc test for comparison of means.

Having established that z-VAD-FMK was not toxic to HaCaT cells, the next step was to incorporate the apoptosis inhibitor into assays with the ferroptosis inhibitors (Figure 3.22). The addition of the pan-caspase inhibitor z-VAD-FMK did not have any significant effect on either $K_2Cr_2O_7$ or $NiSO_4$ induced HaCaT cell death. When used in combination with the ferroptosis inhibitor DFO there was no increase in cell viability seen in comparison to cells that did not undergo pre-treatment with ferroptosis or apoptosis inhibitors. The lack of response seen upon the addition of the apoptosis inhibitor was consistent with the outcome of the Annexin-V/PI assay and cell cycle experiment, in which features of apoptosis were not seen, and that in these conditions neither $K_2Cr_2O_7$ nor $NiSO_4$ appeared to induce an apoptotic form of cell death.

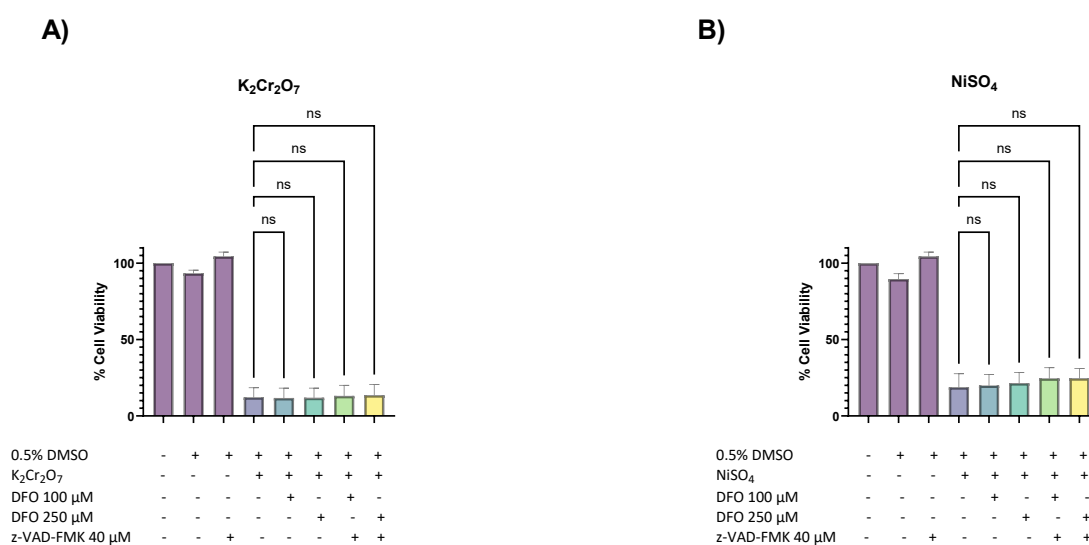


Figure 3.22 Effect of Apoptosis Inhibitors and Ferroptosis Inhibitors on Metal Allergen induced HaCaT Cell Death. HaCaT cells were seeded at a density of 1.0×10^4 cells per well of a 96 well plate. The cells were pre-treated with DFO alone or DFO in the presence of z-VAD-FMK for one hour prior to treatment with (a) $K_2Cr_2O_7$ 100 μ M or (B) $NiSO_4$ 1000 μ M for 24 hours. After this time the alamarBlue assay was carried out to assess cell viability. Results for are expressed as mean $\pm$ SEM. Experiment was performed in triplicate. Data analysed by two-way ANOVA with Tukey's post-hoc test for comparison of means. ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

The lack of apoptosis observed with $K_2Cr_2O_7$ is in contrast with a previously published study by Wang et al (2023) which showed that $K_2Cr_2O_7$ induced apoptosis in HaCaT cells, however their study used concentrations of $K_2Cr_2O_7$ lower than those used here (2-8 μ g/mL)²²¹. While

lower concentrations of $K_2Cr_2O_7$ have been shown to induce apoptosis, it is possible that higher concentrations (100 μM) could induce a necrotic type of cell death. This is particularly interesting as patients are exposed to much higher concentrations of $K_2Cr_2O_7$ during patch testing than are used here (20 mM). The study by Wange et al (2023) also concluded that $K_2Cr_2O_7$ induced apoptosis was ROS mediated²²¹, which aligns with the high ROS and lipid peroxide production seen in HaCaT cells treated with $K_2Cr_2O_7$ in chapter 2. There is also the possibility that $K_2Cr_2O_7$ and other metal allergens could induce both apoptosis and ferroptosis. For example, erastin, a well-known and studied ferroptosis inducer, was also shown to activate caspases and induce apoptosis, while also causing lipid peroxidation and glutathione depletion²⁹³. Interestingly in this study, the use of ferroptosis inhibitors failed to protect cells from erastin treatment, much like the results observed here when $K_2Cr_2O_7$ was treated with ferroptosis inhibitors. Other metals like iron and copper have also been shown to induce both apoptosis and ferroptosis in different cell lines²⁹⁴. It appears that cell death mechanisms are context and concentration dependent, and that at the concentrations and conditions used here, neither $K_2Cr_2O_7$ nor $NiSO_4$ induced apoptosis. It should be noted that the concentrations of metals used in these experiments were lower than those typically applied in patch testing, since higher concentrations were found to be excessively cytotoxic in preliminary experiments. While this limits direct comparison to the *in vivo* patch test setting, the findings remain clinically relevant, as they demonstrate that even sub-patch test concentrations of metal allergens can rapidly compromise keratinocyte viability and induce lipid peroxidation.

While cells could not be rescued using ferroptosis inhibitors, the extent of ROS and lipid peroxidation observed upon HaCaT cell treatment with these metals still indicates a type of necroptotic or ferroptotic cell death. Future research is needed to further establish the relationship between ferroptosis induction and metal allergens.

3.5 Conclusions of Chapter

The aim of this chapter was to characterize the mechanism of cell death induced by metal allergens, by firstly investigating whether it was apoptotic or necrotic in nature, and secondly by investigating the role of ferroptosis in metal allergen induced HaCaT cell death. The Annexin-V/PI assay showed that cells treated with the metals bypassed the early apoptotic stage and indicated a necrotic form of cell death. PI staining was then used to assess DNA fragmentation through the formation of a sub-G₁ peak in the histogram. There was no significant increase in the percentage of cells in the sub-G₁ peak in the metal treated cells in comparison to the untreated cells, indicating that no significant DNA fragmentation was taking place. Cell cycle analysis by PI staining also showed that NiSO₄ induced G₂/M arrest, indicating high levels of cellular stress in HaCaT cells upon treatment with NiSO₄. The Annexin-V/PI assay indicated that the cells were not undergoing apoptosis and were undergoing a necrotic form of cell death.

Having established that the metals most likely induced a necrotic form of cell death, further experiments were conducted to determine whether ferroptosis contributed to this response. Pre-treatment with the ferroptosis inhibitors Fer-1 and DFO did not significantly prevent metal-induced HaCaT cell death, with only minor increases in viability observed. Notably, Fer-1 also failed to rescue cells treated with *t*-BuOOH, suggesting that it was of limited utility in these experiments. In addition, combined pre-treatment with ferroptosis inhibitors and the apoptosis inhibitor z-VAD-FMK did not significantly alter metal-induced cytotoxicity, further supporting the conclusion that cell death did not occur through apoptosis.

This study provides new mechanistic insights into the cytotoxic effects of metal allergens on keratinocytes. Unlike previous reports in other cell types that identified apoptosis as the predominant mechanism, our findings demonstrate that NiSO₄, CoCl₂, and K₂Cr₂O₇ induce a necrotic form of cell death in HaCaT cells, with little evidence of classical apoptosis. However, from these experiments the exact form of cell death could not be conclusively determined. Notably, NiSO₄ exposure was associated with G₂/M cell cycle arrest without DNA fragmentation, suggesting a unique oxidative stress-driven checkpoint response not

previously described in keratinocytes. Furthermore, although metal-induced ROS and lipid peroxidation are consistent with hallmarks of ferroptosis, cell viability could not be rescued by ferroptosis inhibitors, leaving the role of ferroptosis in metal-induced toxicity unresolved. The lack of a protective effect by ferroptosis inhibitors could indicate that the extent of ROS and lipid peroxidation induced by metals overwhelms the protective effects of Fer-1 and DFO.

Overall, future work is needed to fully investigate whether metal allergens can induce ferroptosis, including quantitative measurement of GSH levels and GPX4 levels, both of which should be significantly depleted under ferroptotic conditions, as well as measuring iron accumulation which is upregulated in ferroptosis. Research is also needed to investigate other forms of cell death induced by metals, particularly inflammatory driven mechanisms like necroptosis and pyroptosis. Cells should be pre-treated with necroptosis inhibitors like necrostatin-1 (Nec-1) which rescues cells affected by necroptosis. Necroptotic cells often appear resistant to apoptosis inhibitors like z-VAD-FMK, as shown in the results above, but can be rescued by Nec-1²⁹⁵. To investigate pyroptosis, examination of the levels of Gasdermin-D (GSDMD) should be measured, as GSDMD cleavage by inflammatory caspases is a hallmark of pyroptotic cell death. A reduction in levels of GSDMD and increase in the expression of cleaved N-terminal GSDMD strongly indicates a pyroptotic form of cell death²⁹⁶. Regardless of the mechanism of cell death, it was concluded in Chapter 2 that metal allergens can induce lipid peroxidation in keratinocytes, and so further studies in this thesis focused on the immunological role of lipid peroxidation in the pathophysiology of metal ACD.

Chapter 4: Investigation into the Role of CD1a and CD1d in the Pathophysiology of Allergic Contact Dermatitis

4.1 Introduction

Having established that metal allergens can interact with keratinocyte lipids and drive lipid peroxidation, it was important to investigate how these biochemical changes might translate into immunological consequences relevant to the pathophysiology of ACD. Lipid peroxidation not only alters the structural integrity of cellular membranes but also generates a range of reactive lipid species that could potentially act as novel antigens. Lipid antigens are recognised not by classical MHC molecules but by the CD1 family of lipid antigen-presenting molecules. Within this family, CD1a and CD1d are of particular interest. CD1a is highly expressed on Langerhans cells in the epidermis and has been implicated in the presentation of self-lipid antigens in skin inflammation, and CD1d is reported to have a role in pathophysiology of contact hypersensitisation²⁹⁷. In this chapter, the role of CD1a and CD1d in metal ACD is investigated.

4.1.1 CD1a and CD1d

CD1 molecules are antigen presenting glycoproteins, expressed on the surface of antigen-presenting cells (APCs) and play a major role in the presentation of lipid and glycolipid antigens to distinct subsets of T and natural killer T (NKT) cells²⁹⁸. CD1 proteins are categorized into three groups. CD1a belongs to group 1, along with CD1b and CD1c, and presents lipid antigens to $\alpha\beta$ T cells and $\gamma\delta$ T cells²⁹⁹. An example of this is the CD1 mediated presentation of lysophosphatidicholine (LPC) to CD1a-restricted $\alpha\beta$ T cells, leading to R cells activation and cytokine release³⁰⁰. CD1d belongs to group 2 and presents lipid antigens to invariant natural killer T cells (iNKT cells)³⁰¹. The most well-known example of this is the presentation of the glycolipid α -galactosylceramide (α -GalCer) to iNKT cells by CD1d³⁰². CD1e belongs to group 3 and does not present lipid antigens to T cells, and is instead involved with the processing and loading of lipid antigens onto CD1b³⁰¹. CD1 molecules are structurally similar to MHC molecules, however the CD1 antigen binding groove is narrow and deeply hydrophobic which enables it to present lipid and glycolipid antigens to T cells³⁰³. CD1a and CD1d are the focus of this study due to their prior associations with ACD and contact hypersensitivity. As mentioned previously, CD1a is localised to the skin and has been previously shown to present contact allergens to T cells⁸⁶, and CD1d has been

reported to have a role in the pathophysiology of contact hypersensitivity²⁹⁷. The binding cleft of CD1a and CD1d comprises of two deep pockets. The A' and F' pockets are lined with hydrophobic amino acids which can accommodate the acyl chain of lipid ligands³⁰⁴ (Figure 4.1). CD1a has the smallest binding cleft, and its A' pocket is not directly connected to other pockets and ends abruptly deep in the binding groove, which prevents the binding of compounds with long hydrophobic chains³⁰⁵. In comparison, CD1d has a larger binding groove and a deep A' pocket that can accommodate longer lipid chains³⁰⁶. The structures of the CD1a and CD1d proteins can be seen in Figure 4.1.

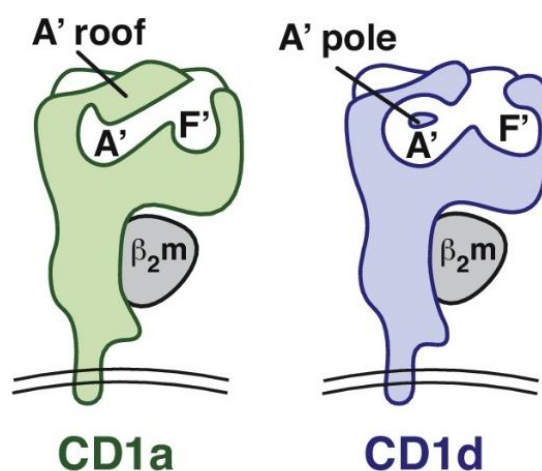


Figure 4.1 The structures of CD1a and CD1d lipid antigen presenting proteins³⁰⁷. CD1a and CD1d both contain two pockets – A' and F', and β_2 -microglobulin (β_2 M), a protein that provides structural stability to CD1 proteins and ensures correct folding and surface expression of CD1 proteins.

CD1 proteins are synthesised in the endoplasmic reticulum (ER) after which they pass through the Golgi apparatus³⁰⁶. CD1a is then trafficked to the early endosomes and the plasma membrane where lipid exchange and antigen loading takes place^{308, 309}. In contrast, CD1d is trafficked more extensively throughout the endosomal network, with antigen loading taking place in the late endosomes and lysosomes³¹⁰.

The frequency of CD1 reactive T cells is reported to be in the range of 1/10 – 1/300 circulating T cells in healthy donors³¹¹. A study by Cotton et al. (2021) estimated the frequency of CD1a-autoreactive T cells to be between 0.10% – 0.14% of skin T cells using

an ELISPOT assay, however tetramer staining showed frequencies of up to 1% in T cells derived from the skin of some donors³¹². de Jong et al. (2010) reported the frequency of CD1-autoreactive T cells among memory T cells in peripheral blood to be between 0.02% – 0.4%³¹³, with a study by Monnot et al. (2023) showing that CD1a-mock tetramers stained 0.03% of T cells in peripheral blood mononuclear cells (PBMC)³¹⁴. The frequency of CD1d reactive iNKT cells in human blood is generally lower than the frequency of CD1a reactive T cells, ranging from 0.01% to 0.1% of T cells³¹⁵. A study by Im et al. (2008) reported that α -GalCer loaded CD1d tetramers recognised 0.19% of T cells in healthy PBMC³¹⁶. In general, tetramer staining resulted in iNKT cell frequencies ranging between undetectable to 0.2% of T cells in human PBMC^{317, 318}.

4.1.2 Recognition of CD1a and CD1d by T Cell Receptors

There are extensive differences in the mechanisms of CD1a and CD1d antigen recognition by T cell receptors (TCRs). De Jong et al (2014) found that T cells responded to CD1a presenting a diverse range of endogenous lipids³⁰⁰. Many of the responses occurred regardless of lipid identity which suggested that the TCR was recognising CD1a itself rather than a particular lipid antigen. Structural modelling showed that the lipid antigens sit deep within the CD1a binding cleft, with minimal protrusion of the polar headgroup, indicating that there is minimal contact between the TCR and the lipid. This recognition mechanism was explored further in a study by Nicolai et al (2020) which revealed that small compounds like farnesol and benzyl cinnamate are fully sequestered within the A' pocket of the CD1a binding cleft and do not protrude through the F' portal. Instead, these small compounds displace self-lipids like sphingomyelins that normally emerge to the outer surface of CD1a and reveal epitopes on the surface of CD1a itself that are recognised by TCRs⁸⁶. This is known as the absence of interference model, in which small lipid antigens are fully sequestered, allowing free TCR contact with the CD1a surface. A further study Wegrecki et al (2022) identified a new mechanism of CD1a-TCR binding. While $\alpha\beta$ T cells bind to the top surface of CD1a near the lipid binding cleft, $\gamma\delta$ T cells bind to the side of the CD1a molecule, parallel to the β -sheet floor and on the opposite side of the antigen-binding cleft. This binding mechanism is completely independent of lipid or antigen identity³¹⁹.

In contrast to CD1a recognition by TCRs, CD1d recognition has been shown to require the presence of a lipid antigen. A study by Borg et al. (2007) detailed how CD1d presenting α -galactosylceramide (α -GalCer) is recognised by iNKT cells³²⁰. The TCR binds CD1d- α -GalCer in a diagonal orientation which crosses over the antigen-binding cleft of CD1d and engages both the CD1d itself and the exposed headgroup of α -GalCer. Modifications to the galactose moiety of α -GalCer or its orientation significantly altered TCR binding and activation. The study concluded that the TCR must recognise a particular configuration of both CD1d and the lipid headgroup of α -GalCer for binding to occur.

4.1.3 CD1a and CD1d in Inflammatory Skin Conditions

CD1a is abundantly expressed on epidermal LCs, which are the major APCs in the skin epidermis, with CD1a-autoreactive T cells in the blood expressing cutaneous lymphocyte antigen and the chemokine receptors CCR4, CCR6 and CCR10 which mediate migration of T cells to skin^{309, 313}. Urushiol, a contact allergen present in poison ivy, is an antigen for CD1a-restricted T cells and has been shown to induce strong CD1a-mediated inflammation. APCs pulsed with urushiol and co-cultured together with T lymphocytes from poison-ivy affected donors resulted in an increase in the production of urushiol-CD1a dependent IL-17 and IL-22 cells in comparison to T lymphocytes from healthy donors³²¹. It was also concluded that CD1a-expressing LCs were essential for the generation of T_H17 cells and skin inflammation³²¹.

A study by Betts et al (2017) further identified contact sensitizers that trigger human CD1-autoreactive T cell responses. APCs pulsed with dinitrochlorobenzene (DNCB) potentiated the response of CD1a and CD1d-autoreactive T cells. However, results showed that newly synthesized CD1 molecules and endogenous lipids are required for DNCB-mediated T cell activation. Further investigation showed that DNCB did not activate T cells directly via CD1 presentation, but rather by modifying the endogenous lipids present, allowing for CD1 and TCR interaction. Finally, it was found that resorcinol, isoeugenol and cinnamaldehyde, fragrance contact allergens present in soaps, detergents and hair dyes, induced a dose

dependent, CD1d-mediated activation of S33d T cells (a clone of CD1d restricted T cell line)³²².

Nicolai et al (2020) tested a wide range of contact allergens for T cell activation via CD1a and identified Balsam of Peru, a collection of compounds which cause contact allergy that area found in perfumes and toiletries as well as a flavouring for food and drink, as potent CD1a-dependent activators of $\alpha\beta$ T cells. Benzyl cinnamate and benzyl benzoate, the two most abundant components of Balsam of Peru, were identified as CD1a-presented antigens, with both compounds inducing CD1a-dependent increases in IFN- γ production⁸⁶. The authors also concluded that there are chemical features that can favour CD1a-dependent T cell activation. These include the presence of aromatic rings, unsaturated bonds and a small molecular size with the optimal size being in the range of 222 to 410 Da.

4.2 Aims and Objectives

In chapter 2 it was shown that metals can induce lipid peroxidation, and the established link between CD1a and CD1d lipid antigen presentation in ACD and contact hypersensitivity suggests that they are plausible pathways by which lipid peroxidation products could activate T cell responses and contribute to the immunopathogenesis of ACD. Furthermore, because CD1a and CD1d naturally bind and present endogenous lipids, they may also provide a potential route for direct T cell activation by the metals themselves.

The aim of this chapter was to evaluate the ability of metal allergens and lipid peroxidation productions to recognize and activate T cells in a CD1a- and CD1d-dependent manner. The objectives to fulfil this aim were

- To investigate T cell recognition in PBMC from healthy and nickel allergic patients by metal and lipid loaded CD1a tetramers and CD1d tetramers
- To investigate T cell activation in PBMC from healthy and nickel allergic patients by metals and lipid peroxidation products presented by CD1a and CD1d transfected APCs.

4.3 Experimental Details

In this chapter, four cell lines were used: HeLa mock-transfected cells and HeLa cells transfected with CD1d, and C1R-mock transfected cells and C1R cells transfected with CD1a. The CD1 transfected cell lines were a gift from Professor Stephen Porcelli, Albert Einstein College of Medicine.

4.3.1 HeLa Cells

HeLa cells were the first immortal human cells to be grown in culture (Figure 4.2). They are an epithelial cell line and were first isolated in 1951 from a cervical carcinoma in a 31-year-old patient. HeLa cells have been the basis of many scientific discoveries and have been previously used in CD1 research³²³. In this research HeLa cells transfected with CD1d (HeLa-CD1d cells) were used to investigate CD1d mediated cytokine production, with HeLa mock transfected cells used as a control (HeLa-Mock cells).

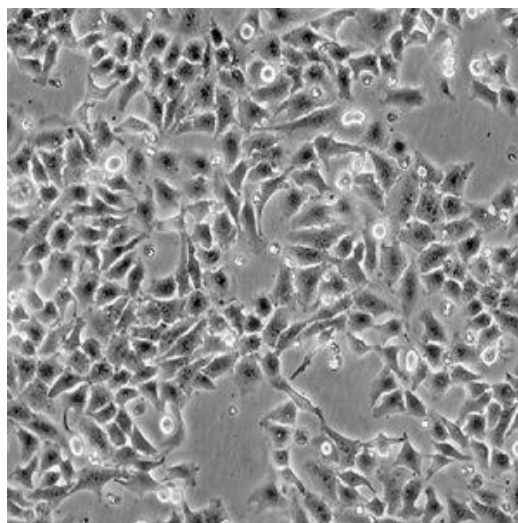


*Figure 4.2 HeLa Cell Culture*³²⁴

4.3.1.1 Thawing of HeLa cells

When not in culture, cells were stored in liquid nitrogen vapour phase at a temperature of -178 °C preserved in 1 mL of a solution containing 10% DMSO and 90% FBS. Cells were thawed in a 37 °C water bath and resuspended in a sterile 50 mL Falcon tube with 10 mL of

DMEM containing 10% FBS in a sterile laminar flow unit. Cells were centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and pellet resuspended in DMEM containing 10% FBS and transferred to a T25 tissue culture flask. Cells were incubated at 5% CO₂ and 37 °C until the desired confluency was reached.

4.3.1.2 Sub-Culture of HeLa Cells

HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) high glucose with Glutamax™ (Gibco, 10566-016) supplemented with 10% FBS (Gibco, 12657029, Brazil), 25 mM HEPES buffer (Gibco, 15630080) and 1% penicillin-streptomycin 10,000 U/mL (Gibco, 15140122). When the cells reached 70% confluency, they were sub-cultured. Media was removed and the cells were washed with 3 mL of room temperature Trypsin-EDTA solution (Sigma, T3924) to prevent interference of the media with trypsinisation. A further 3 mL of Trypsin-EDTA solution was added and the cells were incubated for approximately 5 minutes at 37 °C. 10 mL of culture media was added to neutralise the trypsin, and the cells were transferred to a 50 mL Falcon tube and centrifuged at 900 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 10 – 12 mL of fresh culture media. The cells were transferred to a new T75 flask at a split ratio of 1:3. Cells were sub-cultured at minimum on two independent occasions prior to experimental use following thawing from cryopreservation. The cells were used for a maximum of ten passages before a new batch of cells were thawed from cryopreservation.

4.3.1.3 Cryopreservation of HeLa Cells

After trypsinisation and centrifugation, cells were resuspended in the freezing solution consisting of 10% DMSO and 90% FBS. Thereafter 1 mL of cell suspension was aliquoted into sterile cryotubes and stored in a Mr. Frosty cyro-freezing container in which the cryovials of cells are surrounded by 100% isopropanol. The container was stored at -80 °C overnight, during which the Mr. Frosty lowered the temperature of the cells by 1 °C per minute. After overnight incubation, the cells were then moved to liquid nitrogen at -178 °C for long term storage.

4.3.2 C1R Cells

C1R is a human B-cell lymphoblastoid suspension cell line which lacks surface human leukocyte antigen (HLA) A and B antigens (Figure 4.3). C1R were derived from the Hmy.2 B-LCL cell line. The parental cell line was exposed to gamma radiation and then treated with monoclonal antibodies targeting MHC Class I molecules to produce C1R cells which lack MHC Class I expression. C1R cells have been used previously in studies involving CD1 antigen presentation³²⁵. In this research, C1R cells transfected with CD1a (C1R-CD1a cells) were used to investigate CD1a mediated cytokine production with C1R mock transfected cells used as control (C1R-Mock cells).

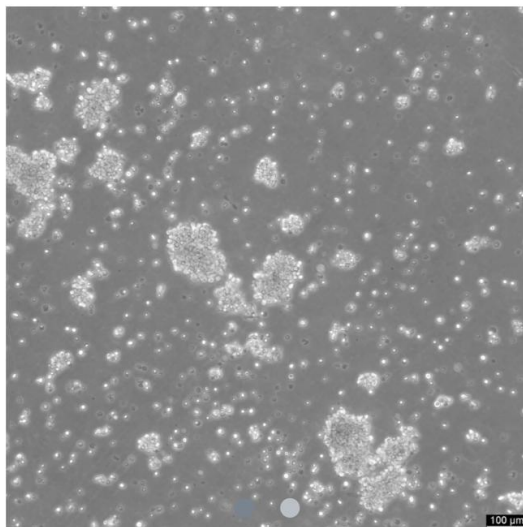


*Figure 4.3 C1R Cell Culture*³²⁶

4.3.2.1 Sub-Culture of C1R Cells

C1R Cells were cultured in RPMI 1640 medium (RPMI) with Glutamax™ (Gibco, 61870010) supplemented with 10% FBS, 25 mM HEPES buffer, and 1% penicillin-streptomycin 10,000 U/mL. The cells were sub-cultured when they reached 70% confluency. Fresh medium was added every 2 to 3 days and culture was maintained at a concentration of between 5×10^5 and 2×10^6 viable cells/mL.

4.3.3 Counting of Cells

All cells used in this chapter were counted in the same way. The cells were counted using Fast-Read plastic disposable haemocytometers (Scientific Laboratory Supplies, DD390498). 10 µL of freshly suspended cells was mixed with 190 µL of Trypan Blue cell viability dye in a 96 well plate. 10 µL of the resulting cell suspension was loaded onto the haemocytometer (Figure 4.4).

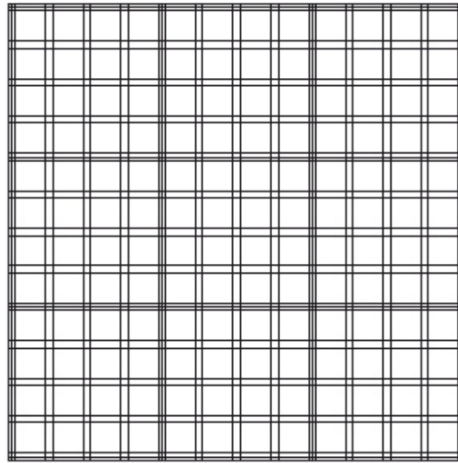


Figure 4.4 A representative Bürker haemocytometer grid

The quantity of cells in two of the larger squares (1 mm x 1 mm x 0.1 mm) was counted, excluding any cells stained with Trypan Blue. The following equation was used to calculate the quantity of cells per mL of suspension.

$$\frac{\text{number of cells in two quadrants}}{10} = \text{number of cells} \times 10^6 / \text{mL}$$

4.3.4 Peripheral Blood Mononuclear Cell Preparation

4.3.4.1 Blood Sample Acquisition

Ethical approval for this study was obtained from the Tallaght University Hospital/St James's Hospital Joint Ethics Committee. Ethical approval was granted in September 2024 (project ID: 2191). Blood samples from healthy donors were acquired from the Irish Blood Transfusion Service (IBTS) at St James's Hospital, Dublin and blood samples from ACD

patients were acquired from patients of the dermatology department in Tallaght University Hospital, Dublin. All patients were allergic to nickel, as confirmed through patch testing. 10 mL of blood was taken from each patient, and the blood samples were prepared immediately after acquisition.

4.3.4.2 Peripheral Blood Mononuclear Cell Preparation

To isolate the peripheral blood mononuclear cells (PBMC) from the blood samples, the blood was first diluted with PBS containing 1% heat inactivated FBS. The healthy blood from the IBTS was diluted in a 1:4 ratio due to its high concentration, whereas the blood from nickel allergic patients was diluted in a 1:1 ratio. The diluted blood was carefully layered onto 7 mL of Lymphoprep™ density gradient medium (STEMCELL Technologies, 18061) in a 20 mL universal tube. The blood sample was then centrifuged at 400 g for 30 minutes with acceleration and brake off. Density gradient centrifugation separates the blood into its components (Figure 4.5).

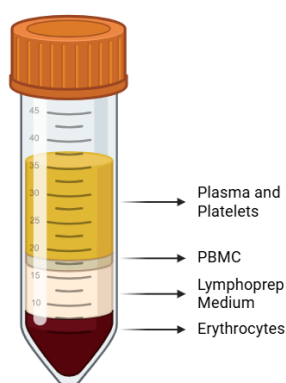


Figure 4.5 The separation of blood components after density grade centrifugation using Lymphoprep.

The top plasma layer was carefully discarded, and the cloudy buffy coat layer containing the PBMC, and the Lymphoprep layer below it, were transferred into a fresh 20 mL universal tube and topped up with PBS containing 1% FBS. The cells were centrifuged for 8 minutes at 1500 rpm with the brake on. The supernatant was discarded, and the cells were washed for a second time using PBS supplemented with 1% FBS. After the second washing, the supernatant was discarded, and the PBMC pellet was resuspended in approximately 1 mL

complete RPMI (RPMI 1640 medium with Glutamax supplemented with 25 mM HEPES buffer, 1% penicillin-streptomycin 10,000 U/mL and 10% heat inactivated FBS). The cells were counted as described in 4.3.3 and were re-suspended at a concentration of $1 - 2 \times 10^6$ cells per mL in complete RPMI containing 1 ng/mL IL-15. The cells were then incubated overnight at 37 °C and 5% CO₂. For long term storage, PBMC were resuspended in the freezing solution consisting of 10% DMSO and 90% FBS. Thereafter 1 mL of cell suspension was aliquoted into sterile cryotubes and stored in a Mr. Frosty cyro-freezing container at -80 °C overnight. After overnight incubation the vials were removed from the Mr. Frosty and transferred to -80 °C for long term storage.

4.3.5 CD1a and CD1d Tetramer Assay

4.3.5.1 Tetramer Loading

CD1a and CD1d tetramers conjugated with PE were obtained from the National Institutes of Health (NIH) tetramer facility. The tetramers were loaded with each of the three metal allergens (NiSO₄, CoCl₂, and K₂Cr₂O₇), two lipid peroxidation products (4-HNE and MDA) and CumOOH. The metals and MDA were prepared in sterile deionized water, and 4-HNE and CumOOH were prepared in DMSO. Farnesol was used as a potential positive control for the CD1a tetramer, and α -GalCer was used as a positive control for the CD1d tetramers. An equivalent amount of sterile, deionized water was added to CD1a and CD1d tetramers and labelled as the unloaded tetramer. A DMSO loaded tetramer was prepared as a vehicle control. All compounds were prepared as 1 mg/mL stock solutions. 8 μ L of test compound was added to a glass vial and made up to 45 μ L with 37°C PBS supplemented with 0.5% 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS). The vial was capped or covered with Parafilm and sonicated at 37 °C for 30 minutes. After sonication, 7.7 μ L of tetramer was added to the vial, along with 1 μ L of 0.5% sodium azide. The tetramers were then incubated at 37 °C and 5% CO₂ for 2 hours, after which the loaded tetramers were stored at 4°C for long term storage.

4.3.5.2 Staining PBMC with Tetramers

The tetramer staining protocol was adapted from a study by Monnot et al (2023).³¹⁴ 0.5×10^6 PBMC per sample were used and added to flow cytometry tubes. The cells are washed with PBS (PBS was added and the cells were centrifuged at 1500 rpm for 8 minutes, after which the supernatant was discarded). The cells are then stained with 100 μ L of eFlour 506-conjugated fixable viability dye (Invitrogen, 65-0866-14), vortexed and incubated for 15 minutes in the dark. The cells were then washed with fluorescence-activated cell sorting (FACS) buffer. FACS buffer consisted of PBS containing 2% FBS. Thereafter, the cells were resuspended in 50 μ L FACS buffer containing 0.5 μ g purified anti-human CD36³²⁷ (Biolegend, 336202). The cells were incubated on ice for 15 minutes. The cells were washed twice with room temperature FACS buffer, resuspended in 25 μ L FACS buffer containing 1 μ L of tetramer and incubated in the dark for 30 minutes at room temperature. 10 μ L of a 1/100 dilution of 1 mg/mL OKT3 (Biolegend, 317301) was added to each tube and the cells were incubated at 37 °C for 10 minutes. The cells were then washed with cold FACS buffer. An antibody cocktail was prepared with antibodies used for surface staining. The details of the antibody cocktail are detailed in table 4.1.

CD3-Pacific Blue	Biolegend, 300431
CD4-FITC	Biolegend, 300506
CD8-APC/Cy7	Biolegend, 344714
CD19-PerCP/Cy5.5	Biolegend, 302230
CD14-PE/Cy7	Biolegend, 301814

Table 4.1 Antibodies and Conjugates used in the Antibody Cocktail

2.5 μ L per test of each antibody was added to the cocktail which was made up in cold FACS buffer to a final volume of 50 μ L per tube. 50 μ L of the antibody cocktail was added to each tube and the cells were incubated on ice for 20 minutes. The cells were then washed twice with cold FACS buffer before being resuspended in 300 μ L of FACS buffer for acquisition via flow cytometry on the same day. If the cells were not to be analysed on the same day as staining, they were fixed with paraformaldehyde. 250 μ L of 4% paraformaldehyde was added to each tube and the cells were incubated for 15 minutes in the dark. The cells were

then washed with FACS buffer, resuspended in 300 μ L FACS buffer and stored at 4 °C overnight before acquisition via flow cytometry.

4.3.5.3 Acquisition of Cells using Flow Cytometry

Cells are acquired using a BD FACSCanto flow cytometry system. 1×10^5 events per sample were acquired and recorded. Alongside the samples, compensation controls and a Fluorescence minus one (FMO) control were performed. The FMO consisted of cells stained with all the antibodies except the PE conjugated tetramer. After acquisition, the data was analysed using FlowJo v 10.10.0. The gating strategy used is displayed in Figure 4.6. The first gate identifies the lymphocytes, and the second gate excludes any doublets in the sample. The third gate identifies the live, viable cells, and the fourth gate excludes monocytes from the analysis. Finally, the last gate identifies the population of interest; T cells that recognise the loaded tetramers.

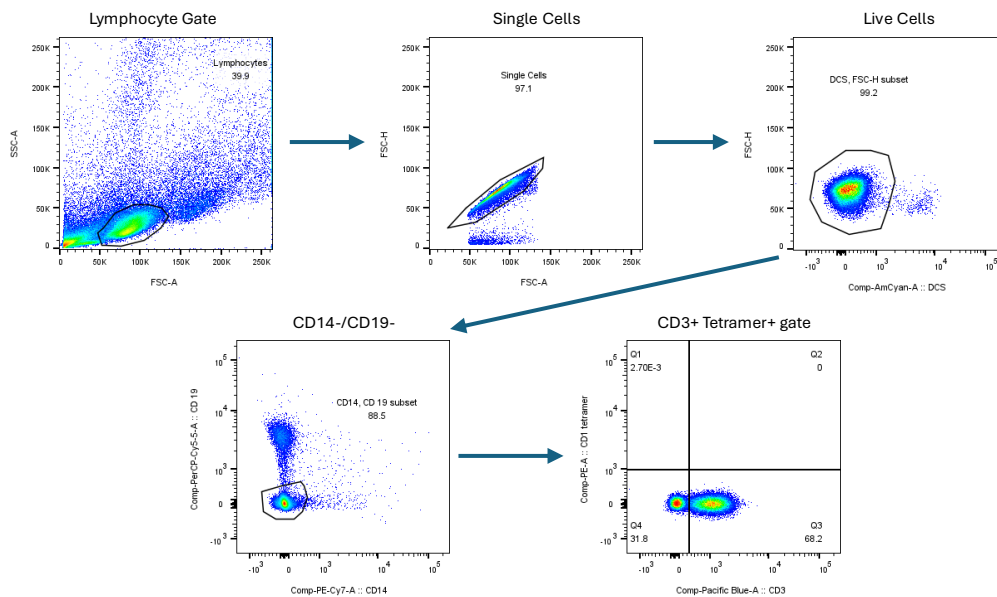


Figure 4.6 Gating Strategy for CD1a and CD1d Tetramer Assays. The cells were first gated on forward scatter (FSC) and side scatter (SSC) to exclude debris. Single cells were then selected, and doublets excluded, using FSC-A versus FSC-H. Live cells were identified as those which stained negatively for fixable viability dye eFlour-506. Within the live cell gate, monocytes were excluded by selecting cells that stained negatively for both CD19 and CD14. Having selected the population of interest, CD3 was plotted against the PE-labelled tetramer to identify T cells that recognized the loaded tetramer.

4.3.6 CD1a and CD1d Functional Assay

The aim of the functional assay was to investigate the ability of metal allergens and lipid peroxidation products to bind to CD1a and CD1d and activate T cells. To do this, four different cell lines were used. Mock-transfected C1R cells (C1R-Mock) and CD1a-transfected C1R cells (C1R-CD1a) as well as mock-transfected HeLa cells (HeLa-Mock) and CD1d-transfected HeLa cells (HeLa-CD1d) were used to present the test compounds to T cells, leading to their activation. The methodology was based on a previously published study³²⁸. T cell activation was measured by the production of selected cytokines. The cytokines measured in this study were interferon- γ (IFN- γ), tumour necrosis factor (TNF) and Interleukin-2 (IL-2). These chosen cytokines are characteristic of the T_H1 profile.

4.3.6.1 Preparation of Stimulator Cells and Addition of Ligands

C1R-mock, C1R-CD1a, HeLa-mock and HeLa-CD1d cells were seeded at 0.1×10^6 cells in 100 μ L culture media per well in a sterile round bottom 96 well plate. The test compounds were then prepared for addition to the stimulator cells. The tested compounds included NiSO₄, CoCl₂, K₂Cr₂O₇, 4-HNE, MDA, and CumOOH. The metals and MDA were prepared in sterile deionized water, and 4-HNE and CumOOH were prepared in DMSO. Similarly to the tetramer assays, farnesol was used as a potential positive control for the CD1a functional assays, and α -GalCer was used as a positive control for the CD1d functional assays. An equivalent amount of water was added to wells of C1R and HeLa cells and labelled as the untreated controls. 0.01% DMSO vehicle controls and FMO controls were also performed. Finally, stimulation controls were performed to ensure the appropriateness of the assay method and that the PBMC were behaving as expected. All compounds were prepared as 1 mg/mL stock concentrations. The compounds were vortexed for 1 minute, heated at 80 °C for 2 minutes and sonicated for 10 minutes. The ligands were then diluted 1/100 in prewarmed RPMI or DMEM. 2 μ L of the diluted ligand was then added to the C1R and HeLa cells in the 96 well plate. This resulted in a final concentration of 200 ng/mL of ligand in each well. The plate was then incubated overnight at 37 °C and 5% CO₂. After incubation

overnight, 0.2×10^6 PBMC in 100 μ L of RPMI were added to each well for co-culture with the stimulator cells.

Stimulation controls consisted of PBMC and RPMI culture medium. There was no co-culture with the stimulator cells in these controls. Three stimulation controls were performed: an unstimulated control in which nothing was added to the PBMC, PBMC stimulated with 50 ng/mL Phorbol 12-myristate 13 acetate (PMA) and 1 μ g/mL ionomycin, and PBMC stimulated with 0.2 μ g/mL anti-CD3 and 0.2 μ g/mL anti-CD28.

After the PBMC were added and the controls prepared, 10 μ g/mL brefeldin A (Biolegend, 420601) was added to every well approximately 8 hours before sample staining, and the plate was incubated at 37 °C and 5% CO₂.

4.3.6.2 Staining of PBMC for Flow Cytometry

After overnight incubation, the PBMC in each well were resuspended and transferred to labelled FACS tubes. The cells were washed in 2 mL PBS, after which 100 μ L of e-flour 506-conjugated Fixable Viability Dye was added and the tubes were incubated for 15 minutes in the dark at room temperature. After washing with FACS buffer, Fc block was added at 3 μ L per sample in 50 μ L FACS buffer, and the cells were incubated for 15 minutes in the dark at room temperature. After incubation the cells were washed with FACS buffer and stained with the surface staining antibody cocktail. This cocktail contained CD3-Pacific Blue, CD4-APC and CD8-APC-Cy7 made up in FACS buffer. All antibodies were added at a concentration of 2.5 μ L per sample in 50 μ L FACS buffer per sample. 50 μ L of the surface staining antibody cocktail was added to each sample and the samples were incubated for 20 minutes in the dark at room temperature. After washing with FACS buffer, the cells were fixed by adding 100 μ L of fixation buffer and incubating the cells for 30 minutes at room temperature. After fixation the cells were washed with permeabilization buffer and then stained with the intracellular antibody cocktail in which the intracellular antibodies were prepared in permeabilization buffer. The antibodies included in the intracellular cytokine cocktail were IFN- γ -PE, TNF-PerCP/Cy5.5 and IL-2-FITC. The antibodies were added at a concentration of 2.5 μ L per test in 50 μ L permeabilization buffer. 50 μ L of the intracellular antibody cocktail

was added to each tube and the samples were then incubated for 30 minutes in the dark at room temperature. The cells were then washed with FACS buffer and resuspended in 300 μ L FACS buffer for flow cytometry acquisition.

4.3.6.3 Acquisition of Cells using Flow Cytometry

Cells were acquired using a BD FACSCanto flow cytometry system. 1×10^5 events per sample were acquired and recorded. Alongside the samples, compensation controls and a Fluorescence minus one (FMO) control were performed. The FMO consisted of cells stained with all the antibodies except the PE conjugated tetramer. After acquisition, the data was analysed using FlowJo v 10.10.0. The gating strategy used is displayed in Figure 4.7. The first gate identifies the lymphocytes, the second gate removes any doublets from the analysis, the third gate identifies the viable cells, and the final gate identifies the population of interest by plotting CD3, which identifies T cells, against the cytokine of interest.

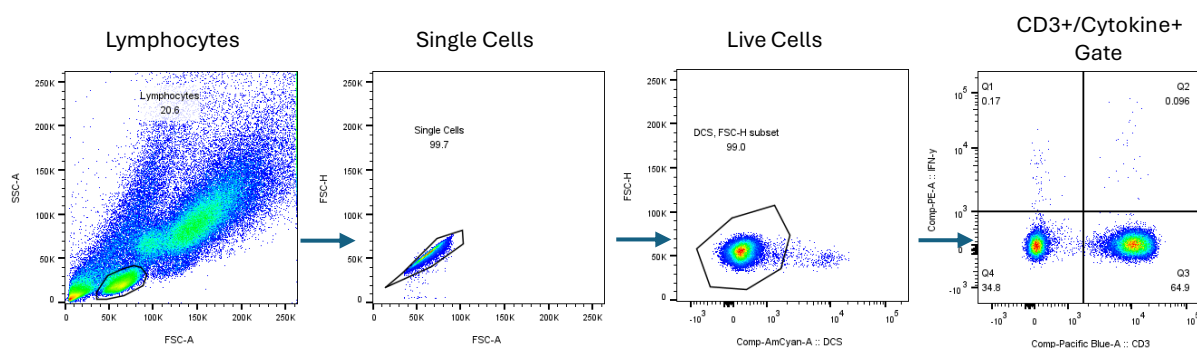


Figure 4.7 Gating Strategy for CD1a and CD1d Functional Assays. The cells were first gated on forward scatter (FSC) and side scatter (SSC) to exclude debris. Single cells were then selected, and doublets excluded, using FSC-A versus FSC-H. Live cells were identified as those which stained negatively for fixable viability dye eFlour-506. CD3 was then plotted against the chosen cytokine antibody to identify cytokine-positive T cells.

4.3.7 Statistical analysis

The results for the tetramer assays were analysed with GraphPad Prism v. 10 via either students paired T test or repeated measures two-way ANOVA. The results for the functional

assays were analysed both as % cytokine positive T cells and as a fold change ratio. The fold change ratio was calculated using the following equation:

$$\frac{\% \text{ of cytokine positive T cells in treated sample}}{\% \text{ of cytokine positive T cells in untreated sample}}$$

The results are then analysed with GraphPad Prism v. 10 via either students paired T test or repeated measures two-way ANOVA. A *p*-value of <0.05 was considered statistically significant.

4.4 Results and Discussion

The results will be discussed and presented by compound. For both the tetramer and the functional assays, farnesol was used as a positive control for CD1a, and α -GalCer was used a positive control for CD1d. For the functional assays, the results are expressed as both % cytokine secretion and as a fold change ratio. FMO controls were carried out for all tetramer assays (Figure A.1) and functional assays (Figure A.4). A stimulation control was carried by treating cells with Phorbol Myristate Acetate (PMA) and ionomycin (Figure 4.8).

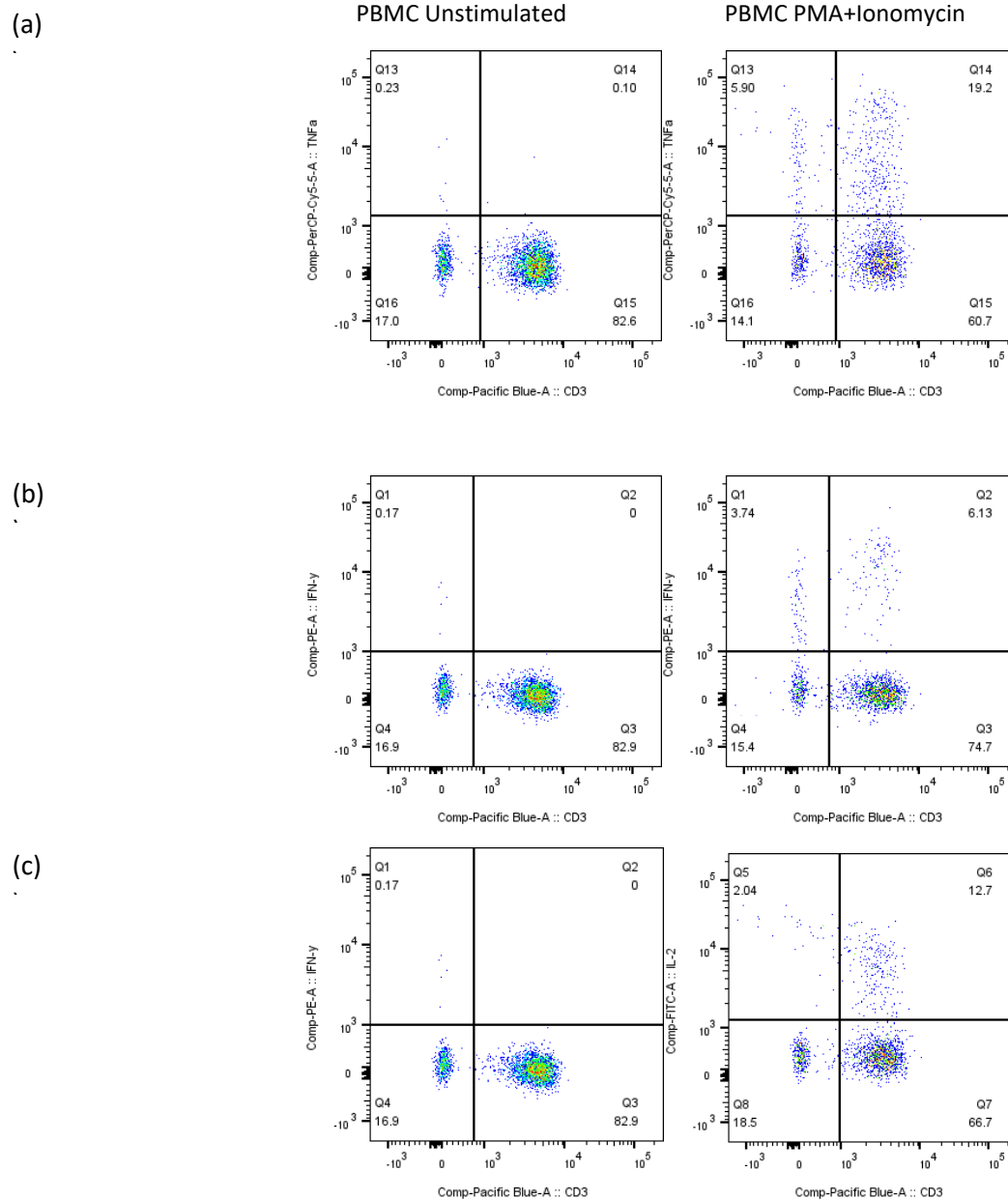


Figure 4.8 Stimulation Control for functional assays 5×10^6 PBMC are untreated or treated with PMA and ionomycin to induce activation. (a) TNF stimulation control, (b) IFN- γ stimulation control, (c) IL-2 stimulation control. Plots representative of one patient.

PMA and Ionomycin stimulation is a powerful, non-specific method to activate immune cells, especially T cells, for studying cytokine production by activating Protein Kinase C (PMA) and increasing intracellular calcium (ionomycin), bypassing natural receptors to trigger strong, simultaneous activation of signalling pathways (like NF- κ B and NFAT) leading to robust release of cytokines like IFN- γ , TNF and IL-2³²⁹. It is commonly used as a positive control to T cell stimulation assays. In this case, It gives a representation of the maximum

percentage of T cells in PBMC that produce each of the cytokines of interest. From figure 4.8 it can be seen that 19.2% of cells in the PBMC sample can produce TNF, 6.13% of cells can produce IFN- γ and 12.7% of cells can produce IL-2. Therefore, the majority of cells present in PBMC cannot produce these cytokines. This gives increased context to the the results obtained with the metals in the stimulation assays in this chapter.

4.4.1 Farnesol and α -GalCer

Farnesol was selected as a positive control due to previously published data that identified it as a CD1a-presented antigen⁸⁶. In this study, farnesol was loaded onto PE-conjugated CD1a tetramers. PBMC from healthy and nickel+ donors were stained with the loaded tetramers and analysed via flow cytometry to identify the number of CD3+ T cells recognized by the loaded tetramers. However, in contrast to previously published results, there was no significant increase in T cell recognition observed by farnesol loaded CD1a tetramers in comparison to DMSO loaded CD1a tetramers in either healthy donors or nickel+ donors (Figure 4.9).

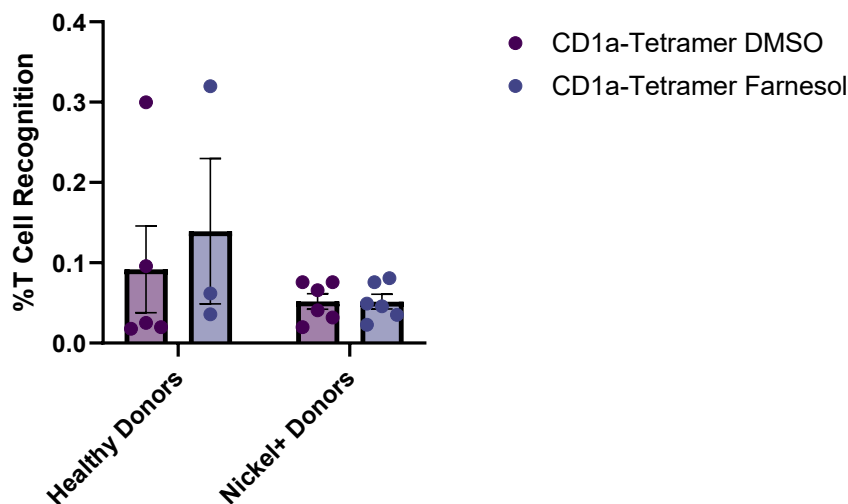


Figure 4.9 T cell recognition by farnesol loaded CD1a tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=6$) were stained with farnesol loaded CD1a tetramers or DMSO loaded vehicle tetramers and incubated for 20 minutes in the dark. PBMC were also stained with a Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means.

There was no increase in T cell recognition by farnesol loaded CD1a tetramers in comparison to the vehicle loaded control tetramers in either the healthy donors or nickel allergic donors. The results indicate that farnesol is not recognized by T cells in these experimental conditions. This was unsurprising, as the paper by Nicolai et al (2020) also found undetectable staining of CD1a-restricted T cells using farnesol loaded CD1a tetramers⁸⁶. Functional assays were carried out to identify the effect of farnesol pulsed C1R-CD1a APCs on T_H1 cytokine positive T cell frequency (Figure 4.10). ACD was long considered to be primarily T_H1 mediated³³⁰, but it is now accepted that a variety of T cell profiles are involved including T_H2 and T_H17 cells, and that the immune response is generally hapten specific³³¹. Nickel has been shown to activate predominantly T_H1 and T_H17 mediated pathways¹²⁸ and thus the T_H1 pathway was the focus of this study.

In a similar trend to the results observed in the tetramer assay, CD1a presented farnesol did not induce an increase in IFN- γ production in PBMC from healthy donors or nickel allergic donors [Figure 4.10(a)]. There was also no significant increase in IL-2 production [Figure 4.10(b)], or TNF production [Figure 4.10(c)] observed in either healthy or nickel+ PBMC that were co-cultured with farnesol pulsed C1R-CD1a APCs, however the standardised fold change ratio showed a minimal non-significant increase in TNF production in nickel+ PBMC.

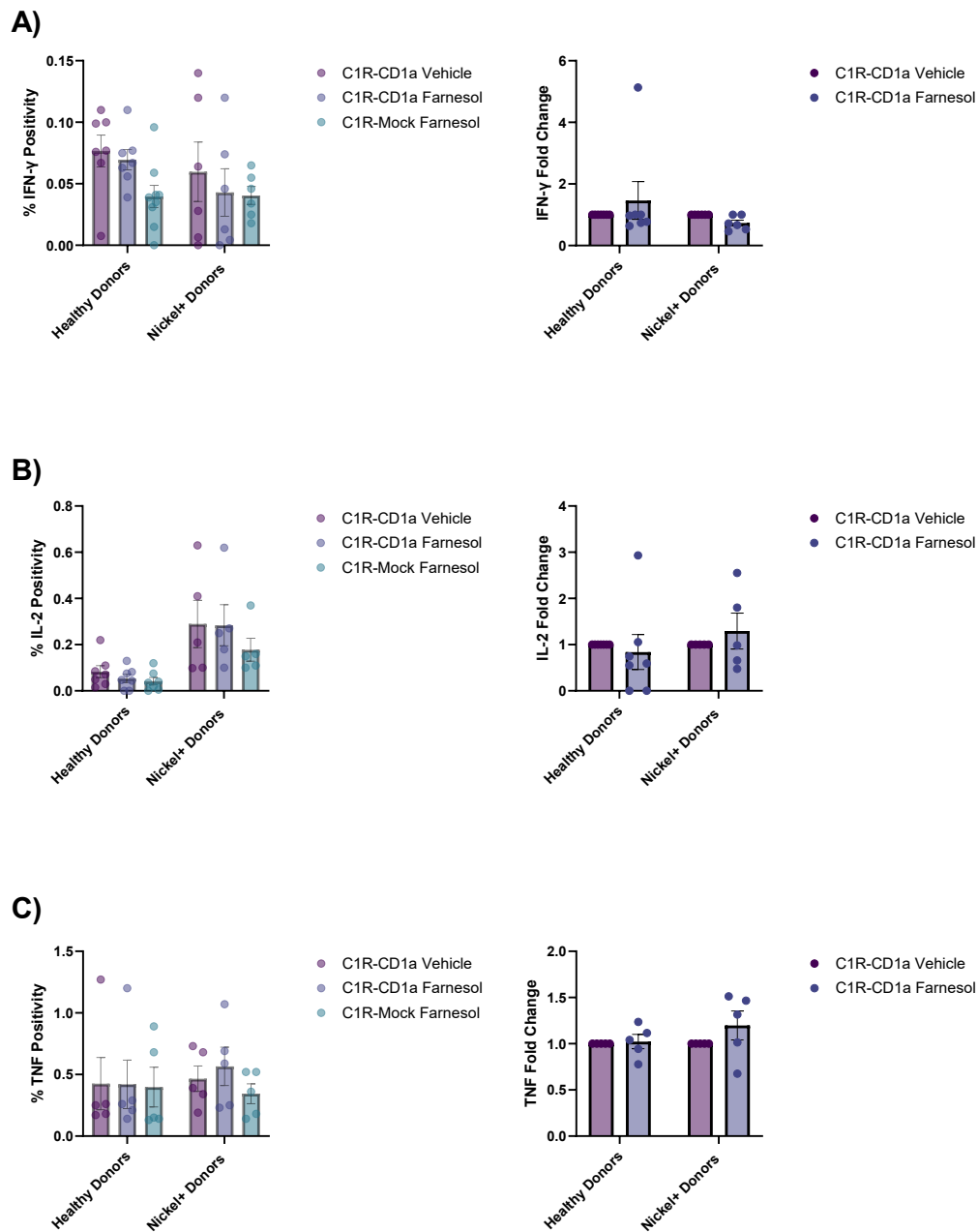


Figure 4.10 Cytokine Production by T cells co-cultured with farnesol pulsed C1R-CD1a APCs. *C1R-CD1a* and *C1R-mock* cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL farnesol overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=5, TNF n=5) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells are quantified. Cytokine positivity is presented as % cytokine positivity in comparison to DMSO controls and mock controls, and also as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data is analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data is analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means.

The lack of both T cell recognition and cytokine production by farnesol in a CD1a-dependent manner comes in contrast to previously published data which saw an increase in IFN- γ production by farnesol in a CD1a dependent manner. However, the differences in results could be explained by the differences in experimental conditions. Farnesol was previously tested using plate bound CD1a rather than CD1a transfected APCs, and a known CD1a autoreactive T cell line, BC2, was used rather than PBMC. In addition, a much higher concentration of farnesol was used to elicit an increase in IFN- γ (150 μ M) in comparison to the 200 ng/mL (0.90 μ M) concentration used here. It is possible that the concentration used in this study was too low to induce cytokine secretion, or that it could not be significantly detected in PMBCs where the percentage population of CD1a autoreactive T cells is so low, rather than a CD1a specific T cell line. In addition, there are relatively few known CD1a antigens thus far and there is only one known study that has used farnesol in CD1a related experiments, indicating that it has not yet been extensively studied using different experimental set ups and conditions. Therefore, the lack of significant T cell recognition or cytokine production observed using farnesol did not affect the procedure or analysis of future CD1a related assays using metals or lipid peroxidation products.

α -GalCer was chosen as a positive control for CD1d tetramer assays and functional assays, due to its extensive use in CD1d related studies since its discovery as a potent CD1d-presented antigen in 1997 by Kawano et al³⁰². It has been previously loaded into CD1d tetramers and used to detect and study iNKT cells³³², and has become the standard reagent to study CD1d antigen presentation, and iNKT cell development and function. Additionally, it has been used in immunotherapy research focusing on cancer and infection^{333, 334}. In this study, α -GalCer was loaded into CD1d tetramers, which were subsequently used to stain PBMC from healthy donors and nickel+ donors. T cells recognized by the α -GalCer loaded tetramers were quantified and analysed (Figure 4.11). α -GalCer loaded tetramers recognized a significantly higher percentage of CD3+ T cells in comparison to DMSO loaded control tetramers in both healthy PBMC and nickel+ PBMC. However, there was no significant difference between the percentage of T cells recognized by α -GalCer loaded CD1d tetramers in PBMC from healthy donors and T cells recognized by α -GalCer loaded

CD1d tetramers in PBMC from nickel+ donors. This suggests that there is no significant increase in α -GalCer/CD1d-restricted T cells in nickel allergic patients in comparison to healthy patients.

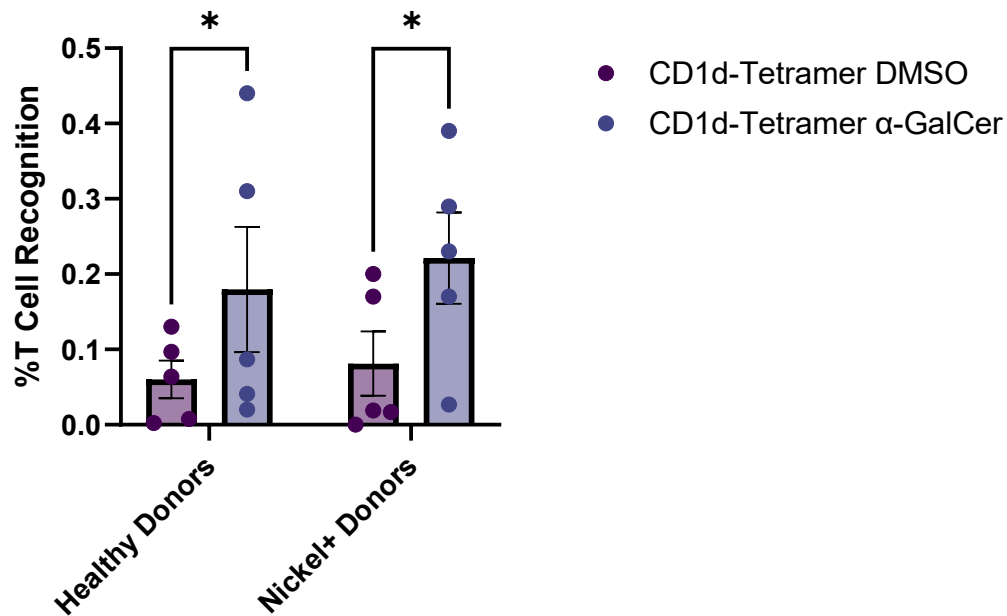


Figure 4.11 T cell Recognition by α -GalCer loaded CD1d Tetramers. 5×10^6 PBMC from healthy donors (n=5) or nickel+ donors (n=6) were stained with α -GalCer loaded CD1a tetramers or DMSO loaded vehicle tetramers and incubated for 20 minutes in the dark. PBMC were also stained with a Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data was analysed by two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. *p<0.05

As a known, potent CD1d antigen, the significant results obtained with α -GalCer loaded tetramers indicated that the method was working and that the tetramers could effectively recognize T cells in PBMC samples. Functional assays were then carried out to investigate if α -GalCer pulsed HeLa-CD1d APCs could increase cytokine production in healthy or nickel+ PBMC (Figure 4.12). CD1d-presented α -GalCer did not increase IFN- γ production in healthy PBMC in comparison to DMSO controls and HeLa-Mock controls. However, there appeared to be an increase in IFN- γ in PBMC from nickel+ donors co-cultured with α -GalCer pulsed CD1d APCs in comparison to DMSO controls and HeLa-mock controls, but the increase was not found to be statistically significant [Figure 4.12(a)]. This increase can also be observed

in the fold change ratio, but the increase is again non-significant. A similar trend was observed for IL-2 production [Figure 4.12(b)]. There was no increase in IL-2 production seen when α -GalCer was presented to PBMC from healthy donors by CD1d in comparison to vehicle controls and HeLa-mock controls. However, α -GalCer appeared to increase IL-2 production in a CD1d dependent manner in nickel+ PBMC. When the results were standardised using the fold change ratio, the increase in IL-2 production in nickel+ PBMC co-cultured with HeLa-CD1d APCs pulsed with α -GalCer was statistically significant in comparison to the vehicle control. Additionally, there was a significant increase in IL-2 production seen in nickel+ donors in comparison to healthy donors. The trend observed with IFN- γ , and IL-2 production continued with TNF production. No increase in TNF was observed with healthy PBMC in the presence of α -GalCer pulsed CD1d APCs, but there did appear to be an increase in TNF production in nickel+ PBMC co-cultured with α -GalCer pulsed APCs in comparison to the vehicle and HeLa-mock controls, albeit the increase was not statistically significant. Standardisation to the fold change allowed this increase in TNF production in nickel allergic patients to be seen more clearly, and it was calculated to be close to significance, with a p -value of 0.09.

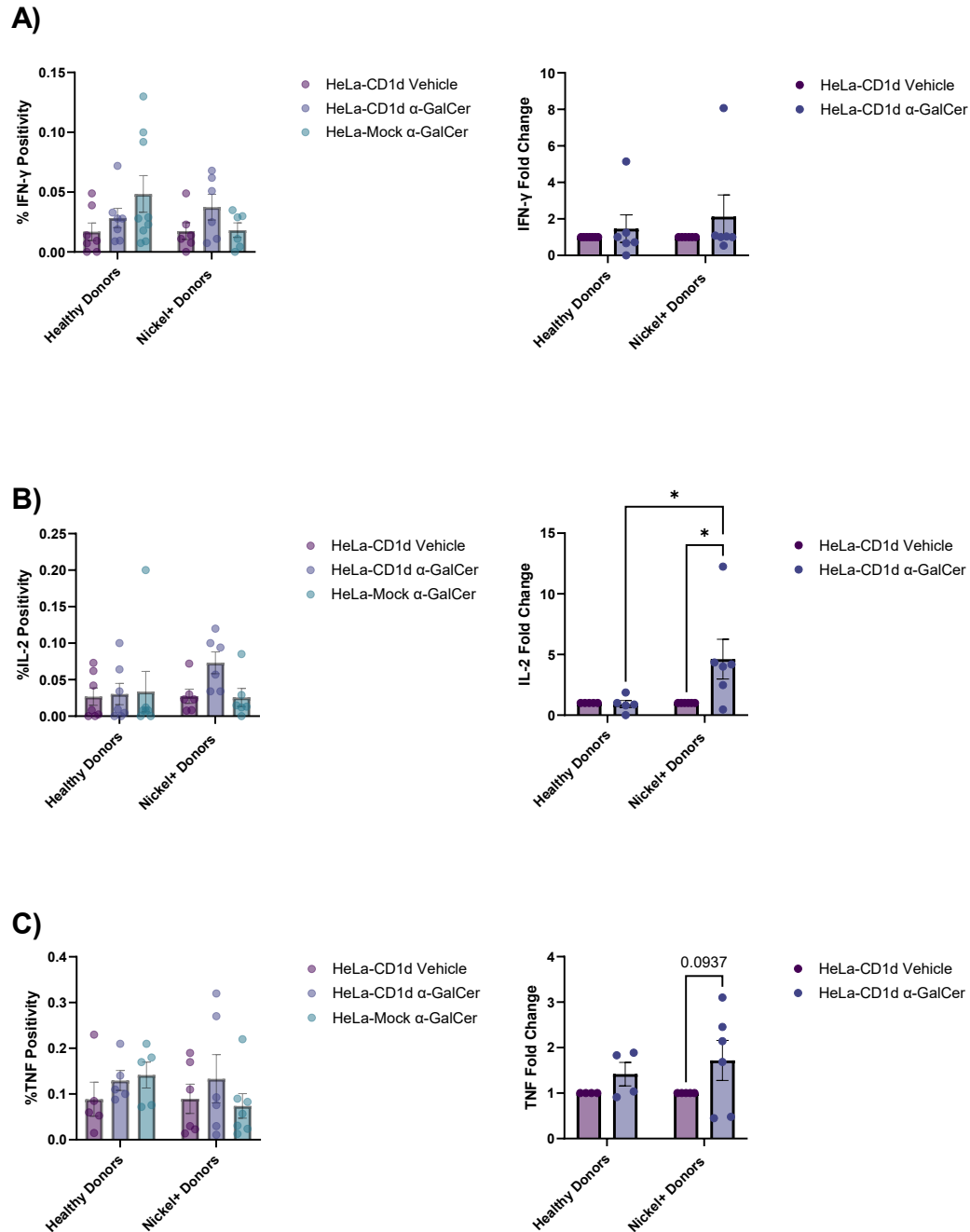


Figure 4.12 Cytokine Production by T cells co-cultured with α -GalCer pulsed HeLa-CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL farnesol overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=7, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=6) per well. Cells were analysed via flow cytometry and frequency of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as (left) % cytokine production in comparison to DMSO controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. Outliers were identified by ROUT analysis and removed. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means.

While there were no increases in cytokine production observed in healthy PBMC, CD1d-presented α -GalCer did appear to induce increases in cytokine production in nickel+ PBMC in comparison to vehicle and CD1d-mock controls, with statistically significant increases being observed when data is standardised using the fold change ratio. It is possible that more significant increases would be seen upon increasing the sample size. The results obtained in the tetramer assay and functional assays reinforced α -GalCer's status as a potent CD1d antigen and correlated with previously published research which showed that α -GalCer (100 ng/mL) could activate T cells. However, the cytokine increases appeared difficult to detect in PBMC samples in which the percentage of CD1d-restricted T cells is very low. The increases observed in nickel allergic patients could suggest a higher percentage of CD1d-restricted T_H1 cytokines in patients with contact allergy in comparison to healthy patients.

Having established the methodology with the α -GalCer positive control, the tetramer and functional assays were then performed with the metals and lipid peroxidation products.

4.4.2 Nickel as a CD1a and CD1d antigen

NiSO₄ has previously been tested as a CD1a antigen in which it was added as an allergen patch in a CD1a plate bound assay, which found no significant increase in IFN- γ production in a known CD1a-reactive T cell line⁸⁶. However, to our knowledge, NiSO₄ has never been tested using CD1a tetramers, and its potential as a CD1a ligand has not been investigated. NiSO₄ was loaded onto CD1a tetramers, which were then used to stain PBMC from healthy donors and nickel+ donors to evaluate CD1a-dependent T cell recognition by NiSO₄ (Figure 4.13). There was a significant increase in T cell recognition by the NiSO₄ loaded tetramers in comparison to the unloaded tetramers in both healthy donors and nickel+ donors, indicating that nickel is a potential CD1a ligand. There was no significant difference between the percentage of T cells recognized by NiSO₄ loaded CD1a tetramers in PBMC from healthy donors and T cells recognized by NiSO₄ loaded CD1a tetramers in PBMC from nickel+ donors. This suggested that there was no significant difference in the percentage of CD1a-

restricted T cells in nickel allergic patients in comparison to healthy patients. Alternatively, due to nickel's status as the most common and prevalent contact allergen, it could be possible that the healthy patients are unknowingly allergic to nickel or have been unknowingly exposed to nickel. As the healthy blood was obtained from the IBTS blood bank, the donors were not screened for allergy to nickel prior to obtaining the blood samples.

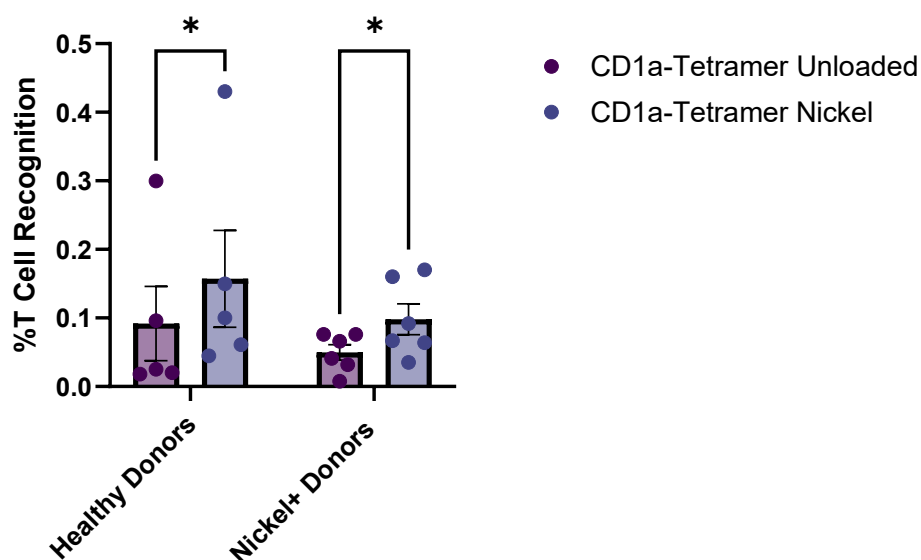


Figure 4.13 T Cell Recognition by NiSO₄ loaded CD1a Tetramers. 5×10^6 PBMC from healthy donors (n=5) or nickel+ donors (n=6) were stained with NiSO₄ loaded CD1a tetramers or unloaded vehicle tetramers and incubated for 20 minutes in the dark. PBMC were also stained with a Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means. *p<0.05

The increase in T cell recognition by NiSO₄ loaded CD1a tetramers in nickel+ PBMC can be seen in the dot plots obtained on the flow cytometer (Figure 4.14). In the dot plots displayed below, representative of one nickel allergic patient, the percentage of cells staining positive for both CD3 and the PE-conjugated tetramer (Q2) was higher with the NiSO₄ loaded tetramer in comparison to the unloaded tetramer. An additional replicate is shown in the appendix in Figure A.2.

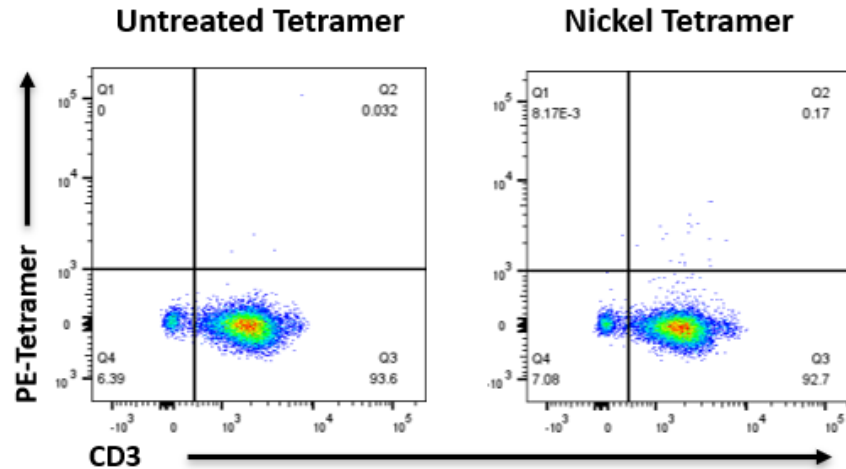


Figure 4.14 Flow Cytometry Plot of T cell recognition by NiSO₄ loaded CD1a tetramer in a Nickel+ Patient.

Having observed nickel's potential as a CD1a ligand through the significant increase in T cell recognition with NiSO₄ loaded CD1a tetramers, functional assays were then performed to assess the ability of NiSO₄ to activate T cells in a CD1a dependent manner. NiSO₄ pulsed APCs were co-cultured with PBMC from healthy donors or PBMC from nickel+ donors, and the production of T_H1 cytokines was measured (Figure 4.15). CD1a-presented NiSO₄ did not induce an increase in IFN- γ positivity in PBMC from healthy donors, but did appear to induce an increase in IFN- γ positivity in PBMC from nickel+ donors [Figure 4.15(a)]. T cells from nickel+ patients co-cultured with NiSO₄ pulsed CD1d transfected HeLa cells appeared to produce more IFN- γ than the T cells cultured with the untreated HeLa-CD1d cells, but the increase was not statistically significant. When the results were standardised using the fold change ratio, the increase in frequency of IFN- γ producing T cells in nickel+ patients was statistically significant. This IFN- γ positivity also appeared to be higher than when T cells were cultured with NiSO₄ pulsed HeLa-Mock cells, with statistical analysis showing that the difference in IFN- γ production was very close to significance ($p=0.0685$), suggesting that the IFN- γ production seen in T cells cultured with NiSO₄ pulsed C1R-CD1a APCs was CD1a dependent. Furthermore, standardisation of the data showed that there was a significantly

higher CD1a dependent production of IFN- γ in PBMC from nickel+ patients than in PBMC from healthy patients.

There also appeared to be a significant increase in CD1a dependent IL-2 production in PBMC from nickel+ donors induced by NiSO₄ treatment, however a similar increase was not observed in PBMC from healthy patients [Figure 4.15(b)]. There was a significant increase in the frequency of IL-2 producing T cells co-cultured with NiSO₄ pulsed C1R-CD1a APCs in comparison to the untreated control and C1R-Mock control. This confirms that the increase in IL-2 production was dependent on both NiSO₄ and CD1a being present. This significant increase was seen in both the raw data, and standardised data. Additionally, there was significantly higher IL-2 production in PBMC from nickel+ patients in comparison to PBMC from healthy patients.

Similarly to IFN- γ and IL-2, there was no increase in the frequency of TNF positive T cells in PBMC from healthy patients [Figure 4.15(c)]. T cells from nickel allergic patients co-cultured with NiSO₄ pulsed C1R-CD1a APCs had an increased frequency of TNF positive T cells than the untreated control. There was also an apparent increase in TNF production in comparison to the mock control, it was not statistically significant. However, a *p*-value of 0.0513, close to significance, suggested that the increase in TNF production was CD1a dependent. Standardization of the results to the fold change ratio confirmed firstly the increased TNF production in nickel+ patients in comparison to the untreated control and confirmed secondly that there was significantly higher frequency of TNF positive T cells in PBMC from nickel allergic patients in comparison to PBMC from healthy patients.

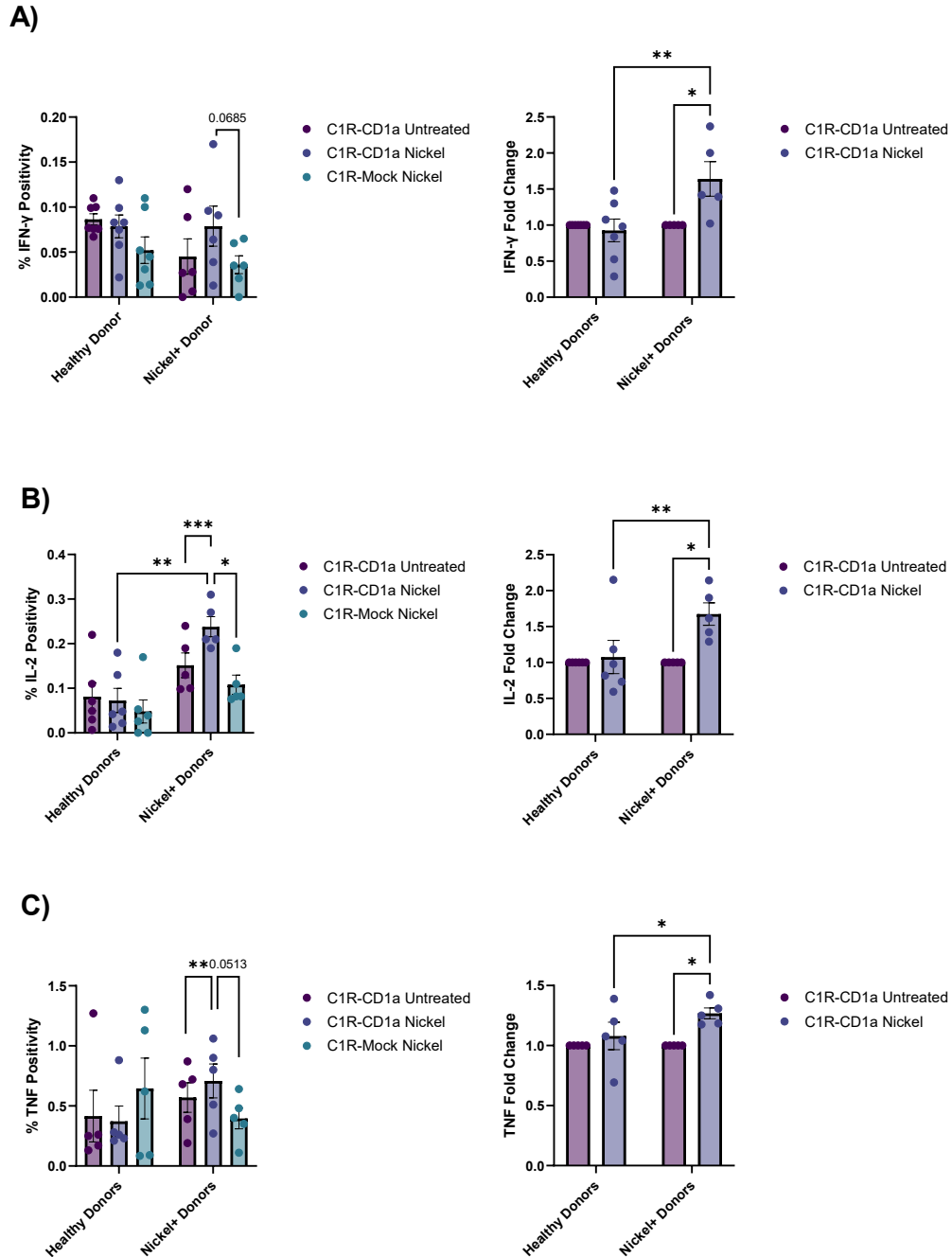


Figure 4.15 Cytokine Production in T cells co-cultured with NiSO_4 pulsed CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL NiSO_4 overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=5, TNF n=5) per well. Cells are analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells are quantified. Cytokine positivity is presented as (left) % Cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The increase in cytokine production by T cells co-cultured with NiSO₄ pulsed C1R-CD1a APCs in nickel+ patients can be seen in the dot plots obtained on the flow cytometer (Figure 4.16) In the dot plots displayed below, representative of one nickel allergic patient, the percentage of cells staining positive for both CD3 and respective cytokine was higher in the T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs, than the C1R-CD1a untreated control and the C1R-Mock control. Frequency of cytokine positive T cells for an additional replicate is shown in Figure A.5.

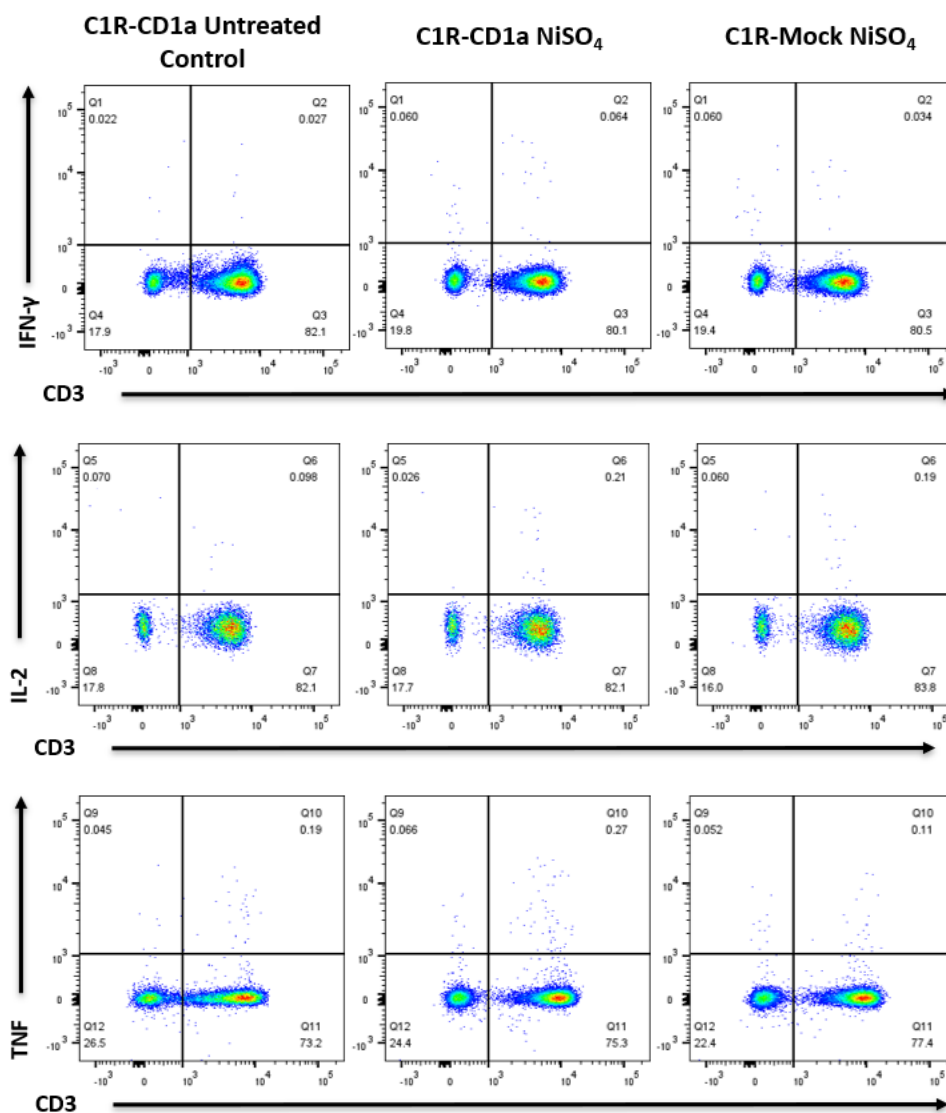


Figure 4.16 Flow Cytometry Plots Showing Cytokine Production by T cells co-cultured with NiSO₄ pulsed C1R-CD1a APCs.

To confirm a CD1a dependent increase in cytokine production, the percentage of multi-functional T cells was quantified, i.e. T cells that are positive for both IFN- γ and TNF after co-culture with NiSO₄ pulsed C1R-CD1a APCs (Figure 4.17). There was an increase in multi-functional T cells in the PBMC of nickel+ patients that were co-cultured with NiSO₄ pulsed C1R-CD1a APCs in comparison to the untreated and C1R-mock controls [Figure 4.17(a)]. Standardisation of the data to the fold change ratio showed that there was significantly more multifunctional T cells present in the PBMC of nickel+ donors than in healthy donors [Figure 4.17(b)].

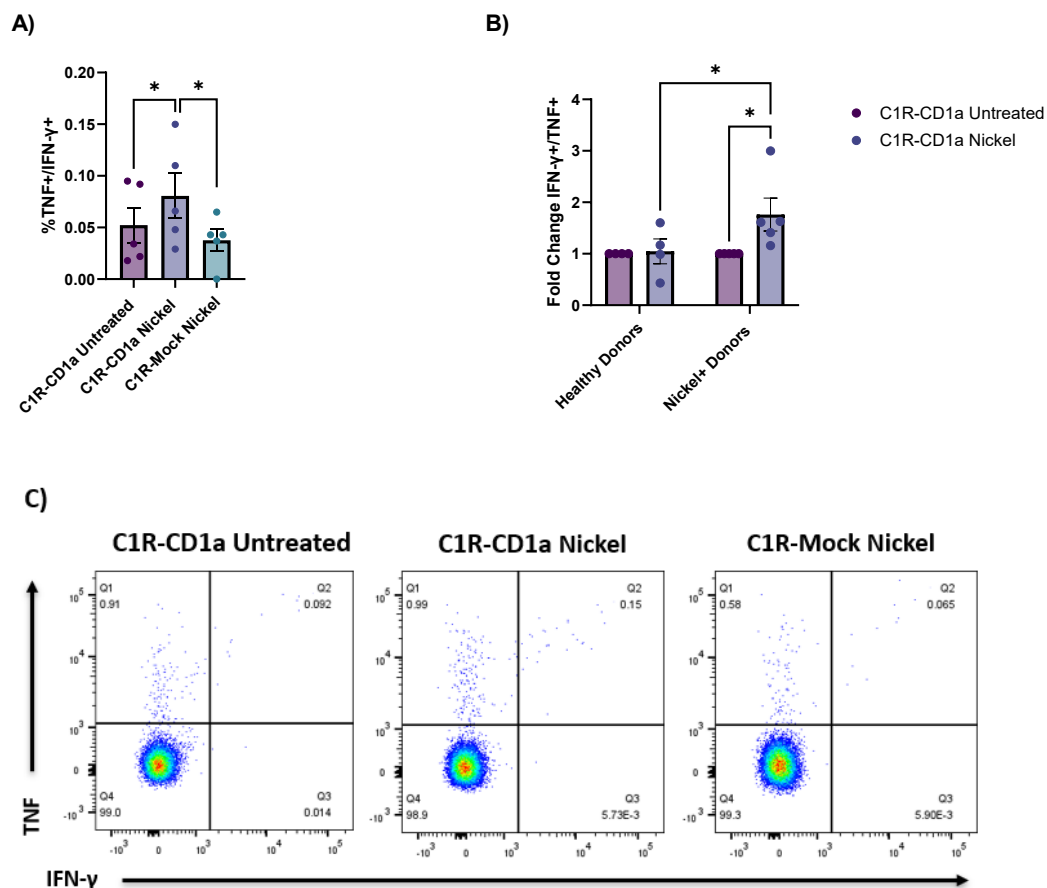


Figure 4.17 Percentage of IFN- γ ⁺/TNF α ⁺ multi-functional T cells in PBMC co-cultured with NiSO₄ pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL NiSO₄ overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (n=5) or nickel+ donors (n=5) per well. Cells were analysed via flow cytometry and T cells staining positive for both IFN- γ and TNF are quantified. A) %TNF- α ⁺/IFN- γ ⁺ T cells produced in PBMC from nickel+ patients co-cultured with NiSO₄ pulsed C1R-CD1a APCs. Data analysed by students paired t test. B) Data standardised to fold change ratio. Data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. C) Flow cytometry plots showing an increase in multifunctional T cells in PBMC co-cultured with NiSO₄ pulsed C1R-CD1a cells. Data presented as mean $\pm$ SEM. *p<0.05 Dot plots for an

additional replicate are shown in Figure A.6. Overall, NiSO₄ appeared to induce CD1a-dependent production of T_H1 cytokines including IFN- γ , IL-2 and TNF, as well as an increase in the number of multifunctional T cells, in the PBMC of nickel allergic patients, but not in the PBMC of healthy patients. These results suggest a potential novel mechanism of action in the pathophysiology of nickel ACD and could provide a new future therapeutic target in the treatment of nickel ACD.

Having established a link between CD1a and nickel ACD, focus then turned to investigating a possible link between CD1d and nickel ACD. NiSO₄ was loaded onto CD1d tetramers, which were then used to stain PBMC from healthy donors and nickel+ donors to evaluate CD1d-dependent T cell recognition by NiSO₄ (Figure 4.18). There was no increase in T cell recognition using nickel loaded tetramers in the PBMC of healthy donors, however nickel loaded CD1d tetramers recognized significantly more T cells in the PBMC of nickel+ patients in comparison to unloaded tetramers. This contrasts with the results obtained with nickel loaded CD1a tetramers, which identified significantly more T cells in both healthy and nickel+ PBMC compared to unloaded CD1a tetramers. This suggested that while CD1a-restricted T cells may be of a similar level in both healthy and nickel allergic patients, there is potentially a higher frequency of CD1d-restricted T cells present in allergic patients than in healthy patients.

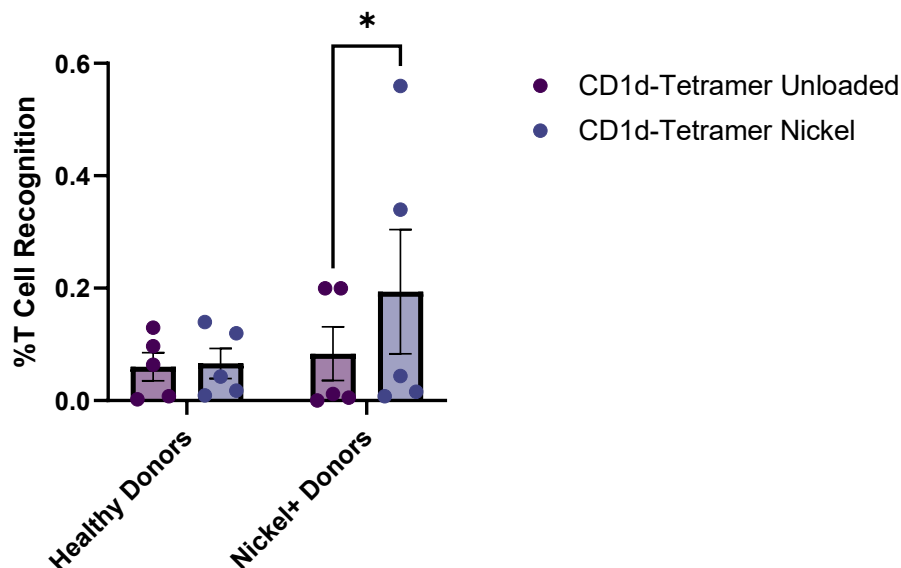


Figure 4.18 T Cell Recognition by Nickel Loaded CD1d Tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=6$) were stained with NiSO_4 loaded CD1d tetramers or unloaded tetramers and incubated for 20 minutes in the dark. PBMC are also stained with Pacific Blue conjugated anti-CD3 antibodies to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means. * $p < 0.05$.

Overall, the CD1d tetramer experiment showed that nickel loaded CD1d tetramers can recognize T cells and suggests that nickel could be a CD1d ligand. This increase can be visualised by examining the flow cytometry dot plots of T cell recognition by nickel-CD1d loaded tetramers in nickel+ PBMC (Figure 4.19). An additional replicate is shown in the appendix (Figure A.3).

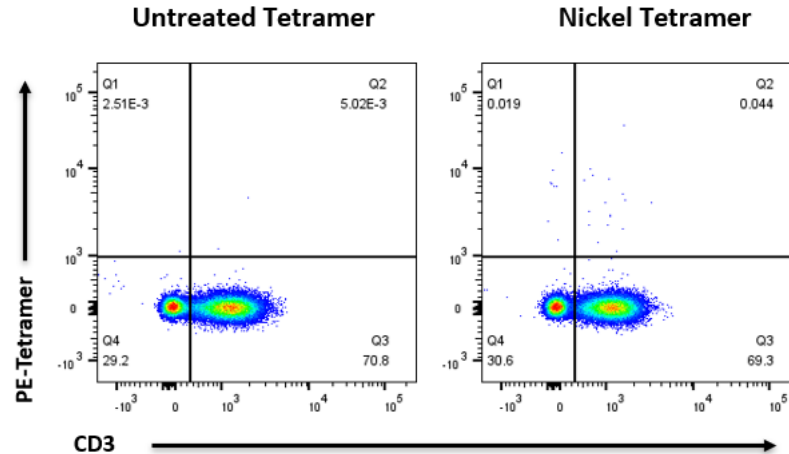


Figure 4.19 Flow Cytometry Plot of T cell recognition by NiSO₄ loaded CD1d tetramer in a Nickel+ Patient. Plots of one nickel allergic patient, representative of n=6.

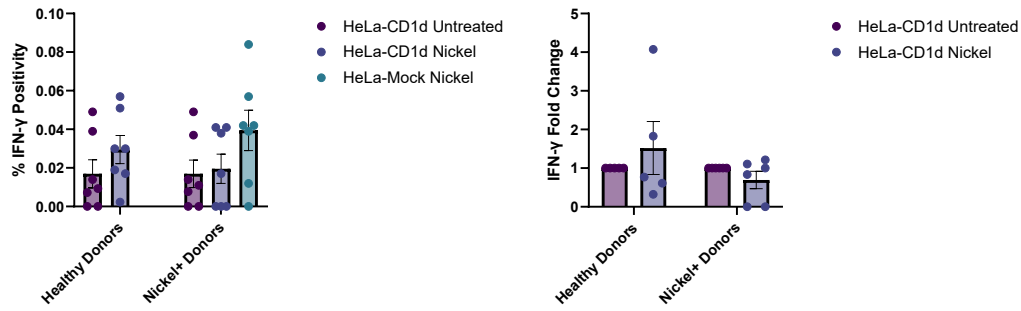
After observing nickel's potential as a CD1d ligand, evidenced by the marked increase in T cell recognition with NiSO₄-loaded CD1d tetramers, functional assays were conducted to evaluate whether NiSO₄ could activate T cells in a CD1d-dependent manner. NiSO₄ pulsed HeLa-CD1d APCs were co-cultured with PBMC from healthy donors or PBMC from nickel+ donors, and the production of T_H1 cytokines was measured (Figure 4.20). Nickel did not appear to induce a CD1d dependent increase in the frequency of IFN- γ positive T cells in either PBMC from healthy patients or PBMC from nickel allergic patients [Figure 4.20(a)]. While there appeared to be an increase in the frequency of IFN- γ positive T cells in healthy PBMC, it was not deemed to be statistically significant, and no increase was observed in nickel+ PBMC. In fact, there appeared to be a higher frequency of IFN- γ positive T cells in the T cells co-cultured with NiSO₄ pulsed HeLa-Mock APCs.

There was also a lack of CD1d-dependent IL-2 production induced by NiSO₄ in healthy PBMC [Figure 4.20(b)]. In contrast, there was a significant increase in IL-2 production in nickel allergic T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs in comparison to the untreated control. However, this increase in IL-2 production was not statistically significant in comparison to the HeLa-Mock control, and so it cannot be confirmed that the NiSO₄ induced increase in IL-2 production was CD1d-dependent. Standardisation of the fold change ratio confirmed the significant increase in the frequency of IL-2 positive T cells in the T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs and showed that there was

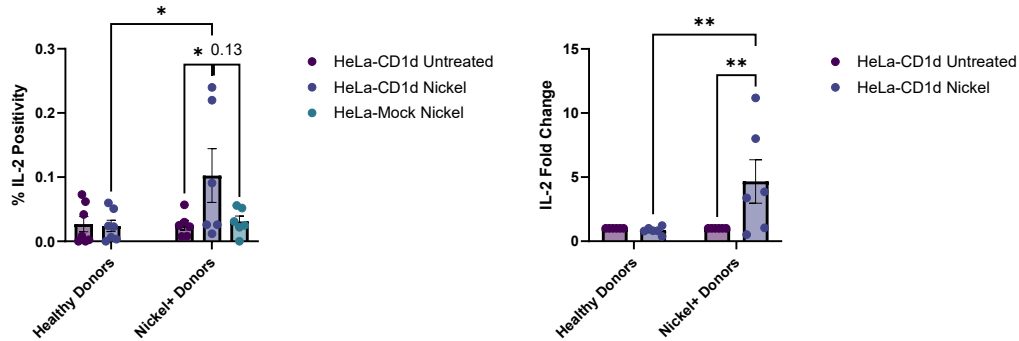
significantly higher IL-2 production in nickel allergic patients in comparison to healthy patients.

There were no significant increases in the percentage of TNF positive T cells in either healthy or nickel allergic PBMC co-cultured with NiSO₄ pulsed HeLa-CD1d APCs according to the raw data (% positivity), however standardisation of the data to the fold change ratio confirmed a significant increase in TNF in both healthy and nickel allergic patients [Figure 4.20(c)]. This increase was confirmed to be CD1d dependent in nickel allergic patients, as the increase in TNF production in T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs was significantly higher than that of the HeLa-Mock control.

A)



B)



C)

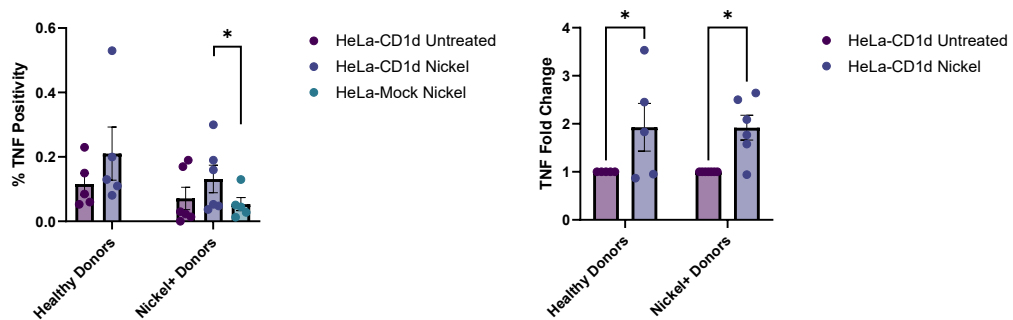


Figure 4.20 Cytokine Production in T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL NiSO₄ overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=6) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells are quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$,

Overall, CD1d presented NiSO₄ increased TNF production in a CD1d-dependent manner, increased IL-2 production (which could not be confirmed as CD1d dependent), but did not induce any increase in IFN- γ production in either healthy or nickel allergic patients. It is unusual to see increases in TNF production but not IFN- γ , although previous studies have shown that certain subsets of T cells can favour TNF production over IFN- γ production, such as $\gamma\delta$ T cell clones³³⁵.

The increase in TNF production and IL-2 production can be visualised on the flow cytometry dot plots obtained (Figure 4.21). In the dot plots displayed below, representative of one nickel allergic patient, the percentage of cells staining positive for both CD3 and the respective cytokine was higher in the T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs, than with the HeLa-CD1d untreated control and the HeLa-Mock control. An additional replicate is shown in the appendix (Figure A.7).

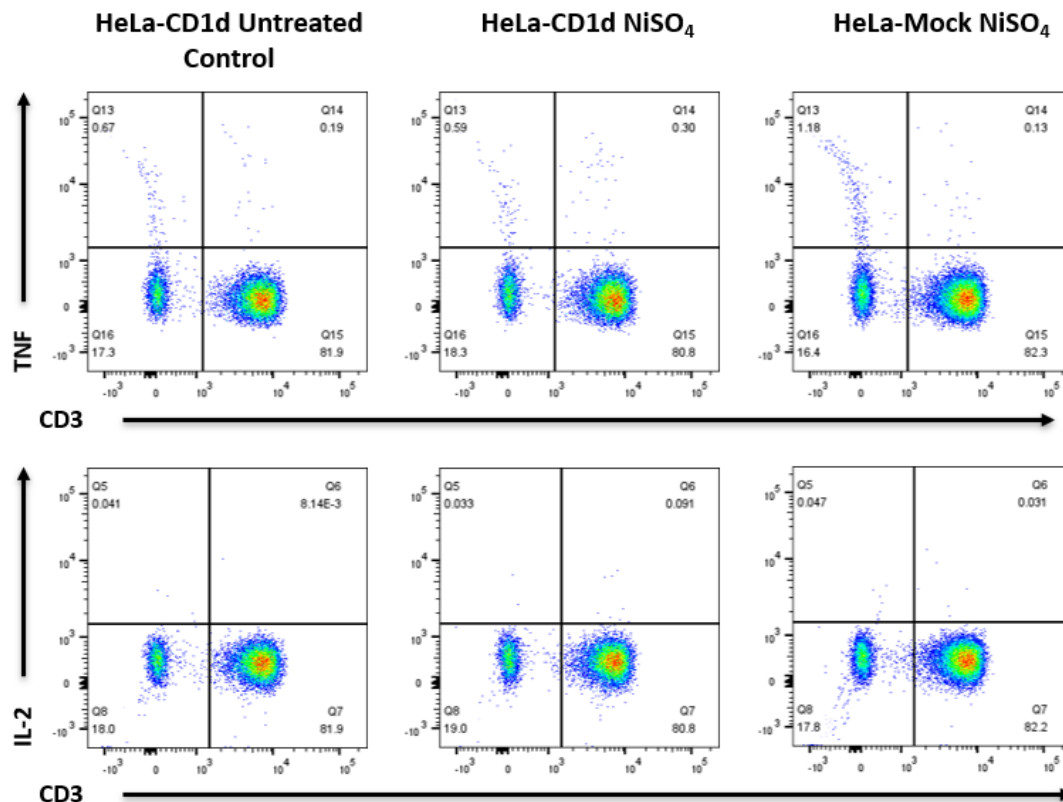


Figure 4.21 Flow Cytometry Plots showing cytokine production in T cells from nickel allergic patients co-cultured with NiSO₄ pulsed HeLa-CD1d APCs.

Overall, T cells from nickel allergic patients recognized NiSO_4 loaded onto both CD1a and CD1d tetramers. CD1a presented NiSO_4 was able to activate T cells and induced production of IFN- γ , TNF and IL-2, whereas CD1d presented NiSO_4 was only able to induce production of TNF and IL-2 in PBMC from nickel allergic patients. The results obtained suggest that nickel acts as a CD1a and CD1d antigen. The CD1 ligands identified so far are all organic molecules, and the role of CD1a and CD1d in metal allergy has not been established. These results are the first that indicate that NiSO_4 is a CD1a and CD1d ligand. The mechanism by which NiSO_4 acts as a CD1a or CD1d ligand, and how it binds to CD1a and CD1d, requires further investigation. The molecular size of NiSO_4 in its hexahydrate form used in these experiments, is 261.95 Da³³⁶, which falls within the optimal size for CD1a antigens of 222 to 410 Da. However, its structure does not contain any other chemical properties that are known to favour CD1a binding, like unsaturated bonds and the presence of aromatic rings⁸⁶. Nickel is also termed as a superantigen, and can directly link TCRs and MHC in a peptide independent manner, therefore a hypothesis is that it binds TCRs and CD1a/CD1d in a similar lipid independent manner³³⁷. Whether nickel binds directly to CD1 proteins or affects other cellular structures that can then bind to CD1 proteins is yet to be seen. Nevertheless the results obtained suggest potential CD1a and CD1d involvement in the pathophysiology of nickel ACD.

4.4.3 Cobalt as a CD1a and CD1d Antigen

Having observed that nickel is a potential CD1a and CD1d antigen, focus then turned to other metal allergens including CoCl_2 . Cobalt was previously evaluated as a CD1a antigen in a plate bound assay with a known CD1a reactive T cell line, which observed an increase in IFN- γ production that was not reproducible⁸⁶. However, it has not been investigated as a CD1a or CD1d antigen using the experimental conditions discussed in this chapter. CoCl_2 was loaded onto CD1a or CD1d tetramers which were subsequently used to stain PBMC from healthy donors and PBMC from nickel allergic donors. Cells that stained positively for CD3 and the PE-conjugated tetramer were quantified and analysed (Figure 4.22). There was no increase in T cell recognition with CD1a-loaded tetramers in either healthy donors or nickel allergic donors, suggesting that cobalt is not a CD1a antigen [Figure 4.22(a)]. CoCl_2

loaded CD1d tetramers appeared to induce an increase in T cell recognition in healthy donors, however this increase was not statistically significant. In contrast, there was a non-significant reduction in T cell recognition in nickel+ donors [Figure 4.22(B)].

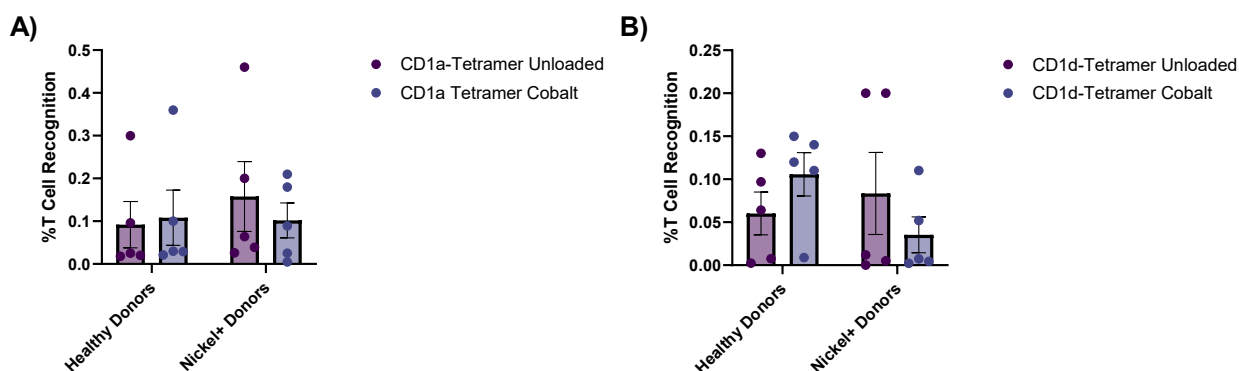


Figure 4.22 T cell recognition by A) CoCl_2 loaded CD1a tetramers and B) CoCl_2 loaded CD1d tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=5$) were stained with A) CoCl_2 loaded CD1a tetramers or unloaded tetramers and B) CoCl_2 loaded CD1d tetramers or unloaded tetramers and incubated for 20 minutes in the dark. PBMC are also stained with Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means

Overall, CoCl_2 did not appear to act as an antigen for either CD1a or CD1d, with a lack of T cell recognition observed with either CoCl_2 loaded CD1a or CD1d tetramers. To further investigate this, functional assays were performed to assess the ability of CoCl_2 to activate T cells in a CD1a or CD1d dependent manner.

CoCl_2 presented by CD1a did not induce an increase in the frequency of IFN- γ positive T cells, in comparison to untreated controls and C1R-mock controls, in both healthy PBMC and nickel+ PBMC [Figure 4.23(a)]. This was confirmed when the data was standardised to the fold change ratio which showed no increases in IFN- γ positivity in either cohort of patient, or also that there was no significant increase in IFN- γ production in healthy patients in comparison to nickel allergic patients.

There also did not appear to be any increase in the frequency of IL-2 positive T cells among T cells co-cultured with CoCl_2 pulsed C1R-CD1a APCs in either PBMC from healthy patients

or PBMC from nickel+ patients, in comparison to untreated controls and C1R-mock controls [Figure 4.23(b)]. There appeared to be a significant increase in the amount of IL-2 produced in T cells co-cultured with CoCl₂ pulsed C1R-CD1a cells in nickel+ patients in comparison to healthy patients, however without a significant difference in IL-2 production in nickel+ patients in comparison to the untreated control in nickel+ patients, the difference in IL-2 production in the two patient cohorts cannot be confirmed. Standardisation to the fold change ratio confirmed that there was no CD1a-dependent production of IL-2 induced by CoCl₂ in either healthy or nickel allergic patients, and that there was no significant difference between the IL-2 levels produced in the two patient cohorts.

Finally, there was no increase in the frequency of TNF positive T cells in PBMC co-cultured with CoCl₂ pulsed C1R-CD1a cells in either healthy or nickel allergic patients, in comparison with untreated controls and mock controls [Figure 4.23(c)]. This was confirmed when the data was standardized to the fold change ratio, which showed a lack of CD1a dependent TNF production in both patient cohorts. In addition, there was no significant difference in the levels of TNF production in nickel allergic donors in comparison to healthy donors.

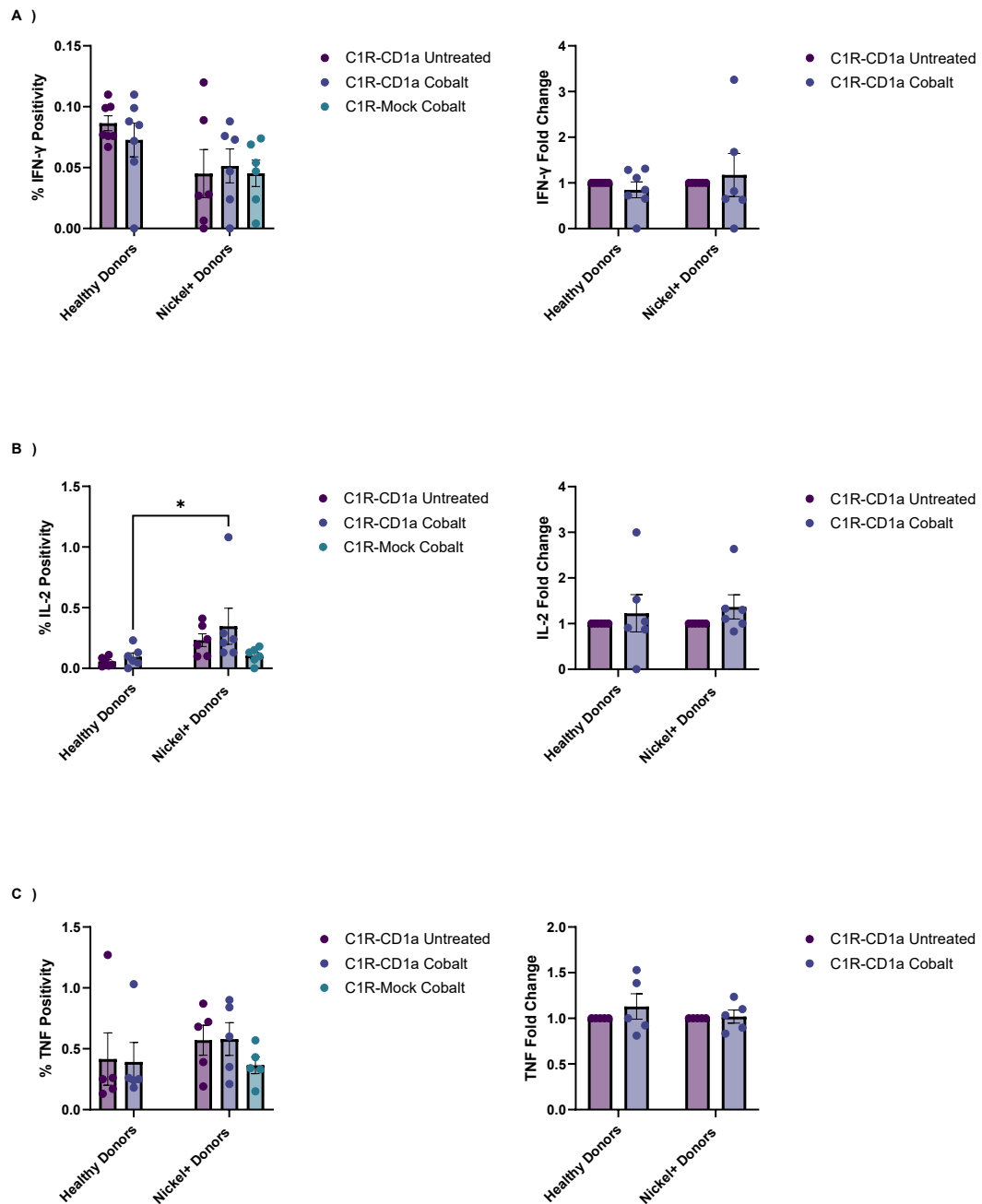


Figure 4.23 Cytokine Production by T Cells co-cultured with CoCl_2 pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL CoCl_2 overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=5) per well. Cells were analysed via flow cytometry and the frequency of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (b) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means.

Overall, there was a lack of CD1a dependent cytokine production induced by CoCl₂ in both healthy patients and nickel allergic patients, which indicated that CoCl₂ does not activate T_H1 cells in a CD1a dependent manner. Thereafter, the ability of CoCl₂ to activate T cells in a CD1d dependent manner and produce T_H1 cytokines was investigated (Figure 4.24).

There appeared to be an increase in the frequency of IFN- γ positive T cells among PBMC from healthy donors, co-cultured with CoCl₂ pulsed HeLa-CD1d APCs in comparison to the untreated control and HeLa-Mock control. However, this increase was not statistically significant until standardisation of the data to the fold change ratio. An increase in frequency of IFN- γ producing T cells was not seen in nickel+ donors, with the fold change ratio showing a significant decrease in the frequency of IFN- γ positive T cells in nickel allergic patients in comparison to healthy patients.

There were no significant changes seen in the frequency of IL-2 positive T cells among T cells co-cultured with CoCl₂ pulsed HeLa-CD1d APCs in healthy or nickel allergic donors, in comparison to the untreated controls and HeLa-mock controls. Standardisation to the fold change ratio confirmed a lack of significant changes in IL-2 positivity in both patient cohorts.

In contrast to IFN- γ positivity, which was increased in healthy patients, but not in nickel allergic patients, TNF positivity was significantly increased in T cells of nickel allergic patients co-cultured with CoCl₂ pulsed HeLa-CD1d APCs in comparison to untreated controls. However, this increase was not significantly higher than the HeLa-mock control, so it cannot be confirmed that the increase in TNF production was CD1d dependent. There was no increase in CoCl₂ induced CD1d dependent production of TNF. Standardisation to the fold change ratio revealed a significantly higher TNF production in nickel allergic patients in comparison to healthy patients.

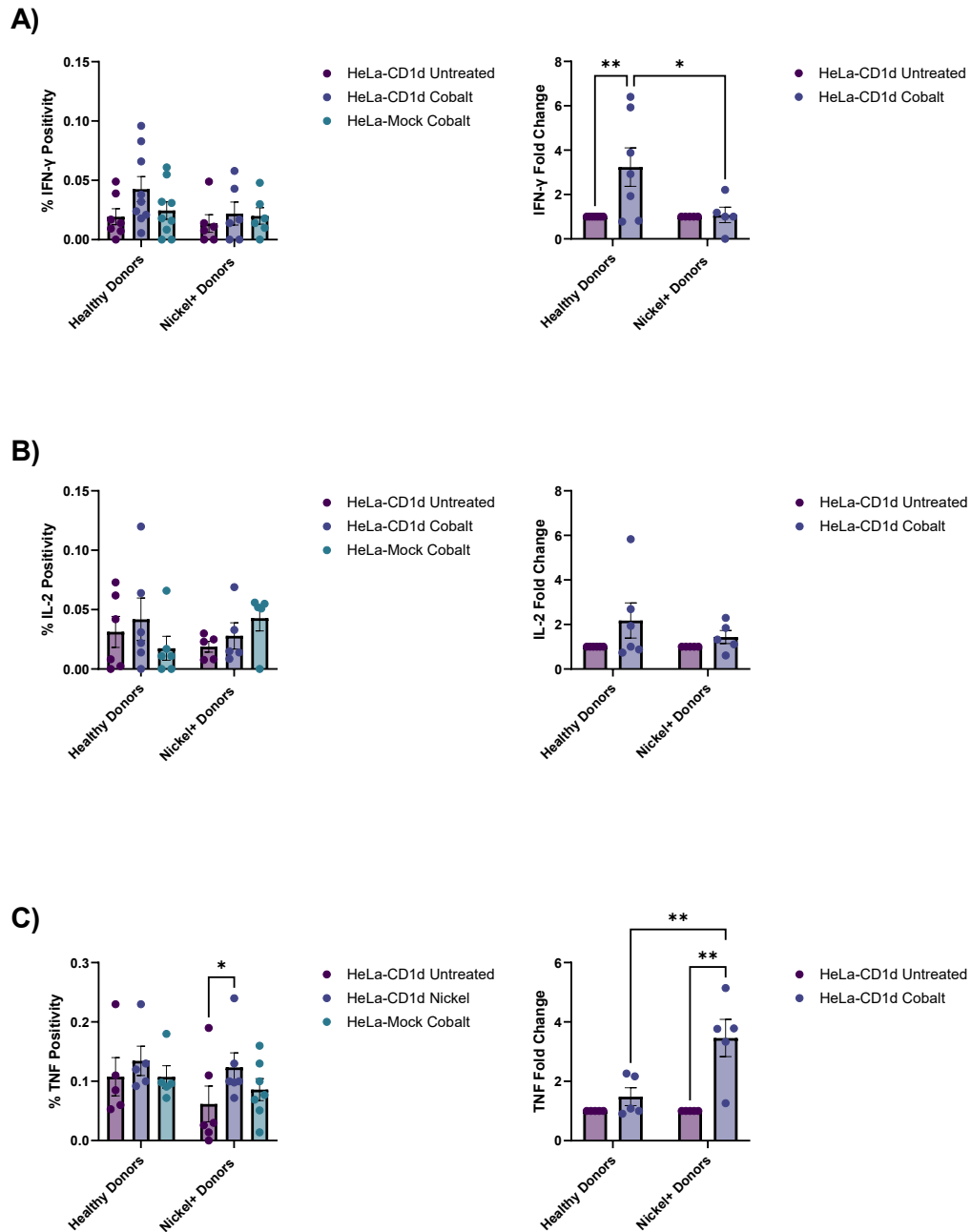


Figure 4.24 Cytokine production in T cells co-cultured with CoCl₂ pulsed HeLa-CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL CoCl₂ overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=5, TNF n=6) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data is analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data is analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$,

Overall, the investigation into CD1d dependent cytokine production induced by CoCl_2 provided some conflicting results. There was no CD1a dependent cytokine production observed, confirming that CoCl_2 is not a CD1a presented antigen. This came as somewhat of a surprise, given the strong CD1a dependent cytokine production observed with NiSO_4 . Cobalt and nickel have historically shared similar characteristics regarding their mechanisms of ACD pathology. Both metals bind directly to TLR4 on T cells leading to their activation, and both metals have been shown to produce a similar cytokine response profile in previous studies^{117, 338}. CoCl_2 appeared to induce some IFN- γ production in PBMC from healthy donors, and some TNF production in nickel allergic donors, however neither could be confirmed as CD1d dependent. It is important to note that the functional assays were carried using PBMC from healthy donors and nickel allergic donors, and it is possible that using PBMC from patients who tested positive for cobalt allergy could lead to more significant results. The lack of T cell recognition or activation by CoCl_2 in a CD1a dependent manner suggest that it is not a CD1d presenting antigen. The data obtained in this study suggest that it unlikely that CD1d has a role in the pathophysiology of cobalt ACD.

4.4.4 Chromium as a CD1a and CD1d Antigen

Having observed contrasting results received with nickel and cobalt as CD1a and CD1d antigens, the assays were then performed with potassium dichromate. Potassium dichromate has also been previously trialled as a CD1a presenting antigen in a plate bound assay using a known CD1a restricted T cell line, which reported no increase in IFN- γ production⁸⁶. It was tested as a CD1a presented antigen using a different experimental set-up in this chapter, as well as being evaluated as CD1d presented antigen.

There was no significant increase in T cell recognition by $\text{K}_2\text{Cr}_2\text{O}_7$ loaded CD1a tetramers either healthy donors or nickel+ donors in comparison to unloaded tetramers (Figure 4.25). This suggested that $\text{K}_2\text{Cr}_2\text{O}_7$ was not a CD1a presented antigen. There was also no increase in T cell recognition observed with $\text{K}_2\text{Cr}_2\text{O}_7$ loaded CD1d loaded tetramers in healthy patients. Due to a lack of increase in T cell recognition in healthy patients and restriction in

CD1d tetramer availability, $K_2Cr_2O_7$ was not tested as a CD1d presented antigen in nickel allergic PBMC.

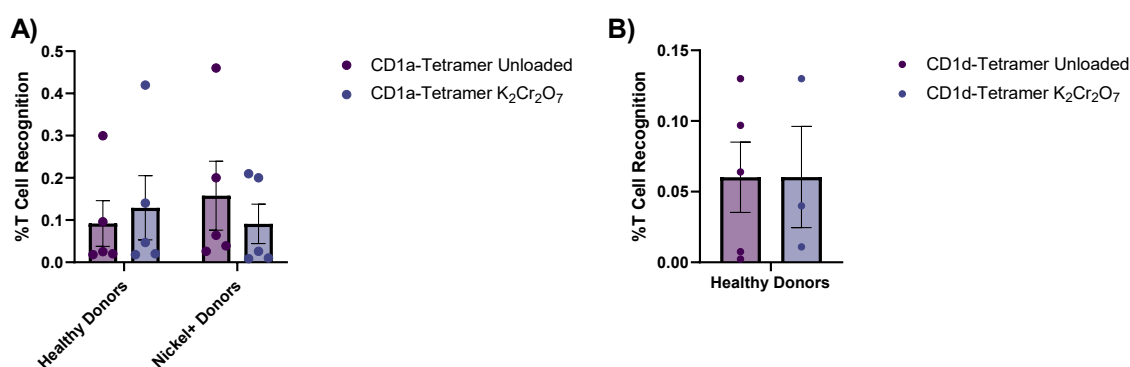


Figure 4.25 T cell recognition by $K_2Cr_2O_7$ loaded CD1a and CD1d Tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=5$) were stained with A) $K_2Cr_2O_7$ loaded CD1a tetramers or unloaded tetramers and B) $K_2Cr_2O_7$ loaded CD1d tetramers or unloaded tetramers and incubated for 20 minutes in the dark. PBMC were also stained with Pacific Blue conjugated anti-CD3 antibodies to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means

Overall, no increase in T cell recognition was observed with $K_2Cr_2O_7$ loaded CD1a or CD1d loaded tetramers, indicating that chromium was not a CD1a or CD1d presented antigen. Following this observation, functional assays were carried out to investigate whether $K_2Cr_2O_7$ activated T cells in a CD1a or CD1d dependent manner and induce the production of T_H1 cytokines.

There was no increase in the frequency of IFN- γ positive T cells observed in T cells from nickel+ donors co-cultured with $K_2Cr_2O_7$ pulsed C1R-CD1a APCs in comparison with untreated controls or C1R-mock controls [Figure 4.26(a)]. In healthy PBMC, there was a significant decrease in IFN- γ positivity induced by CD1a presented $K_2Cr_2O_7$, in contrast to the other metal allergens tested previously. Standardisation to the fold change ratio confirmed a lack of significant increase in the frequency of IFN- γ positive T cells induced by CD1a presented $K_2Cr_2O_7$ in comparison to untreated controls in both healthy patients and nickel allergic patients [Figure 4.26(a)].

There were no significant increases in the frequency of IL-2 positive T cells among T cells co-cultured with $K_2Cr_2O_7$ pulsed C1R-CD1a cells in either healthy or nickel allergic patients. Standardisation of the results to the fold change ratio confirmed the lack of an increase in IL-2 positivity induced by CD1a presented $K_2Cr_2O_7$ [Figure 4.26(b)].

Finally, CD1a presented $K_2Cr_2O_7$ did not induce an increase in the frequency of TNF positive T cells in PBMC from either healthy patients or nickel allergic patients. This was confirmed after standardisation of the data to the fold change ratio [Figure 4.26(c)].

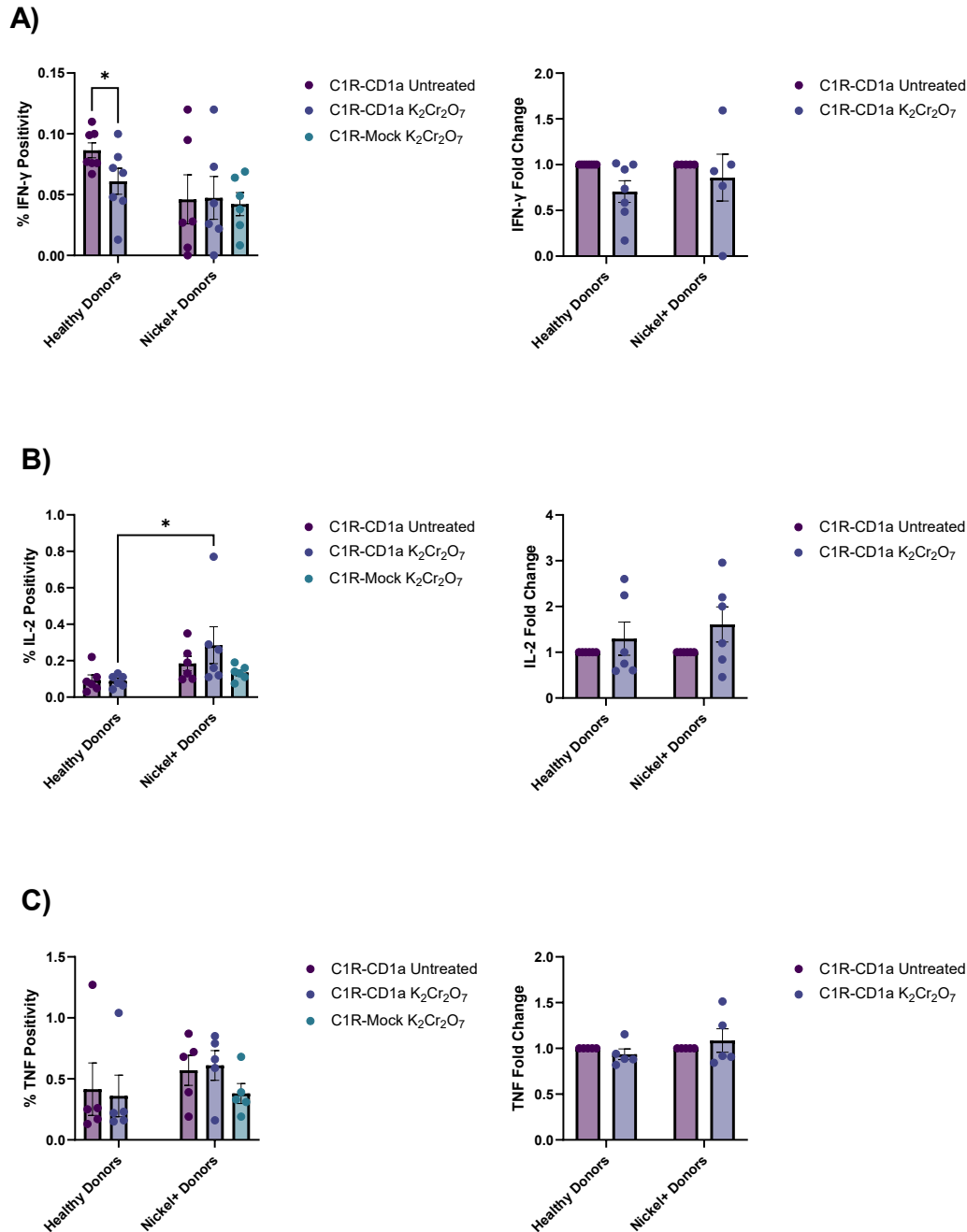


Figure 4.26 Cytokine production in T cells co-cultured with $K_2Cr_2O_7$ pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL $K_2Cr_2O_7$ overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=5) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$.

Overall, there were no increases in T_H1 cytokine production in either healthy or nickel+ T cells co-cultured with $K_2Cr_2O_7$ pulsed C1R-CD1a APCs, suggesting that $K_2Cr_2O_7$ is not a CD1a presented antigen and that CD1a does not play a role in the pathophysiology of chromium ACD.

There was no increase in the frequency of IFN- γ positive T cells in PBMC from healthy patients co-cultured with $K_2Cr_2O_7$ pulsed HeLa-CD1d cells, however a significant increase was observed in nickel allergic patients in comparison to both the untreated control and the HeLa-CD1d mock control. The significant increase in comparison with the HeLa-mock control indicated that the increase in IFN- γ was dependent on both $K_2Cr_2O_7$ and CD1d being present. There was also a significantly higher amount of IFN- γ positive T cells in nickel allergic patients in comparison to healthy patients [Figure 4.27(a)].

There also appeared to be an increase in IL-2 positive T cells in PBMC of nickel allergic patients induced by CD1d presented $K_2Cr_2O_7$ in comparison to the untreated control ($p = 0.055$). However, the increase induced by CD1d presented $K_2Cr_2O_7$ was not significantly higher than that of the HeLa-mock control, which confirmed that the increase in IL-2 positive T cells was not CD1d dependent. Additionally, there was a significant increase in IL-2 positive T cells in nickel allergic patients in comparison to healthy patients [Figure 4.27(b)].

Finally, there was no increase in the frequency of TNF positive T cells in either healthy or nickel allergic PBMC co-cultured with $K_2Cr_2O_7$ pulsed HeLa-CD1d APCs, indicating that T cells in both patient cohorts were not producing TNF in a $K_2Cr_2O_7$ or CD1d manner. Standardisation of the data to the fold change ratio also did not show any significant increases in TNF positivity [Figure 4.27(c)].

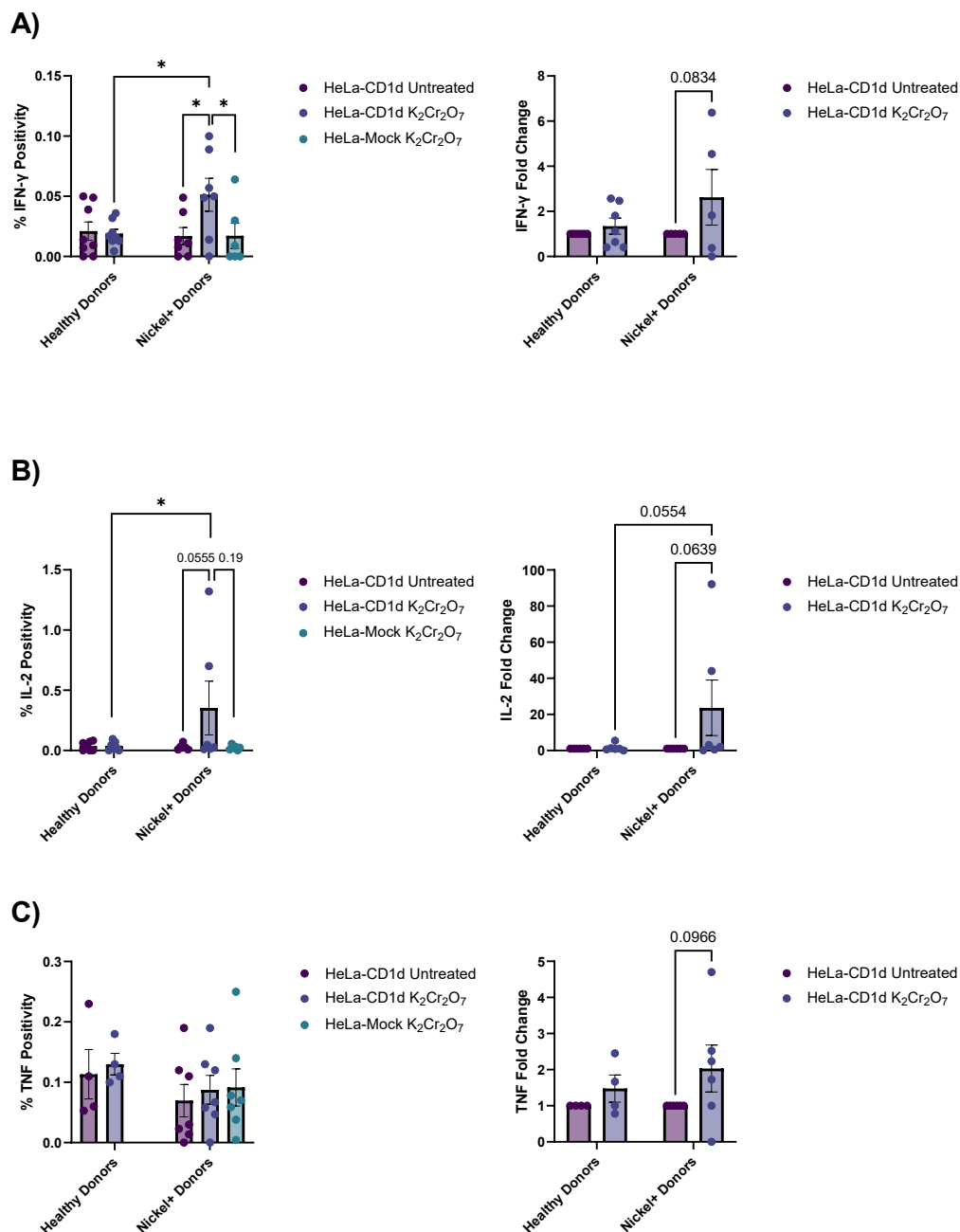


Figure 4.27 Cytokine production in T cells co-cultured with $K_2Cr_2O_7$ pulsed HeLa-CD1d APCs. *HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL $K_2Cr_2O_7$ overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=7, IL-2 n=6, TNF n=7) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as % cytokine positivity in comparison to untreated controls and mock controls, and as a fold change ratio. Data is presented as mean $\pm$ SEM. Outliers were identified by ROUT analysis and removed. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$.*

Overall, CD1d presented $K_2Cr_2O_7$ induced a significant increase in IFN- γ positive T cells in a CD1d dependent manner, but CD1d dependent increases in IL-2 or TNF positive T cells could not be confirmed. $K_2Cr_2O_7$ did not induce an increase in the frequency of cytokine positive T cells in a CD1a or CD1d dependent manner, suggesting that $K_2Cr_2O_7$ was not a CD1a or CD1d presented antigen. While the ability of $K_2Cr_2O_7$ to induce lipid peroxidation was established in previous chapters, if there is any interaction between $K_2Cr_2O_7$ and the endogenous lipids present in CD1a and CD1d, it appears that the potential resulting products formed do not allow for CD1a or CD1d binding to T cells and induction of their activation.

4.4.5 Lipid Peroxidation Products as CD1a and CD1d presented Antigens

4.4.5.1 4-HNE

After establishing the ability of $K_2Cr_2O_7$ to induce lipid peroxidation in the previous chapters of this thesis, lipid peroxidation products were then evaluated as CD1a and CD1d presented antigens. 4-HNE was of particular interest due to the similarities in its chemical structure to other known CD1d presented antigens. 4-HNE contains an $\alpha\beta$ unsaturated aldehyde. As previously mentioned, unsaturated bonds are a chemical feature that is known to favour CD1 binding. Furthermore, an $\alpha\beta$ unsaturated aldehyde functional group is a structural alert for skin sensitization³³⁹, and a known chemical characteristic of a known CD1d presented antigen, cinnamaldehyde³²² (Figure 4.28).

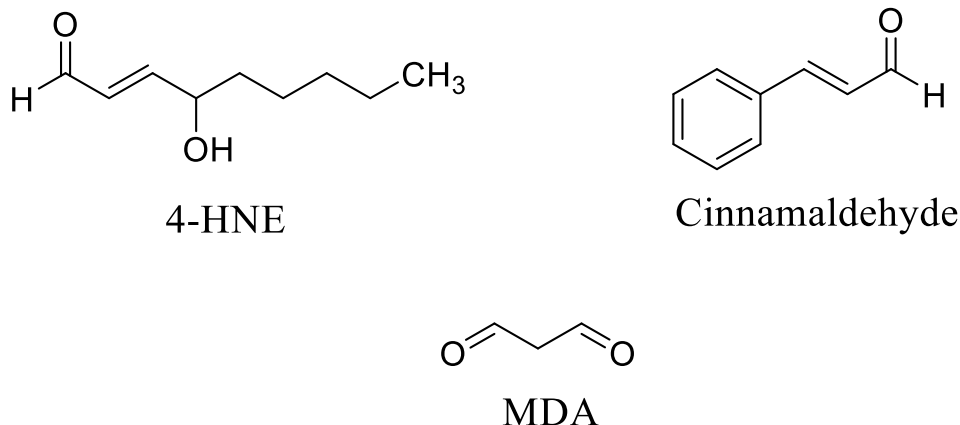


Figure 4.28 Chemical structure of cinnamaldehyde, a known CD1d ligand, and 4-HNE and MDA, lipid peroxidation end products

To evaluate 4-HNE as a CD1a and CD1d presented ligand, CD1a and CD1d tetramers are loaded with 4-HNE and are subsequently used to stain PBMC from healthy patients and nickel allergic patients. Cells that stained positively for CD3 and the PE-conjugated CD1a or CD1d tetramers were quantified and analysed (Figure 4.29).

There appeared to be an increase in T cell recognition with 4-HNE loaded CD1a tetramers in both healthy donors and nickel allergic donors in comparison to DMSO loaded tetramers, however the increase was not statistically significant [Figure 4.29(a)]. The calculated *p*-values were 0.0756 for healthy PBMC and 0.0912 for nickel+ PBMC. It is likely that an increase in sample size could render the increase in T cell recognition by 4-HNE loaded tetramers statistically significant. The increase in T cell recognition indicated that 4-HNE could be a CD1a presented ligand. 4-HNE loaded CD1d tetramers did not result in an increase in T cell recognition in either healthy donors or nickel allergic donors [Figure 4.29(b)].

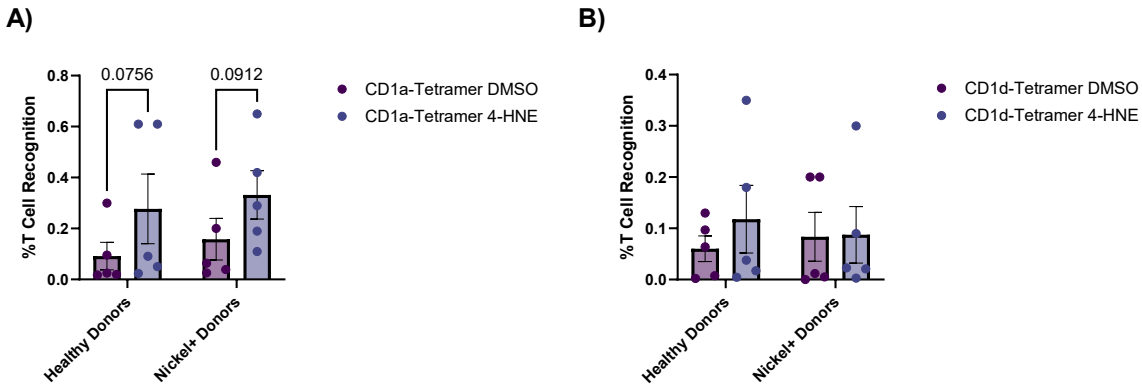


Figure 4.29 T cell recognition by 4-HNE loaded CD1a and CD1d tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=5$) were stained with A) 4-HNE loaded CD1a tetramers or DMSO loaded tetramers and B) 4-HNE loaded CD1d tetramers or DMSO loaded tetramers and incubated for 20 minutes in the dark. PBMC were also stained with Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means

The increase in T cell recognition with 4-HNE loaded CD1a tetramers, and the lack of T cell recognition seen with 4-HNE loaded CD1d tetramers indicated that 4-HNE was potentially a CD1a presented ligand but was unlikely to be a CD1d presented ligand.

There was no increase in the frequency of IFN- γ positive T cells in PBMC co-cultured with 4-HNE pulsed C1R-CD1a APC's in either healthy or nickel allergic patients, in comparison to the untreated controls and C1R-mock controls. Standardisation of the data to the fold change ratio also failed to show an increase in IFN- γ positive T cells in either patient cohort [Figure 4.30(a)]. Additionally, there was no increase in the frequency of IL-2 positive T cells induced by CD1a presented 4-HNE in comparison to the untreated controls and mock controls in either healthy or nickel allergic patients [Figure 4.30(b)]. Furthermore, there was no increase in TNF positive T cells in PBMC co-cultured with 4-HNE pulsed C1R-CD1a APCs, in either healthy or nickel allergic patients in comparison to the untreated controls. However, in nickel allergic patients, there was an increase in TNF positive T cells in comparison to the C1R-mock control. The lack of significant increase in comparison to the untreated control indicated that while CD1a itself can bind to and activate T cells, T cell activation is not enhanced or increased with 4-HNE pulsation of C1R-CD1a APCs [Figure 4.30(c)].

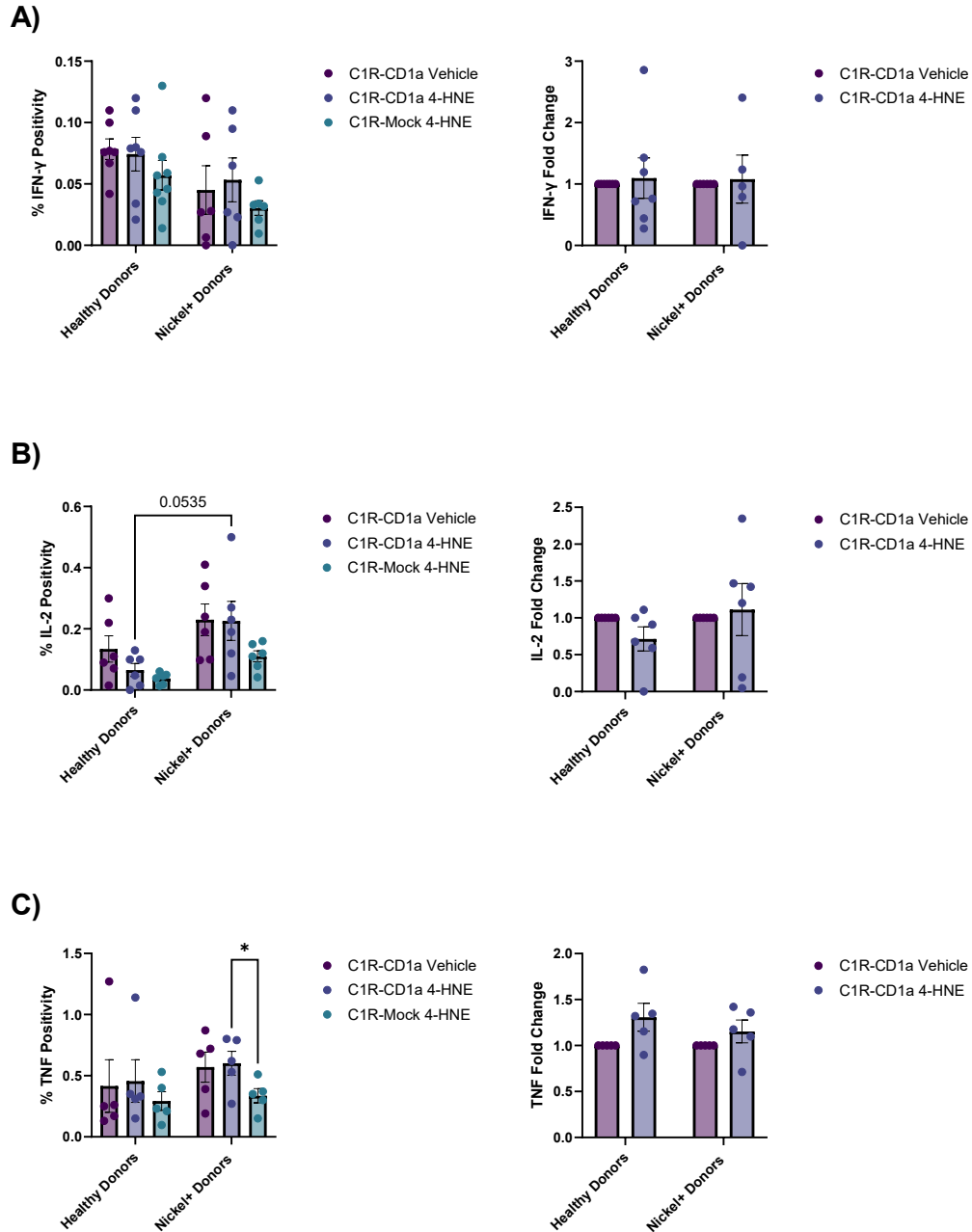


Figure 4.30 Cytokine production by T cells co-cultured with 4-HNE pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL 4-HNE overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=5) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as % cytokine positivity in comparison to vehicle controls and mock controls, and as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means.

Overall, while there appeared to be an increase in T cell recognition with 4-HNE loaded CD1a tetramers, CD1 presented 4-HNE did not appear to activate T cells in either healthy patients or nickel allergic patients, indicating that it was not a CD1a presented antigen. Thereafter, the ability of 4-HNE to activate T cells in a CD1d dependent manner was investigated.

There appeared to be an increase in the frequency of IFN- γ positive T cells among T cells co-cultured with 4-HNE pulsed C1R-CD1a cells in both healthy and nickel allergic patients, in comparison to DMSO controls and HeLa-Mock controls, however the IFN- γ increase was not statistically significant [Figure 4.31(a)]. Standardisation of the data to the fold change ratio also showed a non-significant increase in IFN- γ positive T cells in both patient cohorts in comparison to the DMSO controls. The frequency of IL-2 positive T cells did not seem to increase in T cells stimulated with CD1d presented 4-HNE, in either healthy or nickel allergic donors, in comparison with the DMSO control or HeLa-mock controls [Figure 4.31(b)].

There did not appear to be an increase in the frequency of TNF positive T cells among T cells from healthy patients co-cultured with 4-HNE pulsed C1R-CD1a cells, however there was a significant increase in TNF positive T cells in PBMC from nickel allergic patients, in comparison to DMSO controls and HeLa-mock controls. This indicated that 4-HNE induced TNF production in a CD1d dependent manner in nickel allergic patients [Figure 4.31(c)].

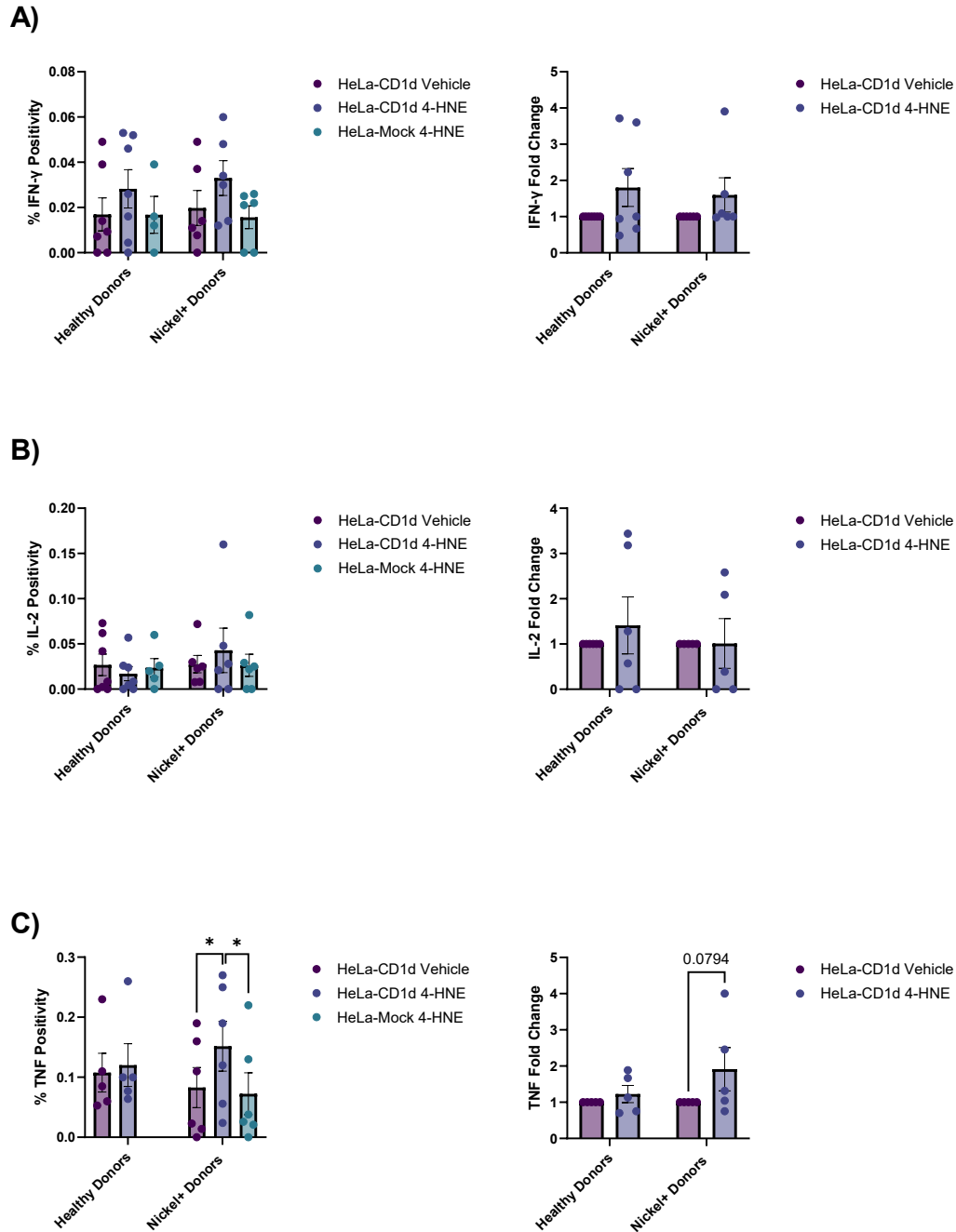


Figure 4.31 Cytokine production in T cells co-cultured with 4-HNE pulsed HeLa-Mock CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL 4-HNE overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=6) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells are quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$

Overall, conflicting results were obtained with the tetramer assays and the functional assays. 4-HNE loaded CD1a tetramers appeared to increase T cell recognition in both healthy and nickel allergic patients, but 4-HNE failed to activate T cells in a CD1a dependent manner and did not induce increases in the frequency of cytokine positive T cells. In contrast, 4-HNE loaded CD1d tetramers did not increase T cell recognition in either patient cohort, but 4-HNE appeared to increase IFN- γ production in a CD1d dependent manner and induced a significant increase in TNF production in a CD1d dependent manner. The contrasting results indicate that further investigation is required into the ability of 4-HNE to act as a CD1a or CD1d presented antigen.

4.4.5.2 MDA

MDA is derived from the peroxidation of polyunsaturated fatty acids and has been used as a biomarker to measure oxidative stress (Figure 4.28)³⁴⁰. Following the finding that metal allergens, particularly $K_2Cr_2O_7$, induced lipid peroxidation in keratinocytes, MDA was evaluated as CD1a and CD1d presented antigen.

There was no increase in T cell recognition with MDA loaded CD1a tetramers in healthy donors, in comparison to unloaded tetramers, and there appeared to be a decrease in T cell recognition with MDA loaded CD1a tetramers in nickel allergic donors in comparison to the unloaded tetramer, which was calculated to be close to significance, with a p -value of 0.0546 [Figure 4.32(a)]. There was no increase in T cell recognition with 4-HNE loaded CD1d tetramers in T cells from healthy patients, however, there did appear to be an increase in T cell recognition in nickel allergic patients, but the increase was not statistically significant in comparison to the untreated control [Figure 4.32(b)].

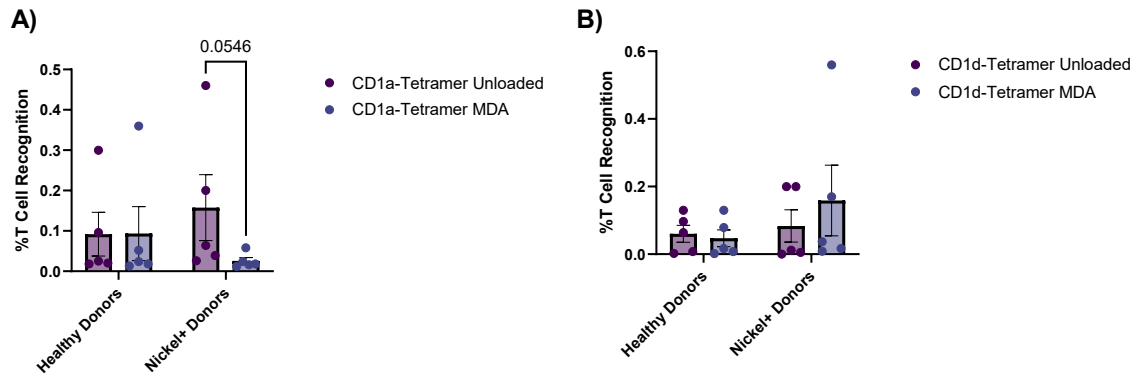


Figure 4.32 T cell recognition by MDA loaded CD1a and CD1d tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=5$) were stained with A) MDA loaded CD1a tetramers or unloaded tetramers and B) MDA loaded CD1d tetramers or unloaded tetramers and incubated for 20 minutes in the dark. PBMC were also stained with Pacific Blue conjugated anti-CD3 antibodies to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means

The lack of significant increases in T cell recognition with either MDA loaded CD1a or CD1d tetramers in healthy or nickel allergic patients, suggested that MDA is neither a CD1a nor CD1d presented antigen.

There was no increase in the frequency of IFN- γ positive T cells from healthy patients co-cultured with MDA pulsed C1R-CD1a cells. There appeared to be an increase in IFN- γ positive T cells in PBMC co-cultured with MDA pulsed C1R-CD1a APCs in nickel allergic patients in comparison to the untreated control, however it was not seen as statistically significant. The IFN- γ positivity increase was significantly higher than the C1R-mock control. Standardisation of the data showed a statistically significant increase in the frequency of IFN- γ positive T cells induced by CD1a-presented MDA in nickel allergic donors. A significant increase in comparison to the untreated control and the C1R-mock control indicated that the IFN- γ production was dependent on the presence of both MDA and CD1a [Figure 4.33(a)].

There was no increase in the frequency of CD1a-dependent, MDA-induced IL-2 positive T cells in samples from healthy donors. In contrast, T cells from nickel-allergic donors showed a significant increase in the frequency of IL-2 positive cells when co-cultured with MDA-pulsed C1R-CD1a APCs compared with untreated controls. This increase was also significant

relative to the C1R-mock controls, confirming that MDA induced IL-2 expression in a CD1a-dependent manner. Furthermore, fold-change analysis demonstrated that the frequency of IL-2 positive T cells was significantly higher in nickel-allergic donors than in healthy donors [Figure 4.33(b)].

There was no increase in the frequency of TNF positive T cells in PBMC from either patient cohort when co-cultured with MDA pulsed C1R-CD1a APCs in comparison with untreated controls or C1R-mock controls. Standardisation of the data to the fold change ratio also did not identify any significant increases in TNF positive T cells [Figure 4.33(c)].

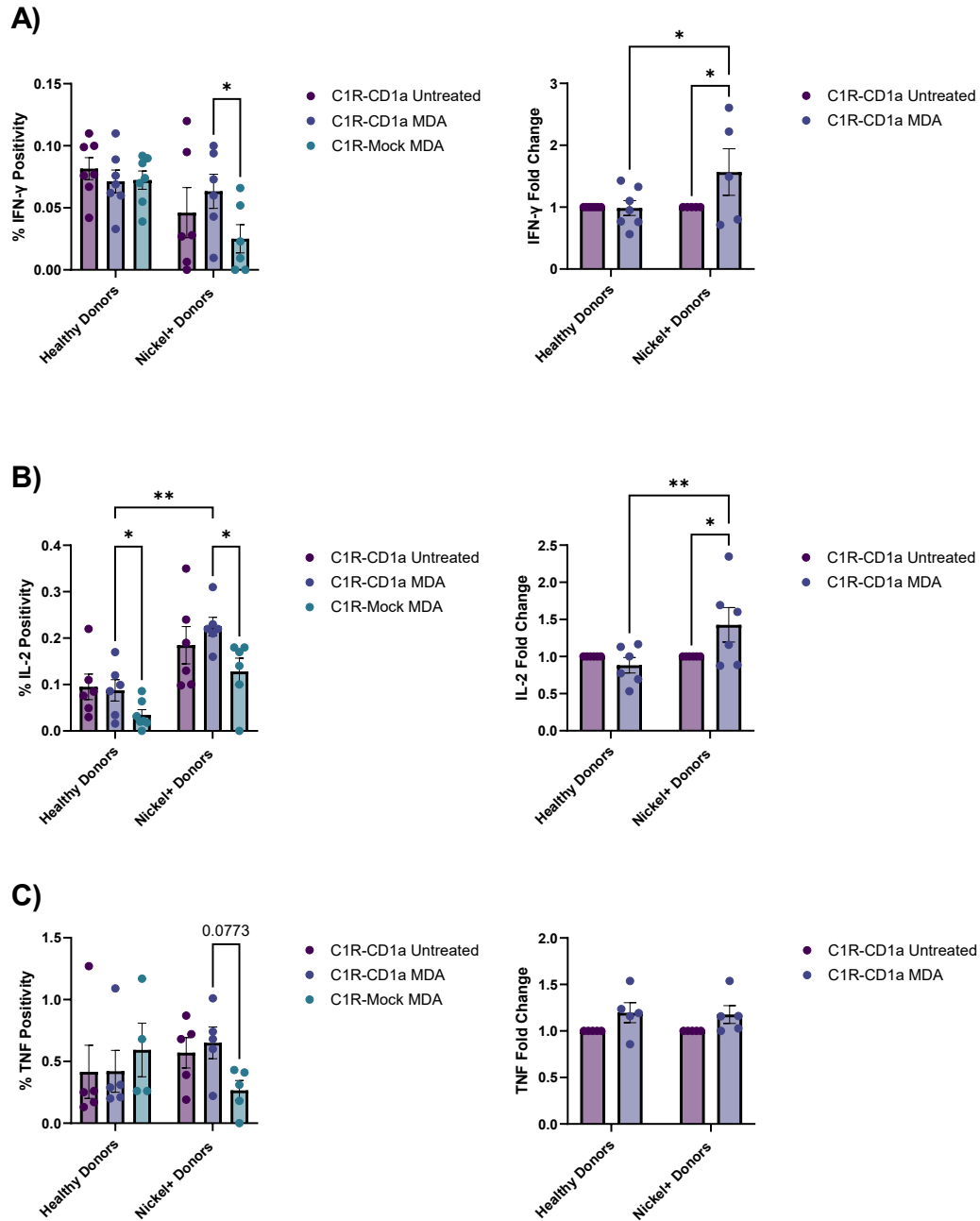


Figure 4.33 Cytokine production in T cells co-cultured with MDA pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL MDA overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=5) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as % cytokine positivity in comparison to untreated controls and mock controls, and as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means.

Overall, MDA appeared to induce IFN- γ and IL-2 production in nickel allergic patients in a CD1a dependent manner, suggesting that MDA could be a CD1a presented antigen. However, the lack of T cell recognition with MDA loaded CD1a tetramers indicated that more investigation was needed into MDA's potential as a CD1a presented antigen. Flow cytometry dot plots displaying IFN- γ and IL-2 production are shown in the appendix (Figure A.8).

Next, MDA was evaluated as a CD1d presented antigen. There was no increase in the frequency of IFN- γ positive T cells observed in PBMC from either healthy patients or nickel allergic patients co-cultured with MDA-pulsed HeLa-CD1d APCs [Figure 4.34(a)]. There was also a lack of increase in IL-2 positive T cells observed in PBMC from either cohort of patients co-cultured with MDA pulsed HeLa-CD1d APCs. Standardisation of the data indicated an increase in IL-2 positive T cells in nickel allergic patients in comparison with the untreated control, however there appeared to a higher frequency of IL-2 positive T cells in the HeLa-mock control, indicating that IL-2 production was not CD1d dependent [Figure 4.34(b)].

CD1d presented MDA did not induce an increase in TNF positive T cells in healthy patients. In contrast, there was a significant increase in TNF positive T cells in nickel allergic patients in comparison to the untreated control, and this effect was also evident after standardisation to the fold-change ratio. However, the increase in frequency of TNF positive T cells induced by CD1d-presented MDA was not significantly greater than in the HeLa-mock control, suggesting that the response cannot be conclusively attributed to CD1d-dependent presentation [Figure 4.34(c)].

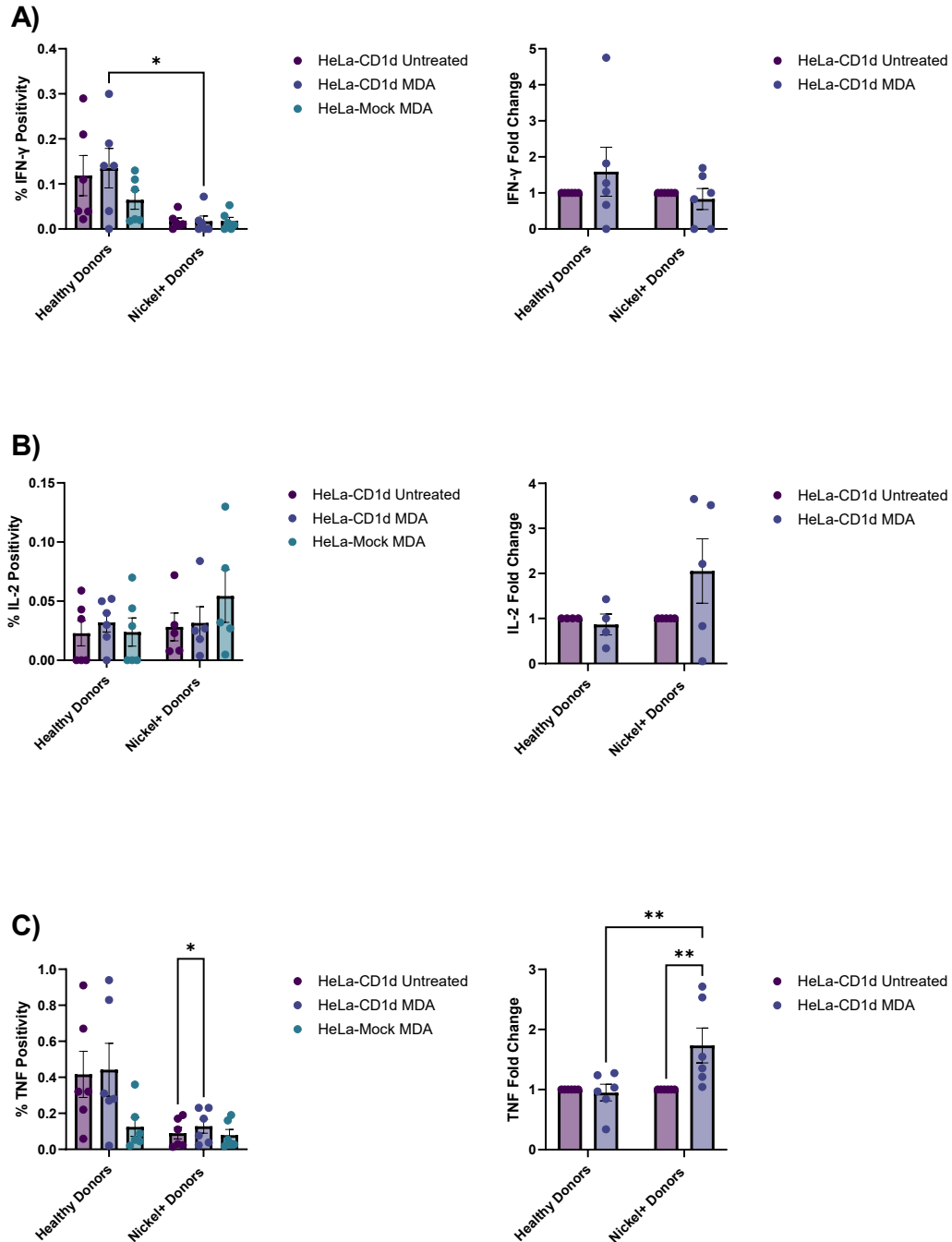


Figure 4.34 Cytokine production by T cells co-cultured by MDA pulsed HeLa-CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL MDA overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=6, IL-2 n=6, TNF n=6) or nickel+ donors (IFN- γ n=6, IL-2 n=5, TNF n=6) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as (left) % cytokine positivity to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production. Data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$

Overall, CD1a-presented MDA was shown to increase the frequency of IFN- γ and IL-2 positive T cells, but not TNF, whereas CD1d-presented MDA displayed the opposite pattern, increasing TNF positive T cells, but not IFN- γ or IL-2. While these functional assays strongly suggest that MDA acts as a CD1a-presented antigen, the absence of detectable T cell recognition using MDA-loaded CD1a tetramers means that further experimental confirmation is required before this conclusion can be definitively established.

The functional assays give a strong indication that lipid peroxidation products can induce T cell activation via CD1a and CD1d mechanisms. 4-HNE appeared to induce T cell activation via CD1d, whereas MDA induced T cell activation via CD1a. Further research is required to confirm these results, by increasing the sample size or by using CD1a and CD1d restricted T cell lines. The indication that lipid peroxidation products could be CD1a and CD1d presented antigens suggests a link between metal induced lipid peroxidation and the immunological mechanisms involved in the pathophysiology of ACD.

4.4.6 CumOOH as a CD1a and CD1d presented antigen

Finally, CumOOH, a compound known to induce lipid peroxidation, was tested as CD1a and CD1d presented antigen. CumOOH was used as a positive control in lipid peroxidation assays in previous chapters, so it was included in the tetramer and functional assays to investigate if it can affect the endogenous lipids present in CD1a and CD1d and enable their binding to and activation of T cells.

There was no increase in T cell recognition by CumOOH loaded CD1a tetramers in either healthy or nickel allergic patients in comparison to the DMSO loaded tetramer control [Figure 4.35(a)]. In fact, there appeared to be a decrease in T cell recognition, similar to that seen with MDA, by CumOOH loaded CD1a tetramers in nickel allergic patients (not statistically significant). CumOOH loaded CD1d tetramers appeared to recognise more T cells in healthy patients in comparison to the DMSO loaded control CD1d tetramer. There was no increase in T cell recognition by CumOOH loaded CD1d tetramers observed in nickel allergic patients [Figure 4.35(b)].

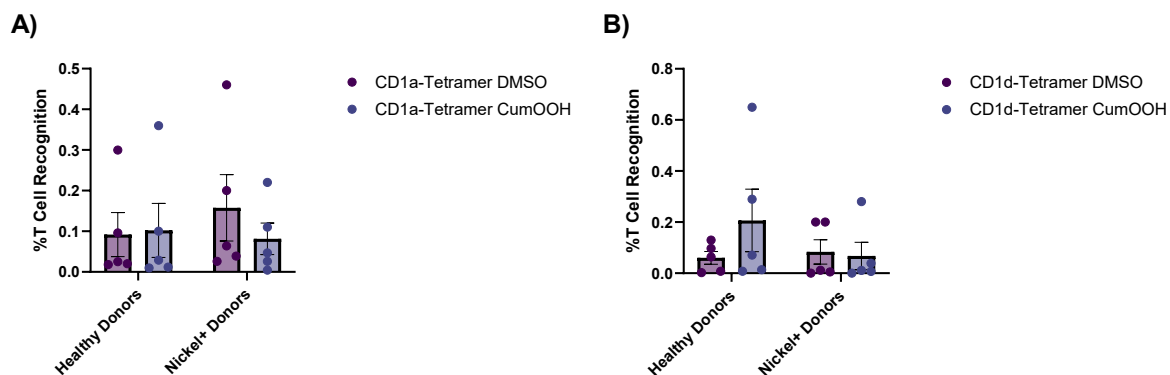


Figure 4.35 T cell recognition by CumOOH loaded CD1a and CD1d tetramers. 5×10^6 PBMC from healthy donors ($n=5$) or nickel+ donors ($n=5$) were stained with A) CumOOH loaded CD1a tetramers or DMSO loaded tetramers and B) CumOOH loaded CD1d tetramers or DMSO loaded tetramers and incubated for 20 minutes in the dark. PBMC were also stained with Pacific Blue conjugated anti-CD3 antibody to identify T cells. Cells were analysed via flow cytometry and cells that stained positive for both CD3 and tetramer were quantified. Data is presented as the mean $\pm$ SEM. Data was analysed by two-way ANOVA with Fisher's post-hoc test for comparison of means.

From the tetramer assays, CumOOH did not appear to be a CD1a or CD1d presented antigen, with the only increase in T cell recognition seen with CumOOH loaded CD1d tetramers in the PBMC of healthy donors.

There was no increase in the frequency of IFN- γ positive T cells from either healthy or nickel-allergic donors co-cultured with CumOOH-pulsed C1R-CD1a APCs compared with DMSO controls or C1R-mock controls. In healthy donors, the frequency of IFN- γ positive T cells was reduced relative to the untreated control [Figure 4.36(a)]. Similarly, there was no increase in the frequency of IL-2 positive T cells in response to CD1a-presented CumOOH in either healthy or nickel-allergic donors, with both groups instead showing a decrease in IL-2 positive T cell frequency when co-cultured with CumOOH-pulsed C1R-CD1a APCs. This reduction in the frequency of IFN- γ^+ and IL-2 $^+$ T cells paralleled the diminished T cell recognition observed with CumOOH-loaded CD1a tetramers in PBMC from nickel-allergic patients [Figure 4.36(b)].

In contrast, the frequency of TNF positive T cells was increased in PBMC co-cultured with C1R-CD1a APCs, in comparison to DMSO controls and C1R-mock controls in nickel allergic patients. Standardisation of the data to the fold change ratio showed that this increase in

TNF positive T cell frequency was statistically significant. The increase in TNF positive T cells in nickel allergic patients was not calculated to be statistically significant in comparison to the C1R mock control, but the *p*-value, at 0.0527, was very close to significance, and suggested that CumOOH increased the frequency of TNF positive T cells in nickel allergic patients in a CD1a dependent manner [Figure 4.36(c)].

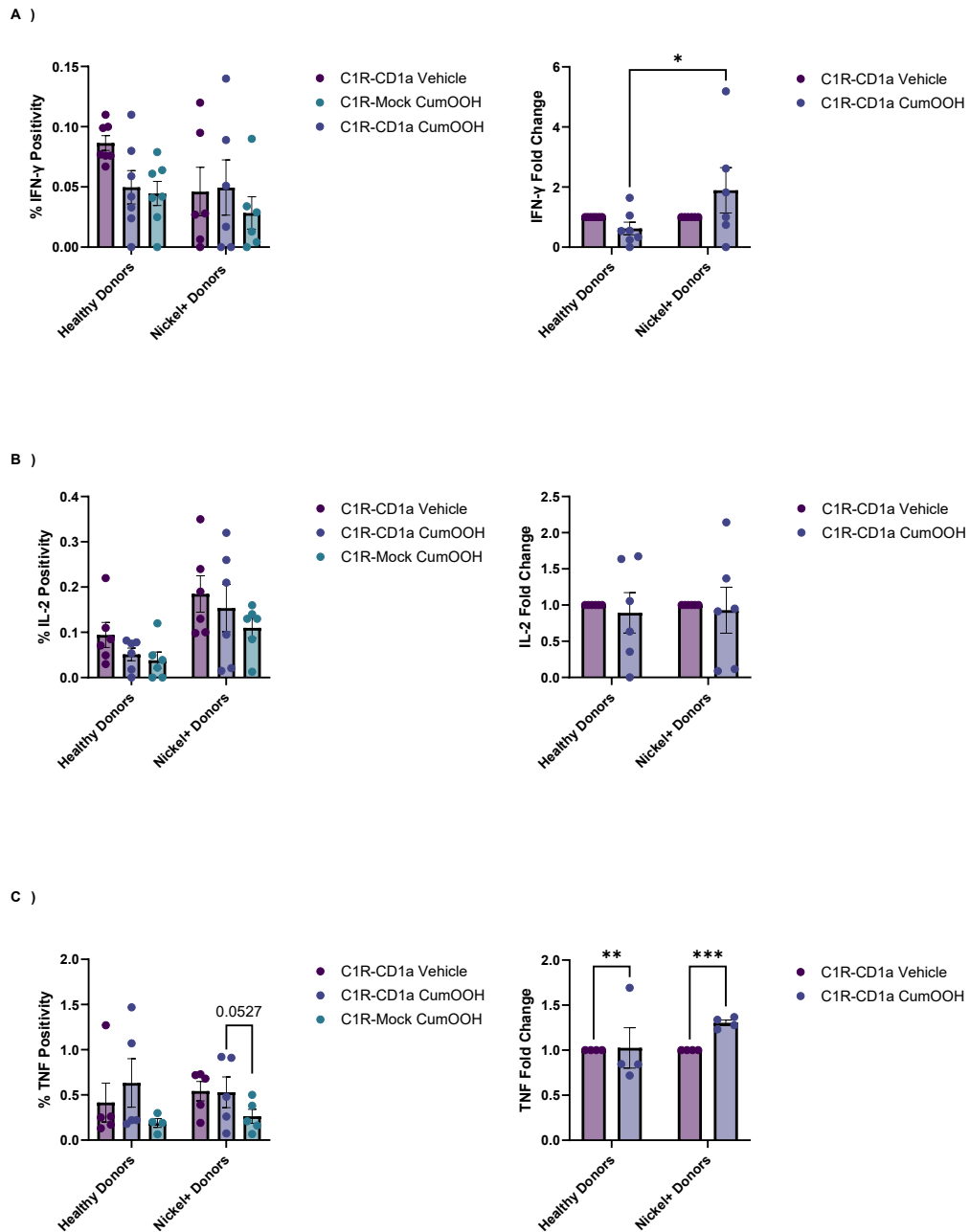


Figure 4.36 Cytokine production by T cells co-cultured with CumOOH pulsed C1R-CD1a APCs. C1R-CD1a and C1R-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL CumOOH overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=5) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells were quantified. Cytokine positivity is presented as % cytokine positivity to untreated controls and mock controls, and as a fold change ratio. Data is presented as mean $\pm$ SEM. Outliers were identified by ROUT analysis and removed. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The frequency of IFN- γ positive T cells was increased in T cells from nickel allergic patients co-cultured with CumOOH pulsed HeLa-CD1d cells, with a near significant increase in compared to DMSO controls and vehicle controls [Figure 4.37(a)]. Standardisation to the fold change ratio showed a close to significant increase in the frequency of IFN- γ positive T cells in nickel allergic patients in comparison to healthy patients. Furthermore, there was no increase in the frequency of IL-2 positive T cells induced by CD1d presented CumOOH in healthy patients. Standardisation of the data showed a close to significant increase in IL-2 positive T cell frequency in nickel allergic patients in comparison to the DMSO treated control. However, the increase was not statistically significant in comparison to the HeLa-mock control. While CumOOH increased the frequency of IL-2 positive T cells, it could not be confirmed that the effect was CD1d dependent [Figure 4.37(b)].

There was no increase in the frequency of TNF positive T cells in PBMC from healthy patients co-cultured with CumOOH pulsed HeLa-CD1d cells. However, the frequency of TNF positive T cells was increased in nickel allergic patients in comparison to the DMSO treated control and HeLa-mock control, which indicated that CumOOH was increasing TNF positive T cell frequency in nickel allergic patients in a CD1d dependent manner. Standardisation to the fold change ratio also showed a significant increase in TNF positive T cell frequency in nickel allergic patients in comparison to healthy patients [Figure 4.36(c)].

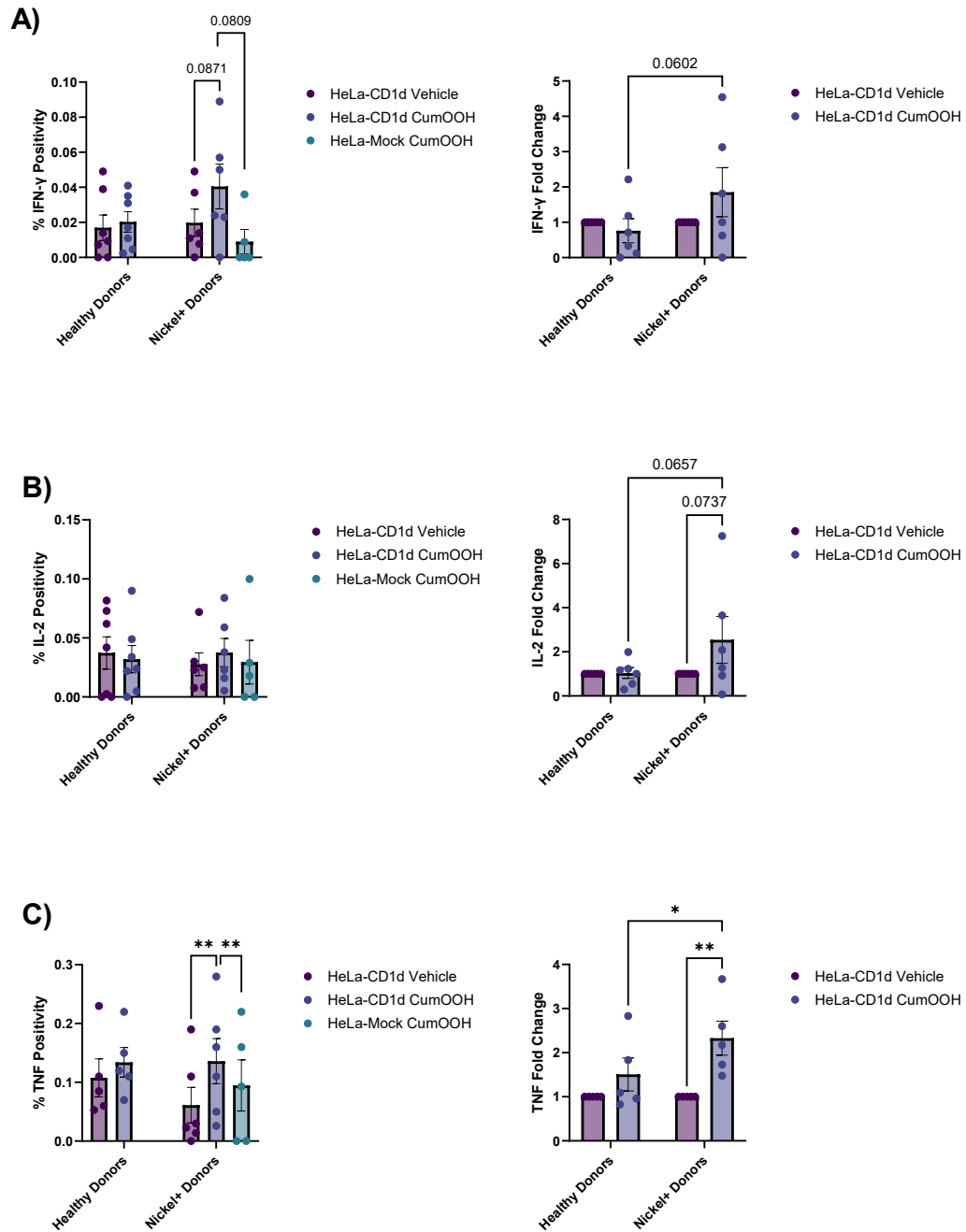


Figure 4.37 Cytokine production in T cells co-cultured with CumOOH pulsed CD1a and CD1d APCs. HeLa-CD1d and HeLa-mock cells were seeded at a density of 0.1×10^6 cells per well in a 96 well plate, pulsed with 200 ng/mL CumOOH overnight at 37 °C and co-cultured with 0.2×10^6 PBMC from healthy donors (IFN- γ n=7, IL-2 n=6, TNF n=5) or nickel+ donors (IFN- γ n=6, IL-2 n=6, TNF n=6) per well. Cells were analysed via flow cytometry and the frequencies of a) IFN- γ , b) IL-2, and c) TNF positive T cells are quantified. Cytokine positivity is presented as (left) % cytokine positivity in comparison to untreated controls and mock controls, and (right) as a fold change ratio. Data is presented as mean $\pm$ SEM. % cytokine production data was analysed by repeated measures two-way ANOVA with Tukey's post hoc test, and fold change ratio data was analysed by repeated measures two-way ANOVA with Fisher's LSD post-hoc test for comparison of means. * $p < 0.05$, ** $p < 0.01$

Overall, cytokine induction was not induced by CumOOH in a CD1a dependent manner, with no increases in IFN- γ , IL-2 or TNF production observed in T cells from healthy or nickel allergic donors co-cultured with C1R-CD1a APCs. However, there was a non-significant increase in IFN- γ production induced by CumOOH pulsed HeLa-CD1d APCs, and a significant CD1d dependent increase in TNF production. The more significant increases in cytokine production seen in a CD1d dependent manner correlate with the CD1d dependent increases in cytokine production seen in T cells co-cultured with K₂Cr₂O₇ pulsed HeLa-CD1d cells. CumOOH is a known lipid peroxidation inducer and K₂Cr₂O₇ was identified as a lipid peroxidation inducer in previous chapters, so the finding that they both produce similar cytokine responses in T cells when presented by CD1d warrants further investigation into the relationship between lipid peroxidation CD1 mediated ACD mechanisms.

A summary of the T cell responses observed in this study are laid out in table 4.2.

	CD1a				CD1d			
	Tetramer Recognition	IFN- γ	IL-2	TNF	Tetramer Recognition	IFN- γ	IL-2	TNF
Farnesol	—	—	—	—				
α -GalCer					↑	↑	↑	↑
NiSO ₄	↑	↑	↑	↑	↑	—	↑	↑
CoCl ₂	—	—	—	—	—	—	—	—
K ₂ Cr ₂ O ₇	—	—	—	—	↑	—	—	—
4-HNE	↑	—	—	—	↑	—	—	—
MDA	—	↑	↑	—	—	↑	—	—
CumOOH	—	—	—	↑	—	—	—	↑

Table 4.2 Summary of results seen for CD1a and CD1d tetramer and functional assays. Arrows indicate a statistically significant increase ($p < 0.05$)

Overall, NiSO₄ had the strongest response in the CD1a and CD1d tetramer and functional assays. NiSO₄ loaded CD1a and CD1d tetramers resulted in an increase in T cell recognition

in healthy and nickel allergic patients and also increased the frequency of cytokine positive T cells in a CD1a and CD1d dependent manner in nickel allergic patients. The other allergenic metals did not appear to act as CD1a or CD1d presented ligands. Outside of the metals, there were some CD1a and CD1d dependent responses seen with lipid peroxidation products. 4-HNE loaded CD1a tetramers recognised more T cells in comparison to unloaded control tetramers, and CD1d presented 4-HNE increased the frequency of IFN- γ positive T cells. Furthermore, CD1a and CD1d presented MDA led to an increase in cytokine positive T cells.

Immunological memory is fundamental to CD1 mediated model of ACD is T cell mediated and involves the activation of naïve T cells into antigen specific memory T cells which are activated after repeated exposure to the allergen. This concept is also applied to CD1 mediated T cell activation in ACD. This is evidenced by an increase in CD1-reactive T cells identified by urushiol loaded CD1a-APCs in patients with poison ivy allergy in comparison to non-allergic patients, leading to increased production of cytokines like IL-17 and IL-22³⁴¹. This suggests prior antigen exposure and antigen-driven expansion, which is a hallmark of immunological memory. The cytokine responses observed in this study are highly relevant to the pathophysiology of ACD. IFN- γ is a key T_H1 cytokine that promotes keratinocyte activation and amplifies inflammatory signalling in the skin, while IL-2 is essential for the activation and clonal expansion of allergen-reactive T cells. TNF further contributes to disease progression by enhancing local inflammation, recruiting immune cells, and driving tissue damage. Together, these cytokines represent central mediators of the T cell-driven inflammatory cascade in skin allergy. Our results suggested the involvement of a CD1a and CD1d dependent cytokine production in the pathophysiology of metal ACD. However, definitive mechanistic proof of CD1-restricted activation will require future studies involving a higher number of patients, and the purification and expansion of tetramer-positive CD1a- and CD1d-restricted T cells. Such approaches would enable detailed functional analysis, clarify the mechanism of CD1-mediated T cell interactions, and provide more evidence that metals like nickel, and lipid peroxidation products can act as novel CD1-presented antigens.

4.5 Conclusions of Chapter

The aim of this chapter was to establish if metals or lipid peroxidation products could recognize and activate T cells in a CD1a and CD1d dependent manner, using CD1a and CD1d tetramers, and C1R-CD1a and HeLa-CD1d APCs. Nickel loaded CD1a tetramers recognised significantly more T cells in both healthy and allergic patients in comparison to the unloaded controls and induced an increase in production of IFN- γ , IL-2 and TNF in a CD1a dependent manner. Nickel loaded CD1d tetramers also recognized T cells in nickel allergic patients and induced production of TNF and IL-2 in a CD1d dependent manner. It was concluded that nickel was acting as a CD1a and CD1d presented antigen in nickel allergic patients or causing an effect that allowed another substance to be presented to T cells, and that the CD1a and CD1d could play a role in the pathophysiology of nickel ACD. This is the first observation of CD1a and CD1d involvement in nickel ACD and contrasts with the only known study investigating nickel as a CD1a antigen which reported no increases in IFN- γ by nickel in a CD1a dependent manner⁸⁶. There was a lack of CD1a or CD1d mediated T cell recognition or activation with cobalt or chromium salts in both healthy and nickel allergic PBMC.

Lipid peroxidation products induced some increases in T cell activation; however not all increases were to a significant extent and could be confirmed to be CD1a or CD1d dependent. MDA increased cytokine production in a CD1a dependent manner, with significant increases in IFN- γ and IL-2 production observed, and 4-HNE appeared to activate T cells in a CD1d dependent manner. Lipid peroxidation products have not previously been investigated as CD1a or CD1d presented antigens, so these observations are the first link between lipid peroxidation and CD1a or CD1d mediated hypersensitivity reactions.

Future research is needed to further investigate CD1a and CD1d related cytokine production in ACD, including increasing the sample size, and blocking CD1a and CD1d T cell receptors to see if cytokine production is reduced or prevented, confirming CD1a and CD1d dependence. Additionally, given that nickel was concluded to be a CD1a and CD1d presented antigen, future work is needed to investigate the exact relationship between

nickel and CD1 proteins and elucidate the mechanism of nickel and CD1 protein binding and how it leads to T cell binding and activation.

Overall, the results in this chapter showed a role for CD1a and CD1d mediated mechanisms in the pathophysiology of ACD and provides a link between lipid peroxidation and the immunological mechanisms of ACD.

Chapter 5: Time-Of-Flight Secondary Ion Mass Spectrometry Analysis of Fragrance Allergen and Preservative Treated Human Skin Samples

5.1 Introduction

In addition to the work completed in this thesis, it was also of interest to study the fate of allergens when applied topically to the skin, and the depth to which they penetrate. The penetration of metal allergens within the skin has been previously studied by the O'Boyle research group¹²⁷. Therefore, in this study the penetration of a preservative and fragrance allergens within ex vivo skin samples, as well as their effect on skin lipids, was investigated. This is the first study of its kind to investigate the penetration of low molecular weight organic allergens within the skin.

5.1.1 2-Methyl-4-isothiazolin-3-one

2-Methyl-4-isothiazolin-3-one (MI) is a low-molecular weight preservative used in an extensive variety of products including cosmetics, household detergents, paints, metalworking fluids, textiles and plastics, amongst others.³⁴² Use of MI together with methylchloroisothiazolinone (MCI) in a 1:3 ratio caused a global epidemic of contact allergy in the 1980s.³⁴³ Subsequently, the use of MI alone was introduced in the early 2000s but MI is also known to be a sensitizer. MI is categorized as a strong sensitizer in the LLNA with an EC3 of 0.4% in acetone: olive oil.³⁴⁴ Exposure to MI/MCI in the general population is widespread, as indicated by detection of a mercapturic acid metabolite of MI and MCI in sixty persons with no specific exposure to MI or MCI/MI.³⁴⁵ Structurally, MI contains a five-membered heterocycle with both a nitrogen and sulfur atom in the ring, also containing a conjugated carbonyl moiety (Figure 5.1). MI is a small electrophilic compound, with the N-S bond reacting with nucleophiles like amino acid residues on proteins forming a hapten, leading to T cell activation and causing skin sensitization³⁴⁶. The incidence of contact allergy to MI has fallen in Europe in recent years, attributable to government regulation limiting concentrations of MI and MCI/MI; however, incidence continues to rise in the USA^{342, 347-349}. In the European Union, MI is not allowed in leave-on products, such as skin lotion, and is limited to 0.0015% in rinse-off products, such as shampoo.³⁵⁰ The pattern and extent of distribution of MI in skin is unknown.

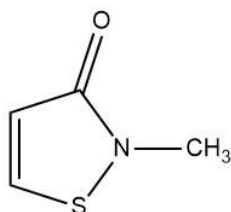


Figure 5.1 Structure of methylisothiazolinone (MI)

5.1.2 Fragrance Allergens

The International Fragrance Agency (IFRA) defines a fragrance as any substance used for its odour properties or malodour coverage³⁵¹. The European Commission reports that 1-3% of the general population are affected by fragrance allergy³⁵². Limonene and linalool are two fragrance terpenes (Figure 5.2). Limonene is the main compound in the essential oils of citrus fruits³⁵³ while linalool is produced by *Lamiaceae* (mints) and *Lauraceae* (laurels, cinnamon, rosewood) plants³⁵⁴. They are added to many common household items and cosmetic products³⁵⁵.

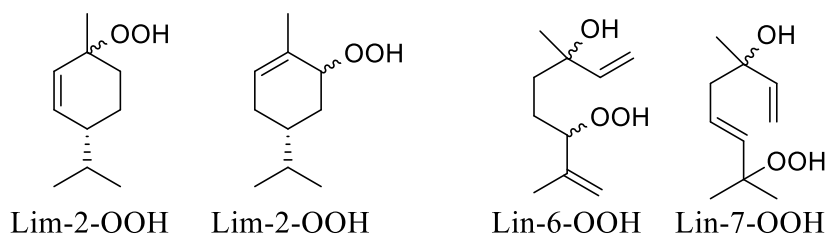


Figure 5.2 Structures of allylic hydroperoxide contact allergens limonene-2-hydroperoxide (Lim-2-OOH), linalool-6-hydroperoxide (Lin-6-OOH), and linalool-7-hydroperoxide (Lin-7-OOH).

A study that used a novel smartphone application identified that 49.5% of tested products contained linalool and 48.5% of tested products contained limonene³⁵⁶. Limonene and linalool are pre-haptens and are classed as occasional contact allergens. Upon exposure to air they undergo oxidation to their respective hydroperoxides, their primary oxidation products, which are significantly more allergenic³⁵⁷. Fragrance hydroperoxides do not act as electrophiles on their own, and do not act in the same way as classical allergens e.g. as a Michael acceptor. Instead, fragrance hydroperoxides bind to proteins via free-radical

mechanisms. Free radicals formed from the hydroperoxide's O-O bond cleavage can react with the nucleophilic amino acid side chains of proteins e.g. cysteine thiols, which leads to the formation of reactive intermediates like epoxides that then covalently modify proteins. This covalently modified protein is presented to T cells and leads to skin sensitisation³⁵⁸.

The LLNA categorizes limonene hydroperoxides (LimOOH) and linalool hydroperoxides (LinOOH) as strong skin sensitizers and are the most potent sensitizers among the autoxidation products of the parent terpenes³⁵⁹.

5.1.3 The Skin Model

In vitro reconstructed human skin models have been widely used in drug testing, cosmetics and toxicology studies as an alternative to animal testing^{360, 361}. They usually consist of human keratinocytes and/or fibroblasts. Despite the recent progress that has been made in the reconstruction of more complex skin models containing other cell types, such models still have limitations, as they are not an exact copy of human skin *in vivo*, and hence the predictability of the human *in vivo* response remains limited. NativeSkin Access[®] human *ex vivo* skin is considered to be highly predictive and cost effective prior to clinical evaluation in humans. NativeSkin Access[®] consists of human skin biopsies obtained after surgery. Hence, it exhibits normal skin barrier function³⁶² (Figure 5.3).

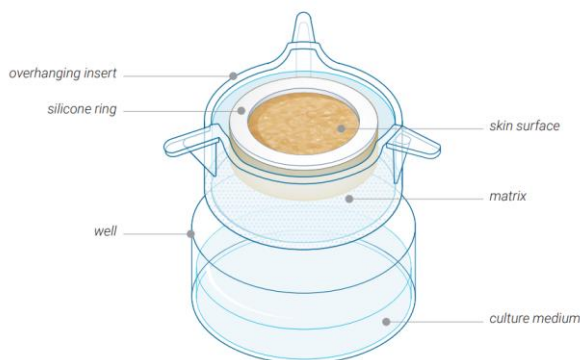


Figure 5.3 The NativeSkin Access[®] *ex vivo* skin model.

5.1.4 Time-Of-Flight Secondary Ion Mass Spectrometry

Mass spectrometry (MS) is a high-throughput analytical detection technique used to get information about the molecular weights and chemical structures of drug metabolites, peptides, carbohydrates and other molecules. The principle of MS depends on the separation of the molecules based on their mass-to-charge (m/z) ratio by ionization of the molecules with high energy electrons to break a molecule into fragments. Fragmentation pattern and m/z values provide information about the molecular weights and chemical structures of the analysed compound³⁶³. MS has many different and complex applications such as in biomarker discovery and proteomics, therapeutic drug monitoring, clinical endocrinology and hormone analysis, microbiology and pathogen identification, and metabolomics³⁶⁴.

While the effects of MI have been studied extensively using e.g. patch testing³⁶⁵, little is known about its distribution in the skin. Studying the distribution of a low-molecular weight compound directly in skin is difficult but recent advances in mass spectrometry imaging (MSI) have demonstrated the possibility to track both allergens and endogenous compounds directly in skin tissue sections.¹⁴ In MSI, ToF-SIMS is a powerful tool to chemically map biological tissues and simultaneously localize both endogenous and exogenous compounds.^{49, 366, 367} ToF-SIMS is an imaging mass spectrometry technique in which a pulsed, energetic “primary” ion beam bombards the surface of a sample and induces a collision cascade, liberating “secondary” ions that are then sent to a time-of-flight (ToF) analyzer for detection³⁶⁸. An argon gas cluster ion beam (Ar-GCIB) can be used to gently sputter the sample (remove the outermost layer), to increase the resolution of the ToF-SIMS images and allow for depth profiling and 3D molecular imaging^{369, 370}. ToF-SIMS is a powerful imaging mass spectrometry technique for cell and tissue analysis³⁶⁶ (Figure 5.4).

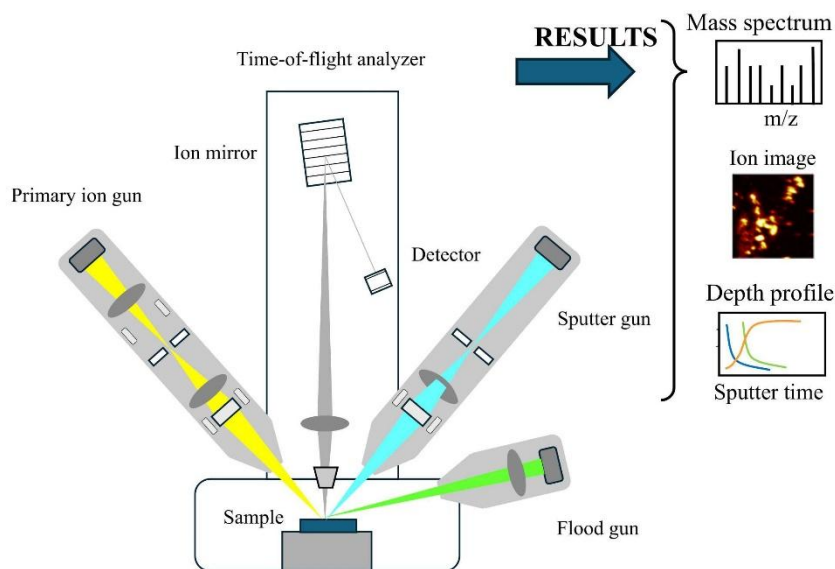


Figure 5.4 Schematic of a dual-beam ToF-SIMS instrument³⁷¹.

The main advantages of ToF-SIMS are its sub-micron, and in some cases sub-100 nm, lateral spatial resolution, high abundance sensitivity, rapid data acquisition, and excellent reproducibility, largely because the technique does not typically require the use of an applied matrix. However, a major limitation of ToF-SIMS is its restricted ability to identify intact molecular species, primarily due to limited mass resolution and mass accuracy at mass-to-charge ratios (m/z) above 150³⁷². While mass spectrometry techniques like LC-MS/MS and GC-MS require analyte extraction and separation, ToF-SIMS analyses solid samples directly and offers exceptional surface sensitivity and allows for imaging of the samples. However, LC-MS/MS and GC-MS offer higher sensitivity and increased quantitative analysis of samples. In comparison to Matrix-assisted laser desorption/ionization (MALDI), another mass spectrometry imaging technique, both techniques have their advantages and are complementary of each other. ToF-SIMS offers higher surface sensitivity and lateral spatial resolution and allows for higher resolution imaging of small molecules like lipids. However, MALDI can provide analysis over a much broader mass range and higher mass accuracy, making it a better choice for analysis of larger molecules like peptides and other proteins³⁷³.

In previous studies, imaging mass spectrometry techniques were used to investigate the effect of contact allergens on skin. MALDI was recently used to show that nickel ions were

confined to the SC and had effects on several lipid species including phosphatidylcholine, sphingomyelin and ceramides in ex vivo porcine skin¹⁶⁸. ToF-SIMS has previously been used to visualise the penetration of metal allergens nickel, cobalt and chromium through the skin³⁷⁴. ToF-SIMS is well suited for the detection and analysis of lipid species in biological species. This is due to the instrument's high lateral resolution and sensitivity³⁷⁵.

5.2 Aims and Objectives

The aim of this chapter is to investigate the penetration of MI and fragrance hydroperoxides within human ex vivo skin tissue, and their effect of the skin lipidome. The objectives to fulfil this aim are:

- To use two different ToF-SIMS systems to determine the effect of Ar-GCIB sputtering on the analysis of ex vivo skin samples
- To use ToF-SIMS to study the uptake and localization of MI in human ex vivo skin tissue (NativeSkin Access[®] model), and follow endogenous lipid changes induced by MI, LimOOH and LinOOH

5.3 Materials and Methods

5.3.1 Allergens

5.3.1.1 Methylisothiazolinone

MI (Merck, CAS: 2682-20-4) was diluted to 0.0175 M (equivalent to the concentration used for diagnostic patch-testing of 0.2% w/v) in phosphate buffered saline (PBS)³⁷⁶. For ToF-SIMS analysis of the MI standard a small amount of MI powder was deposited on a clean silicon wafer.

5.3.1.2 Fragrance Allergens

Hydroperoxides of linalool 1.0% and hydroperoxides of limonene 0.3% (Chemotechnique Diagnostics, Sweden) were obtained as a gift from Yrkesdermatologen, Sahlgrenska Universitetssjukhuset, Gothenburg. These are commercially available products used for patch testing containing autoxidized linalool and limonene. The concentrations are the standard used for patch testing and were recommended for inclusion to the European

Baseline Series by the European Society of Contact Dermatitis (ESCD)^{377, 378}. Hydroperoxides of limonene 0.3% w/w (10 mg) (Chemotechnique, H-032A, lot 21441-5a) or hydroperoxides of linalool 1.0% w/w (10 mg) (Chemotechnique, H-031A, lot 21293A-5a) were added to Vaseline white Ph. Eur (gift from Yrkesdermatologen, Sahlgrenska Universitetssjukhuset, Gothenburg). The hydroperoxide/Vaseline mixture was added to 8mm Finn Chambers on Scanpor.

5.3.2 Ex vivo Skin Tissue Exposure

NativeSkin access (11 mm) (Genoskin, France) was cultured in sterile conditions as recommended by the manufacturers. Briefly, samples were incubated in the provided culture medium for at least one hour at 37 °C, 5 % CO₂, and 95 % relative humidity prior to treatment with MI or fragrance patch test preparations.

MI (Merck) (20 µL, 0.0175 M solution in PBS) was applied to NativeSkin Access for 24 hours. PBS (20 µL) was applied as the vehicle control (n=2). The experiment was performed on two independent occasions with skin samples from two different donors.

Hydroperoxides of linalool 1.0% or hydroperoxides of limonene 0.3% (on 8mm Finn Chambers as above) were applied to NativeSkin Access for 24 hours, ensuring that the patch test mixture was in contact with the skin (n=2). Vaseline white Ph. Eur was applied as the control (n=2). The experiment was performed with skin samples from two different donors.

Both donors were female, aged 42 years old, with skin type 2 according to Fitzpatrick's classification guidelines³⁷⁹. The skin sample from both donors was taken from the abdominal area. Patient 1 had an allergy to latex, and was taking omeprazole, triptan and Nurofen medication. Patient 2 had no allergies and was not on any medication. The Trinity College Dublin Faculty of Health Sciences Ethics Committee approved the study (ref: 220401).

5.3.3 ToF-SIMS Analysis

The ToF-SIMS surface analysis of MI treated skin samples was carried out using two different ToF SIMS 5 instruments (IONTOF GmbH, Münster, Germany). One system was equipped with a 25 keV Bi cluster ion gun as a primary ion source and a 10 keV C60+ source

for sputtering (ToF-SIMS system 1). The other system was equipped with a 30keV bismuth nanoprobe and an Ar-GCIB (ToF-SIMS system 2). For both systems mass spectra in positive ion mode were acquired by using the bismuth primary ion sources. The ToF-SIMS surface analysis of fragrance allergen treated skin samples was carried out using ToF-SIMS system 2. To acquire images with high spatial respective mass resolution, the delayed extraction mode was used.³⁸⁰ The pulsed primary ion currents were in the range of 0.11 pA to 0.17 pA using Bi_3^+ ions. Areas of about $100\ \mu\text{m} \times 100\ \mu\text{m}$ to $200\ \mu\text{m} \times 200\ \mu\text{m}$ on the skin section were selected with a raster of 256×256 -pixels and analyzed with 100 raster scans. For the ToF-SIMS system 2, sputtering with the Ar-GCIB was performed using Ar1500 at 5000eV with an ion current of 4.9 nA.

Three different sections from the specimens of MI and fragrance treated samples and the respective control treated samples were analyzed, respectively. The spectra were internally calibrated to signals of common fragments including $[\text{C}]^+$, $[\text{CH}_2]^+$, $[\text{CH}_3]^+$, $[\text{C}_5\text{H}_{15}\text{PNO}_4]^+$, and $[\text{C}_{27}\text{H}_{45}]^+$.

The MI standards and control skin tissue were analyzed in the spectrometry mode on ToF-SIMS system 2. Here the MI standard was introduced to vacuum and analyzed at multiple time points with a current of 0.4 pA using Bi_3^+ ions.

The software SurfaceLab (version 7.3, IONTOF, Germany) was used to process, record, analyze and evaluate images and mass spectra.

5.3.4 Statistical Analysis

An automated peak selection was used to select m/z values of both the control and MI treated skin in SURFACELAB 7.1 software. The same mass range was selected (m/z 50 to m/z 800) for the SC and the VE. Data was imported to SIMCA software (version 16.0, Umetrics, MKS Instruments Ltd.) as ion intensities normalized to the total ion count. Pareto scaling was performed, and analysis was carried out by principal component analysis (PCA). Data was also analysed using GraphPad Prism 10 for Windows (GraphPad software, USA). A repeated measured two-way ANOVA test was used to analyse the data, with Tukey's multiple comparisons post-hoc test used to compare means. The mean $\pm$ SEM is displayed

on the histograms of the results, and a p value of less than 0.05 was considered to be statistically significant.

5.4 Results and Discussion

The results and discussion will be divided into two sections: MI analysis using ToF-SIMS, and fragrance allergen analysis using ToF-SIMS. This is due to the differences in preparation of the allergens. MI is water soluble and was applied to the skin samples in PBS, whereas the fragrance hydroperoxides are lipid soluble and were applied to the skin samples as patch test formulations prepared in Vaseline.

5.4.1 MI Analysis Using ToF-SIMS

MI is a small molecule, with the molecular formula C_4H_5NOS , a molecular weight of 115 and a vapor pressure of 0.062 mm Hg at 25 °C. Prior to analysis of MI in skin samples, and to understand how MI would behave under vacuum conditions, MI was applied on a silicon wafer and run in ToF-SIMS in both positive and negative polarities (negative data is not discussed), to get an indication about its stability and potential fragmentation patterns.

Spectra from the MI standard (Figure 5.5) showed the molecular ion $[M+H]^+$ from MI at m/z 116.0. The most significant peak in the spectrum was sodium, Na^+ at m/z 23. Sodium is an easily ionized species in ToF-SIMS, and thus it is usually one of the highest peaks in the positive ion mode. The spectra showed many other potential fragments such as m/z 62.9 and m/z 80.9 that are most likely to come from fragmentation or reaction of MI, but these remained unidentified.

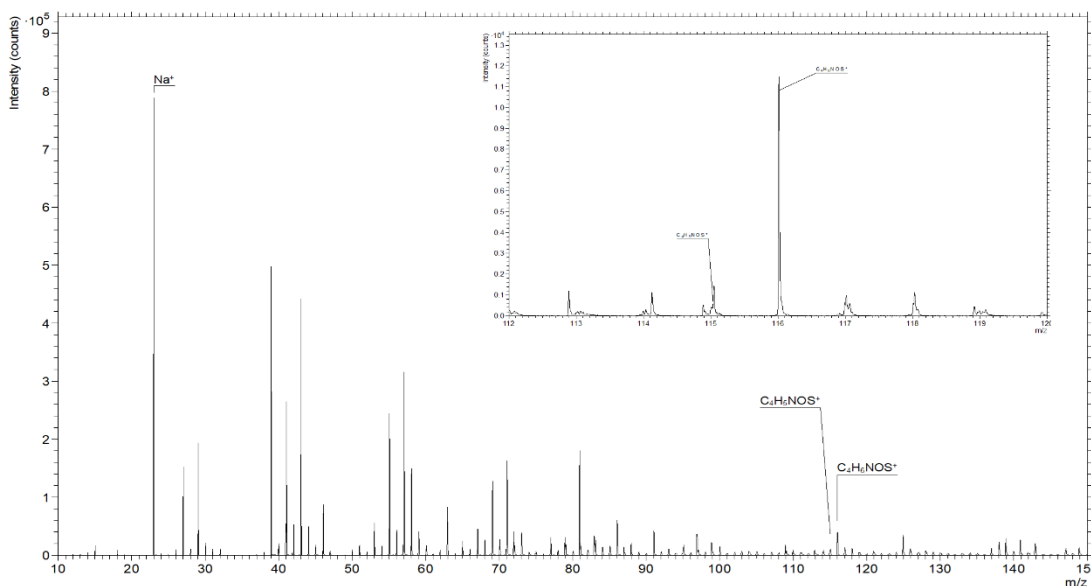


Figure 5.5 ToF-SIMS spectrum in positive ion mode of MI applied on a silicon wafer, showing the molecular ion $[M+H]^+$ from MI at m/z 116.01.

Even with the vapor pressure of MI, the molecular ion remained fully stable in the vacuum of the ToF-SIMS for up to 5 hours. In tissue we expect that MI is less likely to vaporize due to its interaction with the tissue matrix. Hence, we chose to use the m/z 116.0 ion for detection of MI in skin samples as its formation is independent of the matrix being analyzed.

It should however be noted that the ionization of the molecule could be different inside the skin tissue based on the matrix of the sample. In ToF-SIMS, a primary ion beam bombards the surface of the sample and secondary species are ejected from the sample. Only a small fraction of the ejected secondary species are ionized and detected.³⁸¹ The ionization process is strongly affected by the surface and bulk chemistry, i.e. the matrix effect.^{382, 383} In addition, the response of chemicals to the surrounding tissue is very specific and the ion yield of one chemical can be enhanced while the ion yield of another chemical can be decreased. This can make acquired data from biological samples difficult to interpret.^{384, 385}

5.4.1.1 ToF-SIMS analysis of MI in ex vivo human skin without argon sputtering (system 1)

ToF-SIMS was then performed on ex vivo skin tissue samples to study the uptake of MI and the lipid changes that were caused by MI exposure using initially the ToF-SIMS system 1

equipped with the 25 keV bismuth source. This system lacked an argon gas cluster ion source (GCIB), and the obtained data only reflects the very top monolayers of the surface of the skin tissue cross-section. In ToF-SIMS, a pulsed and focused primary ion beam enables mass spectrometry imaging. When using conventional ToF-SIMS instruments, the focusing modes of the primary ion beam can be adjusted to either get a high mass resolution or high lateral resolution. An alternative method to maintain the mass resolution with high spatial resolution is to extract secondary ions from the sample surface with a certain delay after the arrival of primary ions, i.e. so-called delayed extraction³⁸⁰. After identifying the peaks of interest, the delayed extraction imaging method was applied on the skin tissue and the MI molecular ion was located in the MI treated sample as shown in Figure 5.6.

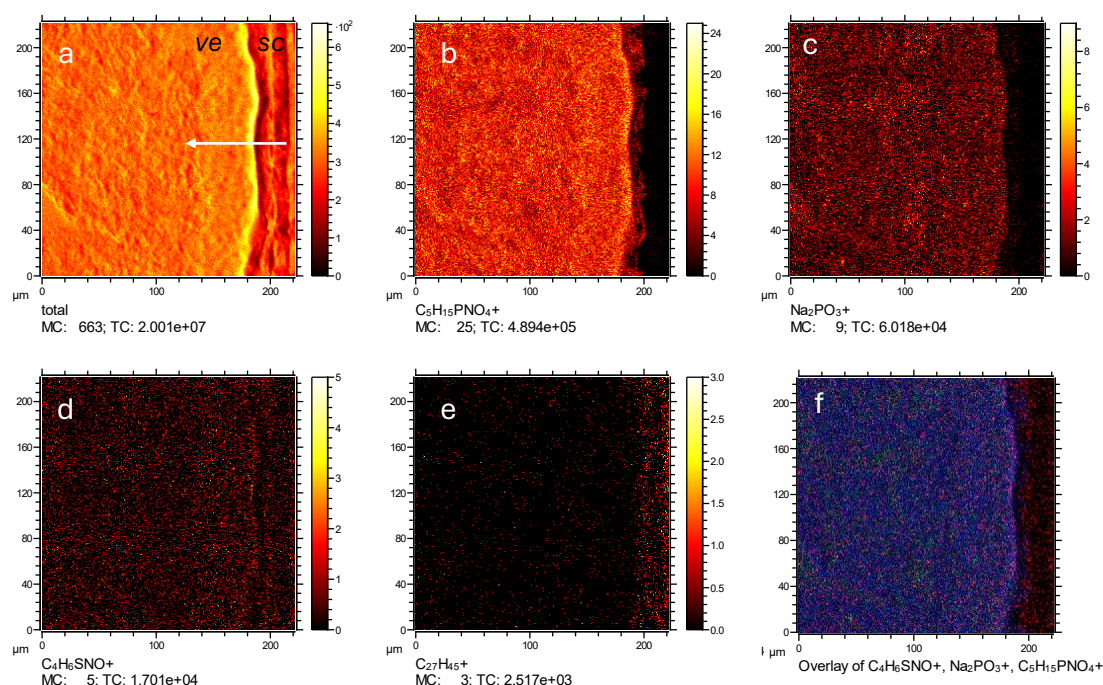


Figure 5.6 ToF-SIMS data of the epidermal region of human ex vivo skin cross section (220 × 220 μm). Data was acquired using system 1. Ion images of (a) total ion: arrow shows direction of penetration of MI into the skin; (b) phosphatidylcholine (PC) head group (m/z 184); (c) Na₂PO₃ nuclear fragment at m/z 124.9; (d) MI molecular ion (m/z 116.0), (e) cholesterol, SC marker (m/z 369); (f) a three-color ion image of the MI fragment (in red, binned x2), m/z 124.9 (in green), and PC (blue, VE marker). VE: viable epidermis, SC: stratum corneum.

Figure 5.6(a) reveals the total ion signal which shows a relatively homogenous signal across all skin layers. The distribution for MI can be seen in Figure 5.6(d) and shows a distribution mainly in the SC but also inside the VE. Very little biologically relevant information could be revealed from the samples except signals from the PC head group [m/z 184.1, Figure 5.6(b)] and cholesterol [m/z 369.3, Figure 5.6(e)]. It was not possible to differentiate any clear morphological cell structures. A spectrum from the skin tissue analyzed using ToF-SIMS system 1 equipped with the 25 keV bismuth source using the spectrometry mode can be seen in Figure 5.7. A background signal from m/z 116.0 using spectrometry mode on this system was also seen.

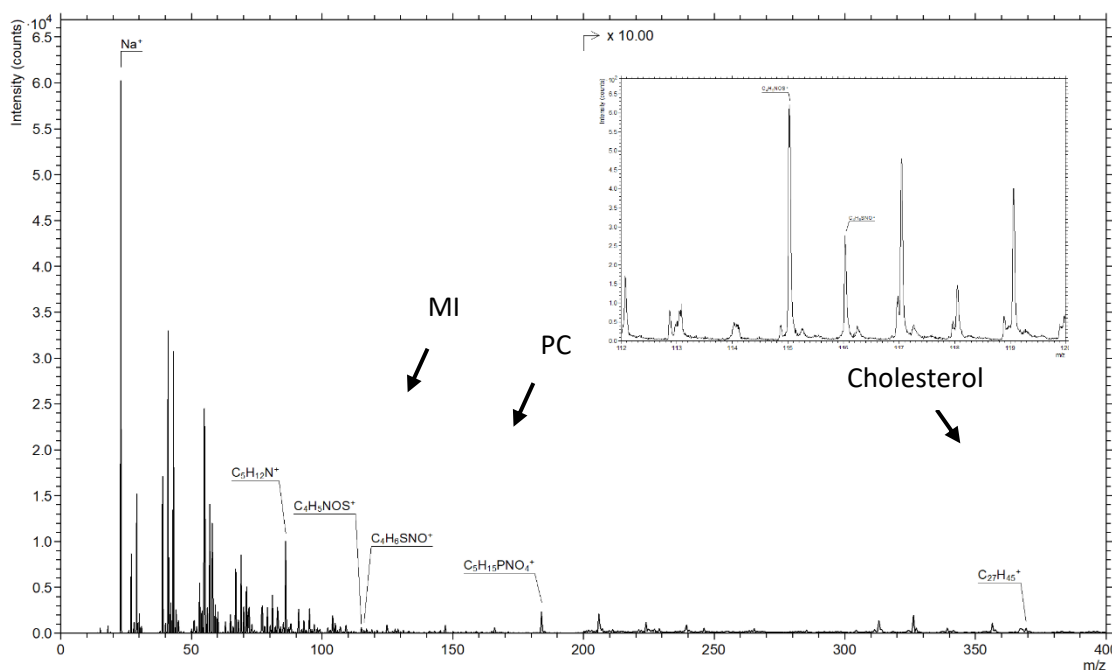


Figure 5.7 ToF-SIMS spectrum in positive ion mode of MI exposed skin tissue without Ar-GCIB sputtering, showing the molecular ion $[M+H]^+$ from MI at m/z 116.0, phosphatidylcholine (PC) head group at m/z 184.1, and cholesterol at m/z 369.3.

5.4.1.2 TOF-SIMS analysis of MI in ex vivo human skin with argon sputtering (system 2)

Additional MI and control samples were run on the ToF-SIMS system 2 instrument using the 30keV bismuth nanoprobe and GCIB sputtering. The use of GCIB argon sputtering in biological ToF-SIMS imaging has significantly improved the technique by enabling the detection of large biomolecules with minimal sample damage, enhancing sputtering yield, and increasing secondary ion intensity.³⁸⁶⁻³⁸⁸ Hence, immediately prior to ToF-SIMS analysis, the samples were sputtered with the Ar-GCIB for 10 seconds (approx. $4\text{E}+14$ ions/cm²) to reveal a fresh surface. The sample was then analyzed in the non-interlaced 3D imaging mode using sequential imaging with the bismuth nanoprobe and GCIB sputtering. The Ar-GCIB sputters the material from the top layer of the sample and allows for improved resolution of the depth profile. The Ar-GCIB is considered superior for biological applications to traditional sputtering sources like oxygen and cesium due to its ability to distribute ionic energy across a cluster of argon atoms, allowing for softer ionization and reduced damage to the sample.³⁸⁸ Several peaks of interest were identified from the ToF-SIMS spectra, including PC headgroup fragment ions at m/z 86.1 and m/z 184.0, and a cholesterol fragment ion at m/z 369.3. Multiple monoacylglycerol fragment ions (m/z 311.1, 313.1, 339.1) and diacylglycerol fragments (m/z 577.2, 601.3, 603.2, 605.3) were also observed as shown in Figure 5.8. The MI fragment was identified at m/z 116.0 as discussed earlier.

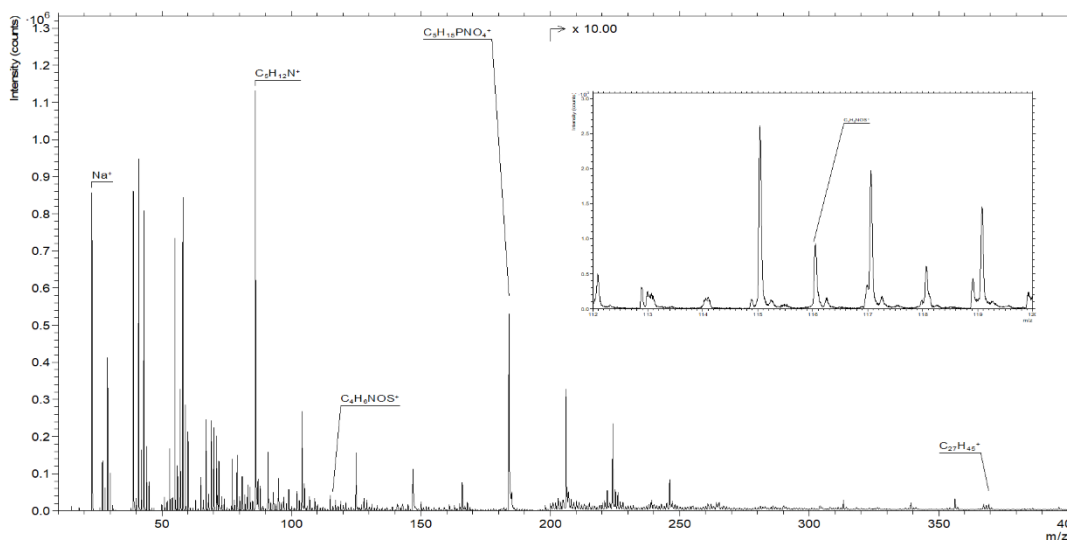


Figure 5.8 ToF-SIMS spectrum in positive ion mode of MI exposed skin tissue after Ar-GCIB sputtering, showing the molecular ion $[M+H]^+$ from MI at m/z 116.0, phosphatidylcholine (PC) head group at m/z 184.1, and cholesterol at m/z 369.3.

The localization of the PC, MI, cholesterol and Na_2PO_3 ions is displayed in Figure 5.9. The images show clear cellular structures in the VE, including nuclei and phospholipid membranes. This image is comparable to previously published images which visualized GenoSkin biopsies using light sheet fluorescence microscopy. These images show a mature SC and the dermal-epidermal junction which is comprised of keratinocytes. Similar cellular structures can be seen more clearly using this improved ToF-SIMS mass spectrometry imaging technique compared to the initially used ToF-SIMS system 1. Non-homogenous skin structures have also been noted by others using OrbiSIMS and GCIB sputtering.⁵⁰

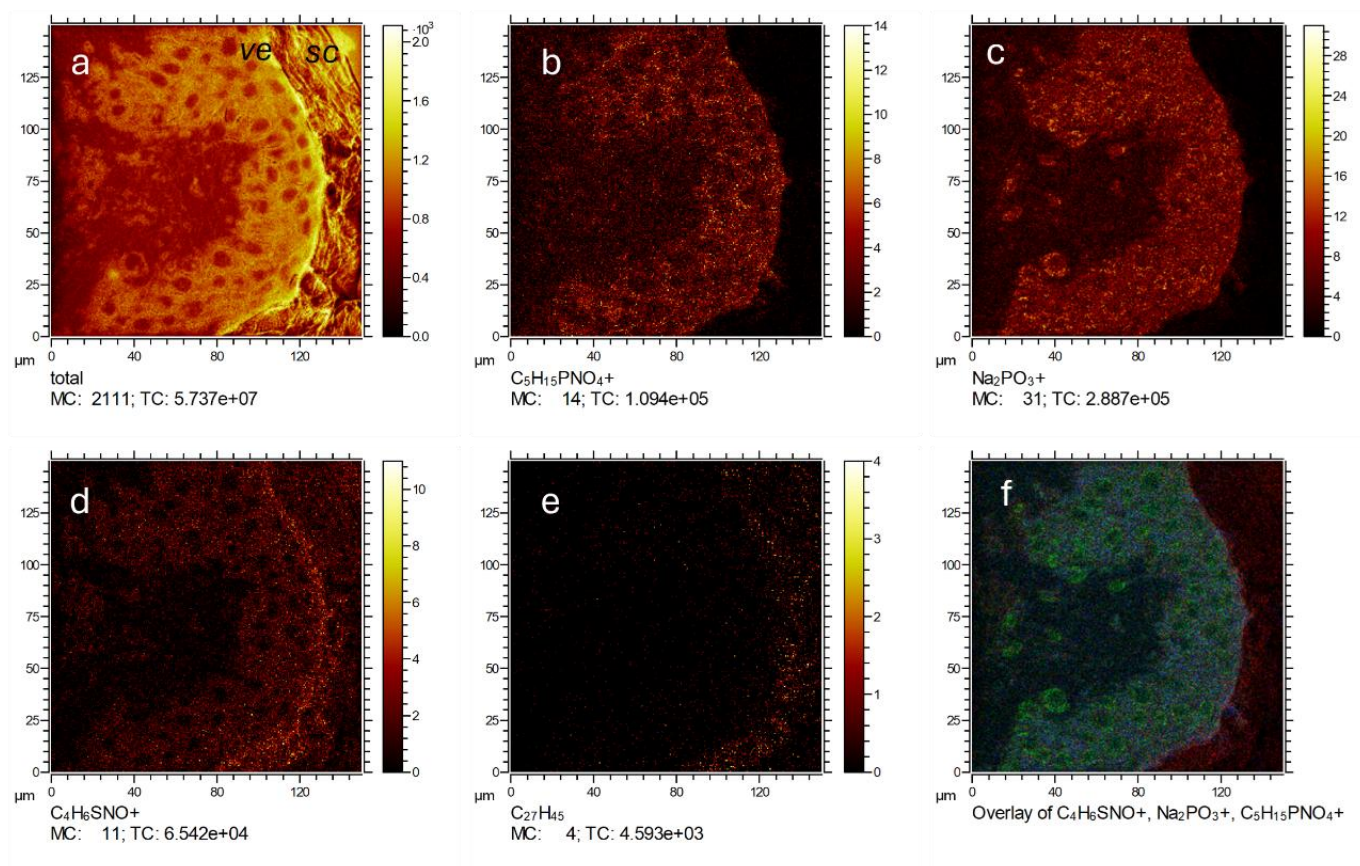


Figure 5.9 ToF-SIMS data of the epidermal region of human ex vivo skin cross section ($150\ \mu\text{m} \times 150\ \mu\text{m}$) after 10s Ar GCIB sputtering to $4E+14$ ions/cm² (system 2). Ion images of (a) total ion (b) phosphatidylcholine (PC) head group (m/z 184) (c) Na_2PO_3 nuclear fragment at m/z 124.9 (d) MI molecular ion (m/z 116.0), (e) cholesterol, SC marker (m/z 369); (f) a three-color ion image of the MI fragment (in red), Na_2PO_3 m/z 124.9 (in green), and PC (blue, VE marker). VE: viable epidermis, SC: stratum corneum.

In Figure 5.9(c), the nuclear fragment at m/z 124 can be seen, and is also represented in green in the overlay image in Figure 5.9(f). This was only detected in the upper layers of the VE, most likely the SG and SS and was at its highest intensity in keratinocytes. This indicates that this ion corresponds to a compound that is mainly restricted to the nucleus. Surrounding the cells was the PC headgroup fragment [m/z 184.1, Figure 5.9(b)]. This fragment was also only seen in the VE, corresponding with previously published studies that conclude that levels of phospholipids are negligible in the SC.¹⁹ The fragment appeared at its highest intensity on the surroundings of the m/z 124 signal from the cells, indicating that this fragment contributes to the phospholipid barrier. The MI ion appeared as a brighter band at the end of the SC layer but also showed a localization similar to the PC headgroup with a low continuous signal across the SC and VE (red in Figure 5.9(f)). The signal from cholesterol could be seen mainly in the SC as shown in Figure 5.9(e). Analysis of PBS exposed control tissue, also sputtered with Ar-GCIB for 10 seconds, revealed a low background signal at m/z 116.0 and a larger adjacent peak at m/z 116.1 that could potentially influence the signals in the MI exposed tissue. As this signal was relatively low, and the achieved mass resolution allowed separation of m/z 116.0 and m/z 116.1 the influence was considered to be non-significant as demonstrated in Figure 5.10.

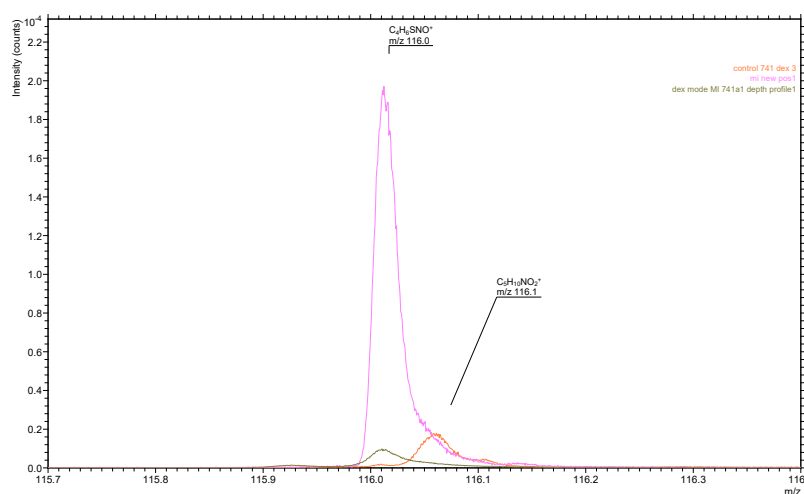


Figure 5.10 ToF-SIMS spectrum of MI in positive ion mode of sputtering, showing the molecular ion $[M+H]^+$ from MI at m/z 116.0 from the reference sample in pink, the $[M+H]^+$ from MI at m/z 116.0 in the MI exposed skin tissue after Ar-GCIB sputtering in green and the peak at m/z 116.1 in the PBS exposed control skin tissue in orange

The distribution of m/z 116.0 in the control tissue is shown in Figure 5.11.

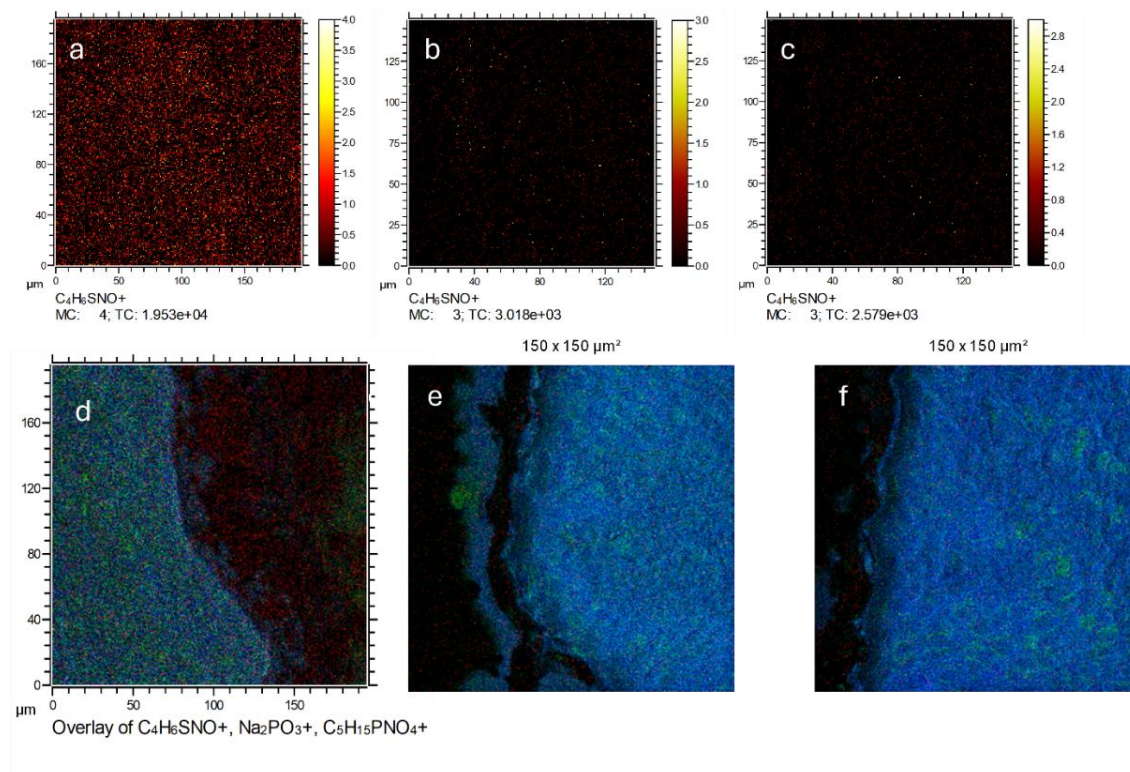
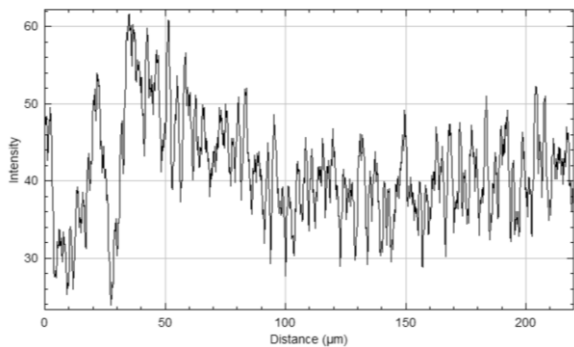


Figure 5.11 ToF-SIMS analysis of m/z 116 in skin tissue sections. (a) ToF-SIMS image of m/z 116.0 in PBS-exposed control skin (system 1, spectrometry mode, no Ar-GCIB sputtering). (b, c) ToF-SIMS images of m/z 116.0 in PBS-exposed control skin after 10 s of Ar-GCIB sputtering (system 2, delayed extraction mode, shown for two independent tissue samples). (d) RGB overlay of m/z 116.0 (MI, red), m/z 124.9 (Na_2PO_3^- , green), and m/z 184.1 (phosphatidylcholine headgroup, blue) in PBS-exposed skin (system 1, spectrometry mode). (e, f) RGB overlay of the same ions in MI-exposed skin after 10 s of Ar-GCIB sputtering (system 2, delayed extraction mode, shown for two independent tissue samples).

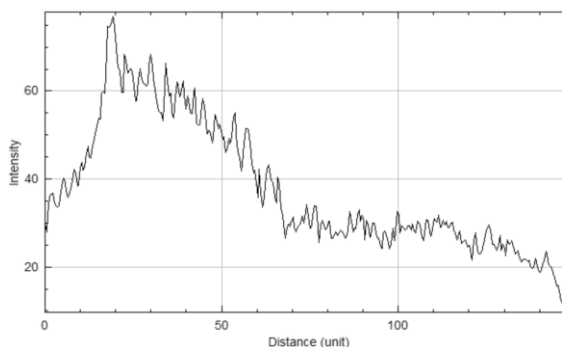
A linescan was generated which shows the intensity of the molecular ion from MI across the skin sample using ToF-SIMS system 1 and 2 (Figure 5.12). The linescan shows the initial accumulation of MI in the SC, and then a substantial increase in intensity at about 30-40 μm into the skin sample, representing the penetration of MI into the VE. The increase corresponds to the more intense MI signal that appears to penetrate the keratinocytes in the upper layers of the VE. The intensity of the ion then levels out and decreases slightly as the scan reaches the depths of 130 - 140 μm , representing the lower layers of the VE. The results from the line scan from the TOF-SIMS system 2 using Ar-GCIB sputtering gives a clearer view of the penetration and shows an overall higher level of the ion also within the VE that was more difficult to see without GCIB sputtering. The initially high signal of MI on

the SC is however lower after GCIB sputtering, indicating that sputtering affects the SC layer differentially than the VE layer.

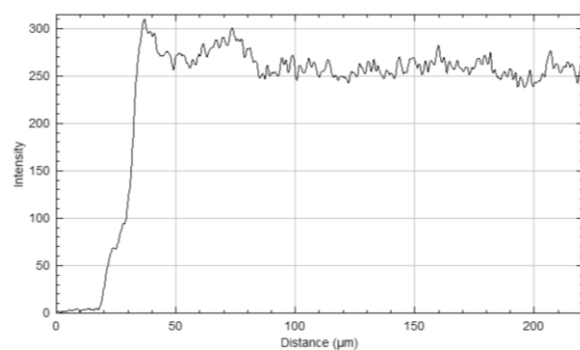
(a) (i)



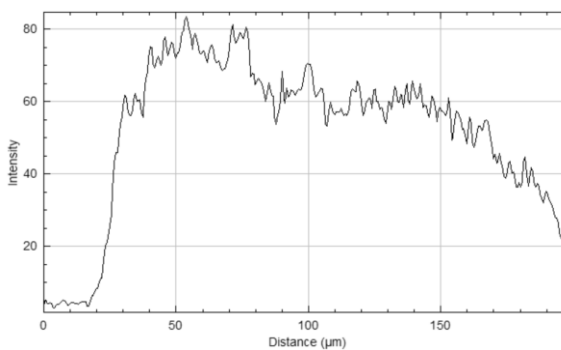
(b) (i)



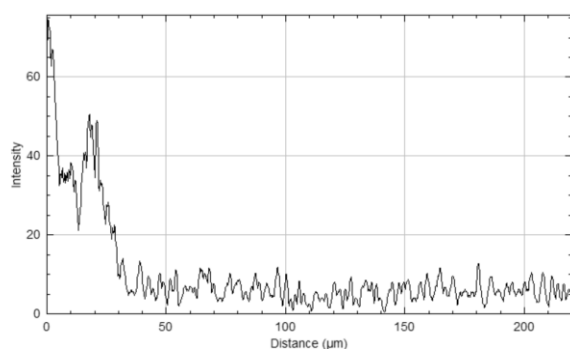
(a) (ii)



(b) (ii)



(a) (iii)



(b) (iii)

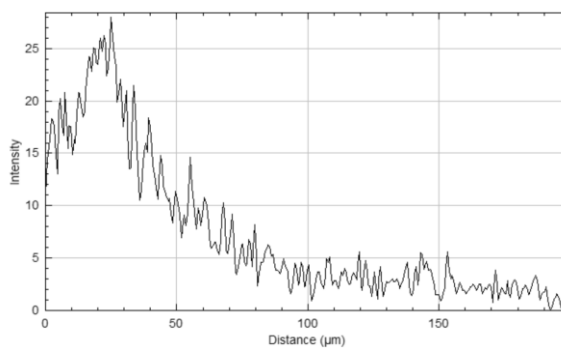


Figure 5.12. Linescan of the MI molecular ion (m/z 116.0), showing penetration of MI as a function of penetration depth (distance) by ToF-SIMS system 1 (a) and using ToF-SIMS system 2 with Ar-GCIB sputtering (b). (a) Line scans using system 1 showing the intensity of (i) MI ion at m/z 116, (ii) PC headgroup ion at m/z 86 to identify the VE, and (iii) cholesterol ion at m/z 369 to identify the SC. (B) Line scans using system 2 showing the intensity of (i) MI ion at m/z 116, (ii) PC headgroup ion at m/z 86 to identify the VE, and (iii) cholesterol ion at m/z 369 to identify the SC.

This effect on the diminishing cholesterol signal in the SC could also be seen when applying a higher Ar ion dose to the skin tissue. Figure 5.13 shows another example of MI exposed tissue where a higher Ar sputter dose of 5.3×10^{14} ions/cm² was applied. The signal from cholesterol in the SC is fully depleted [Figure 5.13(e)]; however, the contrast of the keratinocyte nuclei was much improved [Figure 5.13(c)]. The MI signal can still be detected in the SC and the penetration into VE can also be seen adjacent to the keratinocyte nuclei [Figure 5.13(d) and 5.13(f)].

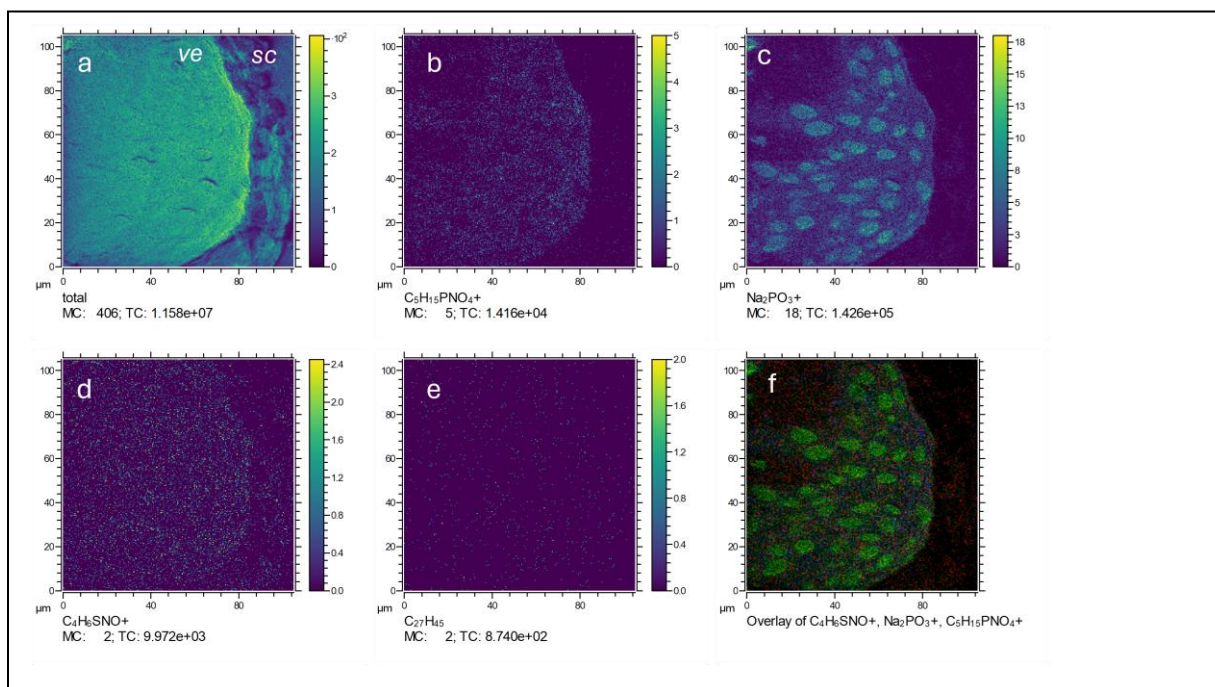


Figure 5.13. ToF-SIMS data of the epidermal region of human ex vivo skin cross section ($105 \mu\text{m} \times 105 \mu\text{m}$) after Ar GCIB sputtering to 5.3×10^{15} ions/cm². Ion images of (a) total ion (b) phosphatidylcholine (PC) head group (m/z 184) (c) Na_2PO_3 nuclear fragment at m/z 124.9 (d) MI fragment (m/z 116.0), (e) cholesterol, SC marker (m/z 369); (f) a three-color ion image of the MI fragment m/z 116.0 (in red), Na_2PO_3 m/z 124.9 (in green), and PC m/z 184 (blue, VE marker). VE: viable epidermis, SC: stratum corneum.

5.4.1.3 Effect of MI treatment on endogenous skin lipids

To investigate the effect of MI treatment on endogenous skin lipids, and due to the differences in lipid composition in different layers of the skin, the SC and VE were analyzed separately. The cholesterol fragment at m/z 369.3 was used as a marker of the SC region, and the PC headgroup fragment at m/z 86.1 was used to identify the VE. Principal component analysis (PCA) suggested that lipid changes were more prominent in the SC rather than the VE of MI-treated samples. A PCA scores plot that analyzed m/z values in the

range of m/z 50 – 650, identified differences in the chemical composition of the SC of the control (PBS) samples and the treated MI samples [Figure (5.14(a))]. The clustering of the MI treated samples (green) in a different quadrant compared to control samples (blue) indicates differences in the chemical makeup of these samples. A PCA loadings plot indicated at which m/z values the most variability between samples was observed. The loadings plot identified changes in PC headgroup fragment at m/z 86.1 (highlighted in blue), as well as some potential differences in diacylglycerol (DAG) content at an m/z of 577.1 (highlighted in red) [Figure 5.14(b)].

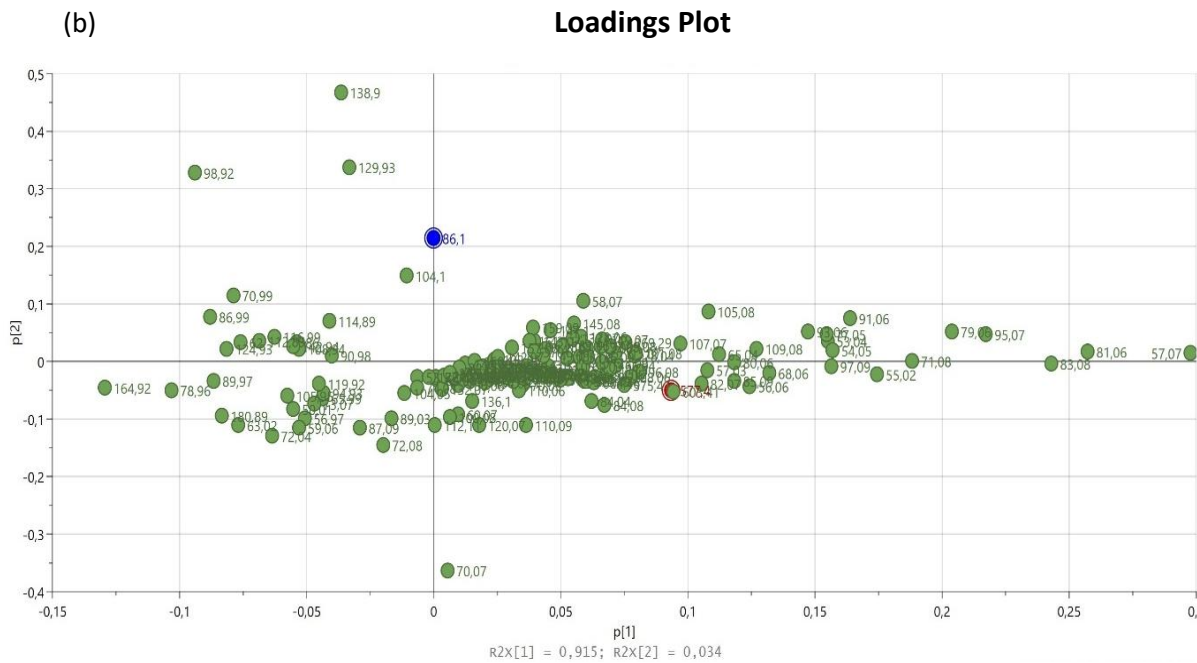
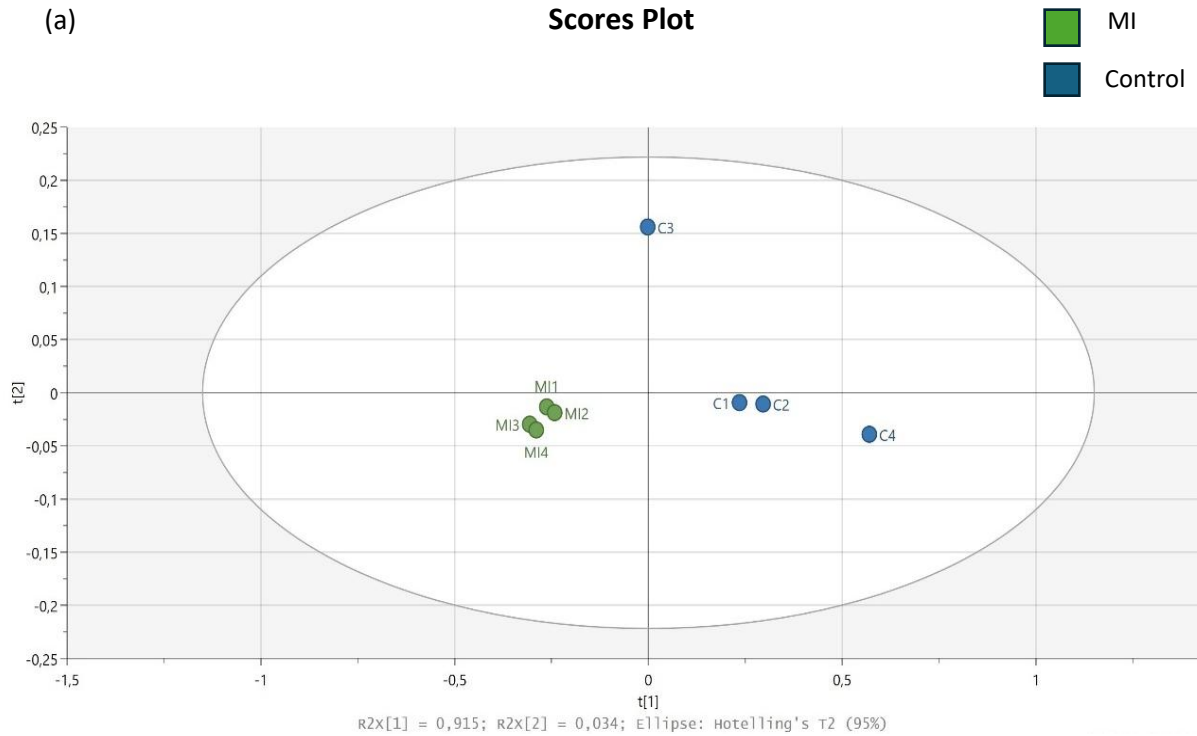


Figure 5.14 (a) 2D score plot and (b) loadings plot for PCA of ToF-SIMS data of the SC of MI-treated ex vivo human skin (green) and control (PBS-treated) ex vivo skin (blue). Data was extracted and analyzed as normalized intensities to total ion count. Green = MI treated, Blue = PBS treated

To investigate lipid changes in more detail, a selection of ion peaks corresponding to different lipid species were selected and analyzed. Lipid species analyzed included PC headgroup (m/z 86.1 and m/z 184.0), cholesterol (m/z 369.3), monoacylglycerols (MAGs) (m/z 311.1 and m/z 313.1) and DAGs (m/z 577.1 and m/z 601.3). The differences in intensities of the peaks at these m/z values in the MI treated samples and controls were analyzed and significant decreases in intensity were seen across many lipid species (Figure 5.15).

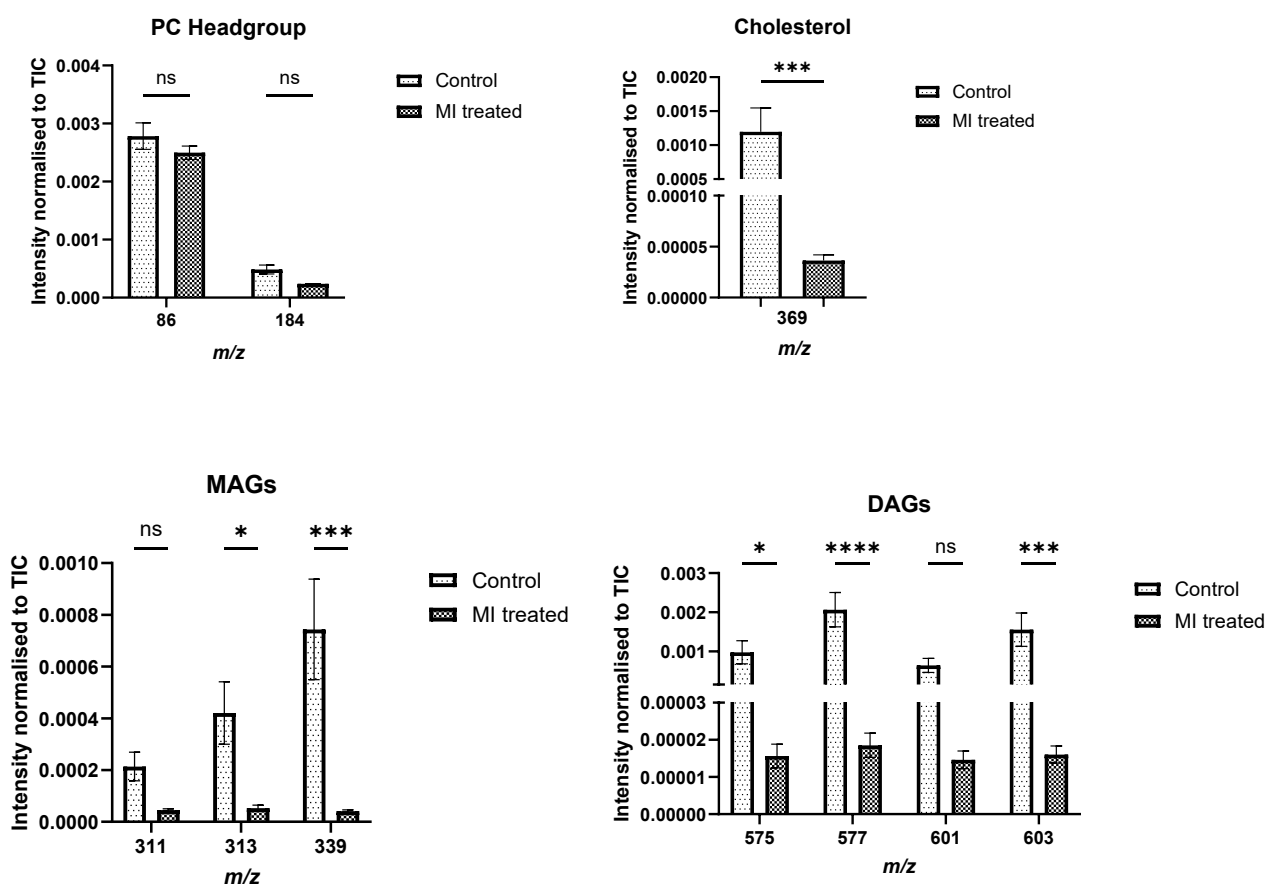
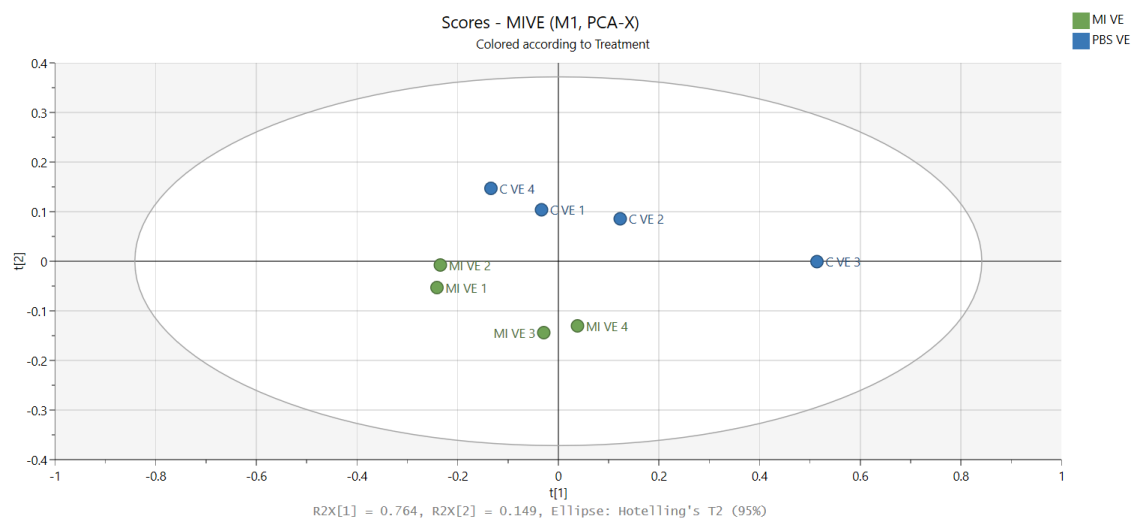


Figure 5.15 SC skin lipid analysis for PBS control treated and MI treated ex vivo human skin. Data was normalized to total ion count in positive ion mode. Skin was treated with 0.0175 M MI in PBS. Control = PBS treated skin samples. Data was analyzed by two-way ANOVA, with Šidák post-hoc test for multiple comparisons. Cholesterol data was analyzed by unpaired students T test. * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = no significance. $n = 4$.

The intensity of PC headgroup fragments at m/z 184.1 and m/z 86.1 decreased after treatment with MI. The decrease correlates with previously published data which showed a decrease in another phospholipid, phosphatidylethanolamine (PE), in bacterial cultures treated with MI³⁸⁹. In addition, another sensitizing preservative, sorbic acid, has also been documented to induce a decrease in PC levels³⁹⁰. A decrease in PC headgroup fragment intensity has also been previously documented in the SC after treatment with metal allergens³⁹¹. MI is known to induce an increase in ROS levels³⁹², which could lead to increased oxidative stress and lipid peroxidation, which could explain the reduction in PC levels observed above. Statistically significant decreases in intensity were also seen across the cholesterol, MAG, and DAG lipid species. DAG ions could also represent triacylglycerols (TAG), as TAG is fragmented in ToF-SIMS to produce mainly DAG ions³⁹³. As the concentration of MI used in these experiments was equivalent to the concentration used in the patch testing for MI allergy, these lipid changes are of clinical relevance.

In the VE, a scores plot suggested differences in the chemical composition of MI treated skin samples in comparison to the control PBS treated samples. The MI samples (Figure 5.16(a), green) were clustered together and away from the PBS treated samples (Figure 5.16(a), blue), indicating differences in the chemical make-up of these skin samples. A loadings plot identified variances at many m/z values, including the m/z 86.1 PC headgroup ion, which was positioned away from the center of the graph, which indicated differences in the PC headgroup intensities between the MI treated samples and the PBS treated samples.

A)



B)

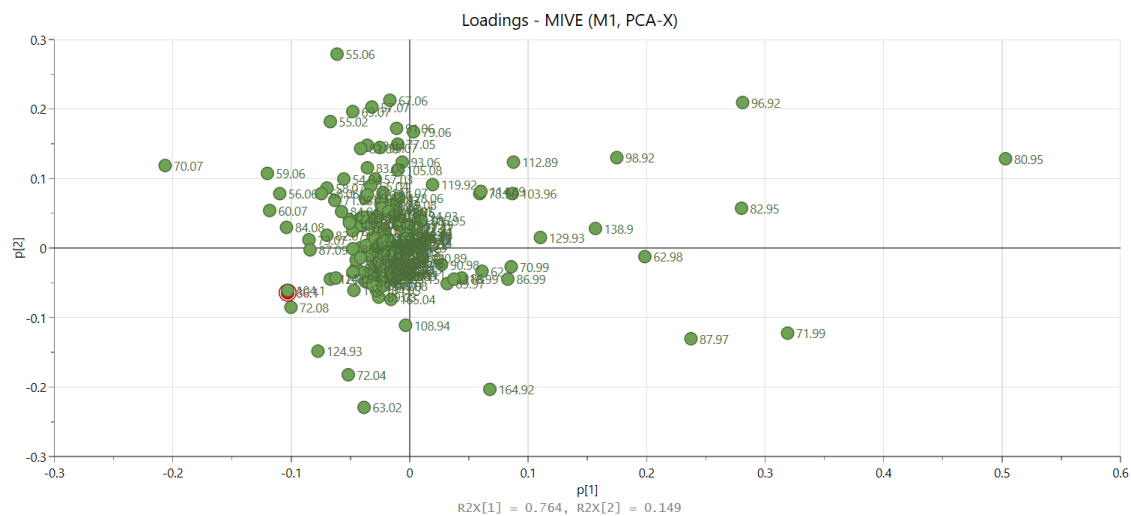


Figure 5.16(a) 2D score plot and (b) loadings plot for PCA of ToF-SIMS data of the SC of MI-treated *ex vivo* human skin (green) and control (PBS-treated) *ex vivo* skin (blue). Data was extracted and analyzed as normalized intensities to total ion count.

To investigate the lipid changes in more detail, a selection of ion peaks corresponding to specific lipid species were selected and analysed. The ions were selected based on indicated differences in the PCA loadings plot and additional ions of interest, as investigated in the

SC. There was a significant increase in the intensity of the PC headgroup ion at m/z 86.1 in the VE of the MI treated samples in comparison to the VE treated samples, but no significance was observed in the m/z 184 PC headgroup ion. A decrease in cholesterol intensity was observed, however it was not statistically significant. There were no significant changes in the intensity of the MAG ions in MI treated samples in comparison to PBS treated samples, however significant decreases in the intensity of the DAG ions were observed in MI treated samples. This correlated with the decrease in DAG intensity observed in the SC of MI treated samples.

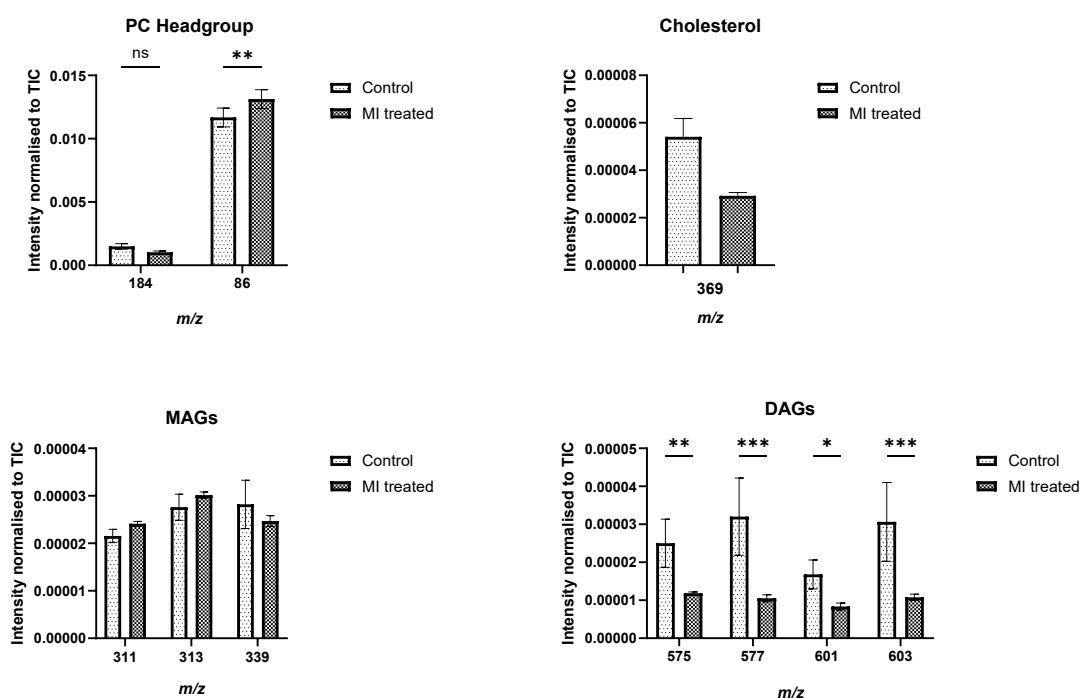


Figure 5.17 VE skin lipid analysis for PBS control treated and MI treated ex vivo human skin. Data was normalized to total ion count in positive ion mode. Skin was treated with 0.0175 M MI in PBS. Control = PBS treated skin samples. Data was analyzed by two-way ANOVA, with Šidák post-hoc test for multiple comparisons. Cholesterol data was analyzed by unpaired students T test. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, ns = no significance. $n = 4$.

5.4.2 LimOOH and LinOOH Analysis using ToF-SIMS

For analysis of limonene hydroperoxide (LimOOH) and linalool hydroperoxide (LinOOH) using ToF-SIMS, the patch test preparations for these allergens are applied to ex vivo skin

samples (hydroperoxides of linalool 1.0% w/w, and hydroperoxides of limonene 0.3% w/w). LimOOH and LinOOH ions produced using ToF-SIMS were firstly identified, allowing for the subsequent tracking of the fragrance allergens through the skin samples. Previous work using LC/ESI-MS/MS of LimOOH identified the most abundant fragment ions as the molecular ion peak at m/z 169.2, the $[M+H-H_2O]^+$ peak at m/z 151.2, and the $[M+H-H_2O-CH_2-CH_3COOH]^+$ peak at m/z 93.1³⁹⁴. LC-MS analysis with ESI or APCI ionisation identified the ions at m/z 169.1 and m/z 151.2 as the most abundant LimOOH fragments³⁹⁴. A study investigating the stability of LimOOH using LC-MS identified the $[M+H]^+$ peak at m/z 169.1 as a minor ion, and the $[M+H-H_2O]^+$ peak at m/z 151.1 as the most dominant peak³⁹⁵. These peaks were chosen and examined on the LimOOH spectra obtained from the ToF-SIMS analysis. The mass spectrum peaks and distribution of ions within the skin sample are compared to the Vaseline treated skin samples. Differences in the mass spectra were identified in both the m/z 169.1 peak and the m/z 151.1 peak, with both present in the hydroperoxides of limonene treated skin sample at higher intensities than in the Vaseline control treated sample. Examination of the ToF-SIMS images showed that both ions in the LimOOH treated skin samples appeared to accumulate in the SC [Figure 5.18(a) and 5.18(c)]. These differences in both the mass spectra and the ToF-SIMS images indicate the ions at m/z 169.1 and 151.1 in the LimOOH treated skin samples are representative of LimOOH fragments.

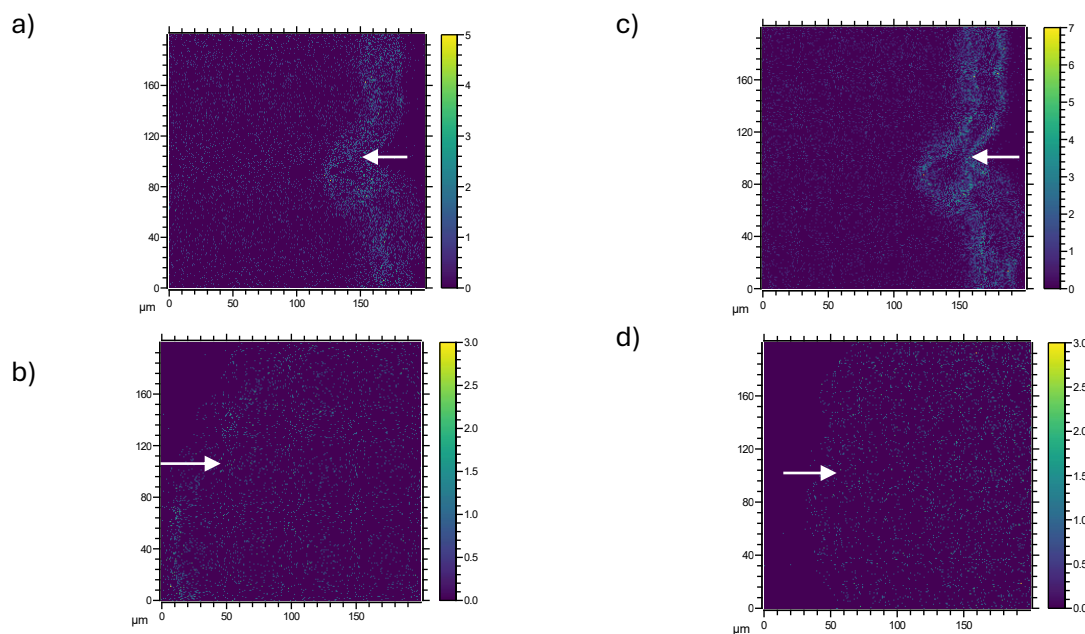


Figure 5.18 ToF-SIMS data of the epidermis of human ex vivo skin cross section (200 × 200 μm). $C_{10}H_{17}O_2^+ (M+H)^+$ ion at m/z 169.1 in (a) skin treated with hydroperoxides of limonene (0.3%) and (b) Vaseline treated skin for 24 hours. $C_{10}H_{15}O^+ (M+H-H_2O)$ ion at m/z 151.1 in (c) skin treated with hydroperoxides of limonene (0.3%) and (d) Vaseline treated skin for 24 hours. Experiment was carried out with $n=2$ patients, with four replicates in each hydroperoxides of limonene treated sample and three replicates in the Vaseline treated samples. Arrows indicate direction of penetration.

A similar approach was taken to identify LinOOH ion fragments in hydroperoxides of linalool 1.0% treated skin samples. LC/ESI-MS/MS of LinOOH identified the $[M+H]^+$ peak at m/z 187.2, the $[M+H-H_2O]^+$ peak at m/z 169.1 and the $[M+H-3H_2O-CH_2=C=CH_2]^+$ peak at m/z 93.0³⁹⁶. The m/z 187.2 and 169.1 peaks were identified in the spectrum of the hydroperoxides of linalool 1.0% treated skin samples and compared with the corresponding peaks in the spectrum for the Vaseline treated skin sample. The ToF-SIMS images of the ions in both samples were also compared (Figure 5.19). The images showed that there were differences in the distribution of the ions at m/z 187.2 and m/z 169.1 in the skin samples compared to the Vaseline treated control samples. The differences seen in distribution of the ions indicate that the ions at m/z 187.2 and m/z 169.1 in the hydroperoxides of linalool 1.0% treated skin samples correlate to LinOOH fragments.

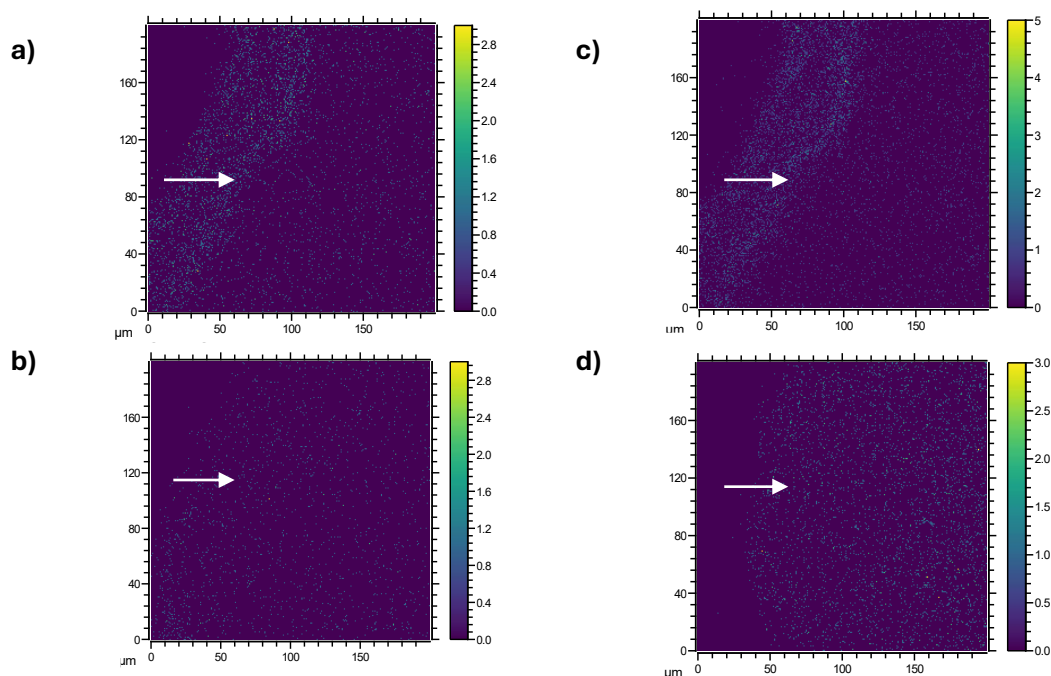


Figure 5.19 ToF-SIMS data of the epidermal region of human *ex vivo* skin cross section ($200 \times 200 \mu\text{m}$). $\text{C}_{10}\text{H}_{19}\text{O}_3^+$ ($\text{M}+\text{H}$) $^+$ ion at m/z 187.2 in (a) skin treated with hydroperoxides of linalool (1.0%) and (b) Vaseline treated skin for 24 hours. $\text{C}_{10}\text{H}_{17}\text{O}_2^+$ ($\text{M}+\text{H}-\text{H}_2\text{O}$) $^+$ ion at m/z 169.1 in (c) skin treated with hydroperoxides of linalool (1.0%) and (d) Vaseline treated skin for 24 hours. The experiment was carried out with $n=2$ patients, with four replicates in each hydroperoxides of linalool treated sample and three replicates in the Vaseline treated samples. Arrows indicate direction of penetration.

5.4.2.1 Distribution of limonene hydroperoxide and linalool hydroperoxide in skin

Following identification of LimOOH and LinOOH ions, the distribution of the allergens in the skin samples were assessed. To indicate the positions of the SC and the VE, lipid ions known to have primary residence in these areas were used. Cholesterol, at m/z 369, was found primarily in the SC³⁹⁷ and phosphatidylcholine is primarily found around keratinocytes³⁹⁸, and so the PC headgroup fragment at m/z 86 was used to identify the VE. The overlay images clearly identified the presence of clear skin structures (Figure 5.20). The PC headgroup fragment at m/z 86 (blue, Figure 5.20) illustrates the phospholipid membranes of the keratinocytes in the VE. The circular shape and location of the ion at m/z 124, surrounded by the m/z 86 (PC) ion, suggests that it represents a nuclear fragment.

Both hydroperoxides follow a similar pattern regarding penetration through the skin, primarily confined to the SC with some penetration into the upper layers of the VE. Line scans of cholesterol were used to identify the SC, which was present at a depth of 10 – 60

μm into the skin sample. Line scans of PC were used to identify the VE, which was present at 50 – 200 μm into the skin samples. Line scans measuring ion intensity through the 200 μm depth of the skin sample confirmed where LinOOH and LimOOH were at their highest intensity [Figure 5.20(b) and 5.20(d)]. The LinOOH ion fragment (m/z 187.2) was at its highest intensity at a depth of 20-80 μm from the skin surface, which represents the SC which is usually around 10 -20 μm in thickness in normal healthy skin³⁹⁹ [Figure 5.20(b)]. Analysis of the PC headgroup fragment which was used as a marker for the VE [Figure 5.20(e)], showed that the VE is positioned at 60-70 μm into the skin sample. Comparisons with the linescan for the LinOOH fragment showed some overlap beginning at 60 μm into the skin sample, indicating that there is some penetration of LinOOH into the upper layers of the VE, with the intensity then decreasing further into the VE. Similarly, the LimOOH ion fragment (m/z 151.1) was at the highest intensity between 30-50 μm into the skin sample, indicating the localisation of the ion to the SC [Figure 5.20(d)]. The intensity of the LimOOH ion decreases between 50-80 μm into the skin samples. The VE of the sample begins at 50 μm into the sample as indicated by the linescan for the PC headgroup ion [Figure 5.20(f)], which indicated that LimOOH was penetrating into the upper layers of the VE. The images of the hydroperoxide ions in the skin coupled with the linescans confirmed that, upon application to the surface of the skin in a patch test preparation, LinOOH and LimOOH remain primarily in the SC, with penetration into the upper layers of the VE

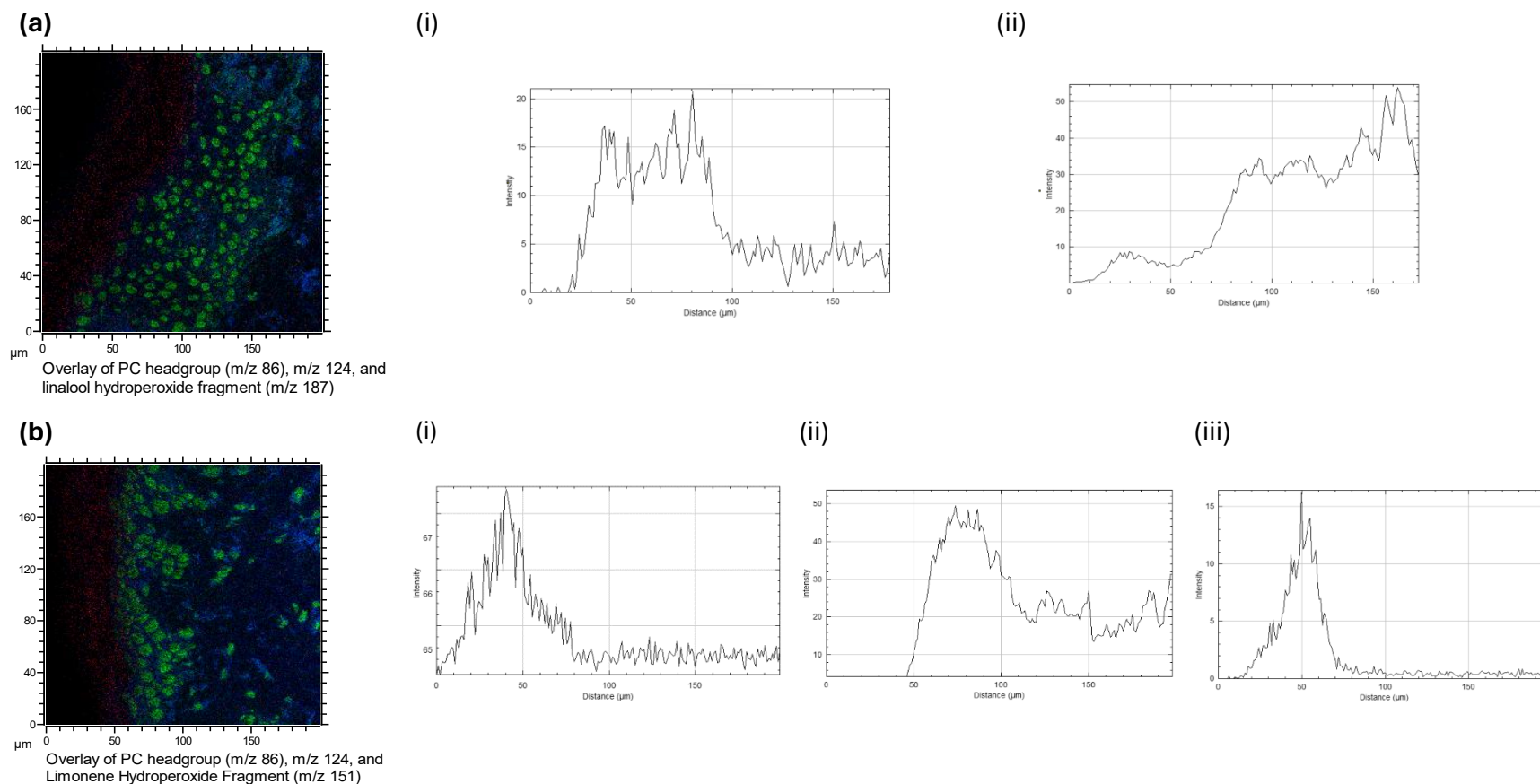


Figure 5.20 Overlay Images and Linescans of LimOOH and LinOOH treated skin samples. **(a)** ToF-SIMS overlay images (200 x 200 μm) of ex vivo skin samples treated with hydroperoxides of linalool 1.0%. Red = LinOOH fragment at m/z 187, green = nuclear fragment at m/z 124, blue = phosphatidylcholine (PC) headgroup at m/z 86. White arrow indicates direction of penetration. **[a(i)]** Line scan of LinOOH fragment ion at m/z 187.2 showing intensity across the skin sample. **[a(ii)]** Line scan of PC headgroup ion at m/z 86 in ex vivo skin sample treated with hydroperoxides of linalool. **(b)** ToF-SIMS overlay images (200 x 200 μm) of ex vivo skin samples treated with hydroperoxides of limonene 0.3%. Red = LimOOH fragment at m/z 151.1, green = nuclear fragment at m/z 124, blue = phosphatidylcholine (PC) headgroup at m/z 86. **[b(i)]** Line scan of LimOOH fragment ion at m/z 151.1 showing intensity across the skin sample. **[b(ii)]** Line scan of PC headgroup ion at m/z 86 in ex vivo skin sample treated with hydroperoxides of limonene. **[b(iii)]** Line scan of cholesterol ion at m/z 369 in ex vivo skin sample treated with hydroperoxides of limonene. Images are representative of n=2 donors with four sections of skin analysed in each patient for LimOOH and LinOOH.

5.4.2.2 Skin lipid changes induced by hydroperoxides of limonene and hydroperoxides of linalool

Lipid composition varies significantly throughout different layers of the skin, and thus to investigate the effect of hydroperoxides on the skin lipid composition, the SC and VE were analysed separately. The cholesterol fragment at m/z 369 was used as a marker for the SC, and the PC headgroup fragment at m/z 86 was used to identify the VE. To firstly identify whether there was any variability in the chemical composition of the SC of skin treated with hydroperoxides of linalool (1.0%), hydroperoxides of limonene (0.3%), and Vaseline, partial least squares discriminant analysis (PLS-DA) was carried out (Figure 5.21). Unsurprisingly, given the penetration depth of the hydroperoxides discussed above, more prominent lipid changes were seen in the SC rather than the VE, using PCA. Skin samples analysed by PLS-DA showed clusters based on treatment, with the hydroperoxides of limonene 0.3% treated samples grouped away from the hydroperoxides of linalool 1.0% treated samples, and both sets of fragrance treated samples grouped away from the Vaseline control treated samples (Figure 5.21). The clustering of samples away from each other, without any overlap, indicates that there are differences in the chemical composition of the SC in the three classes of samples. PLS-DA has been previously used to analyze the effects of metal allergens on skin chemical composition, which indicated differences in the composition of the SC upon treatment with the metal allergens nickel, cobalt, and chromium³⁹¹. Our analysis indicated that lipid changes occurred in the SC upon exposure to patch test preparations of hydroperoxides of limonene and linalool.

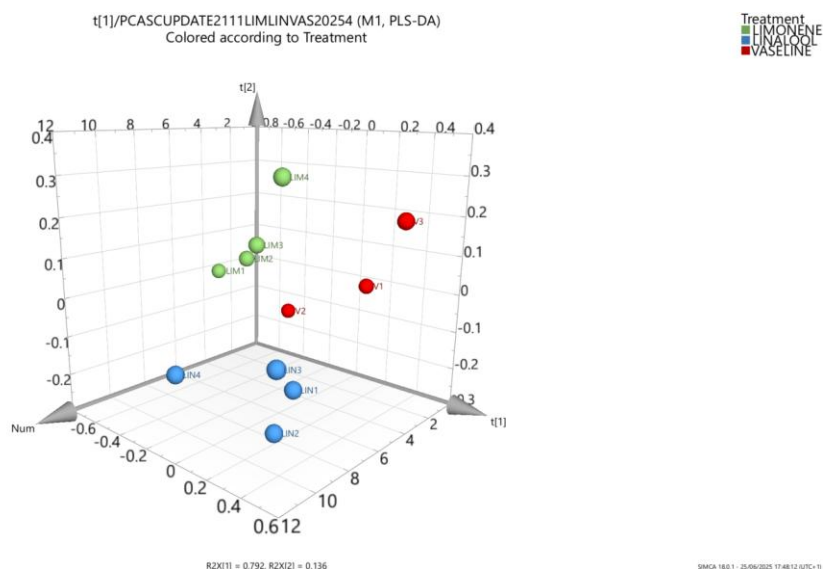


Figure 5.21 Partial least squares discriminant analysis (PLS-DA) of SC composition of Vaseline (red), hydroperoxides of linalool 1.0% (blue) and hydroperoxides of limonene 0.3% (green) treated ex-vivo human skin. Data was extracted and analyzed as normalized intensities to total ion count. Range from m/z 50 – m/z 605 analysed.

The same analysis did not show clustering by treatment when the VE was analysed, indicating that the lipids of the VE were unaffected by treatment with hydroperoxides of limonene and linalool (Figure 5.22). Following this, specific lipids were studied in more detail.

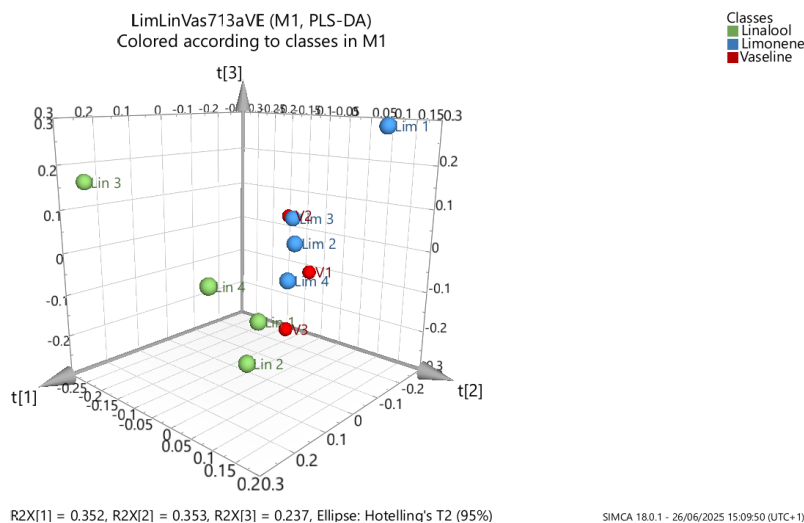
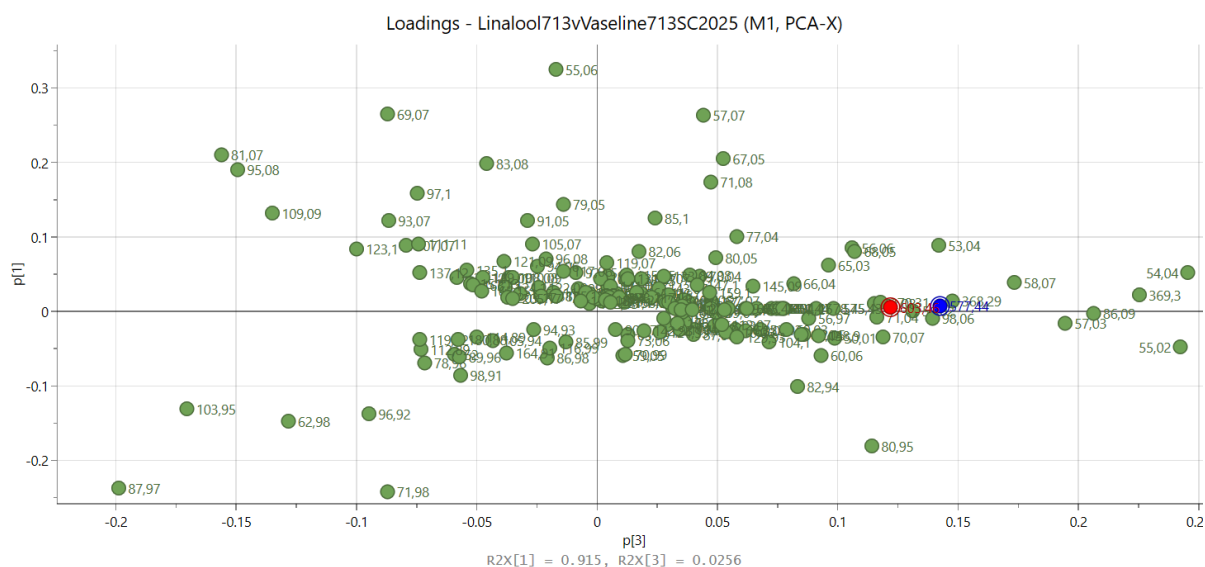


Figure 5.22 Partial least squares discriminant analysis (PLS-DA) of VE composition of Vaseline (red), hydroperoxides of linalool 1.0% (green) and hydroperoxides of limonene 0.3% (blue) treated ex-vivo human skin. Data was extracted and analyzed as normalized intensities to total ion count. m/z 50 – m/z 605

A PCA scores plot [Appendix II, Figure A2.1 (a)] identified differences between the chemical composition of the SC of the Vaseline treated skin samples and hydroperoxides of linalool 1.0% treated skin samples. The blue LinOOH samples are clustered together and away from the green Vaseline control samples. Component 2 successfully separated the LinOOH samples from the control samples, indicating differences in the chemical make-up of the SC upon treatment with LinOOH. PCA analysis was also carried out to identify any variation in the composition of Vaseline exposed skin and hydroperoxides of limonene 0.3% treated skin [Appendix 2. Figure A2.1(b)]. As with treatment with hydroperoxides of linalool 1.0%, more prominent lipid changes were observed in the SC in comparison to the VE. A scores plot was able to differentiate between the Vaseline treated samples in blue, and the hydroperoxides of limonene 0.3% treated samples in green. Component 2 separated the samples, and the clustering of the blue samples away from the green indicate variability in the chemical composition of the SC of the control and hydroperoxides of limonene 1.0% treated samples.

A PCA loadings plot was also generated for each treatment condition and the Vaseline controls which indicated the m/z values at which there was the most variability within the samples (Figure 5.23). The analysis indicated variability between the hydroperoxides of linalool 1.0% treated skin samples and control skin samples at m/z 603.46 and m/z 577.44 which represented two diacylglycerol (DAG) fragments, as well as in the PC headgroup fragment at m/z 86.09 and a cholesterol fragment at m/z 369.3. In the hydroperoxides of limonene 0.3% treated samples, variability was also detected for fragments at m/z 603, 577, 369 and 86.

a)



b)

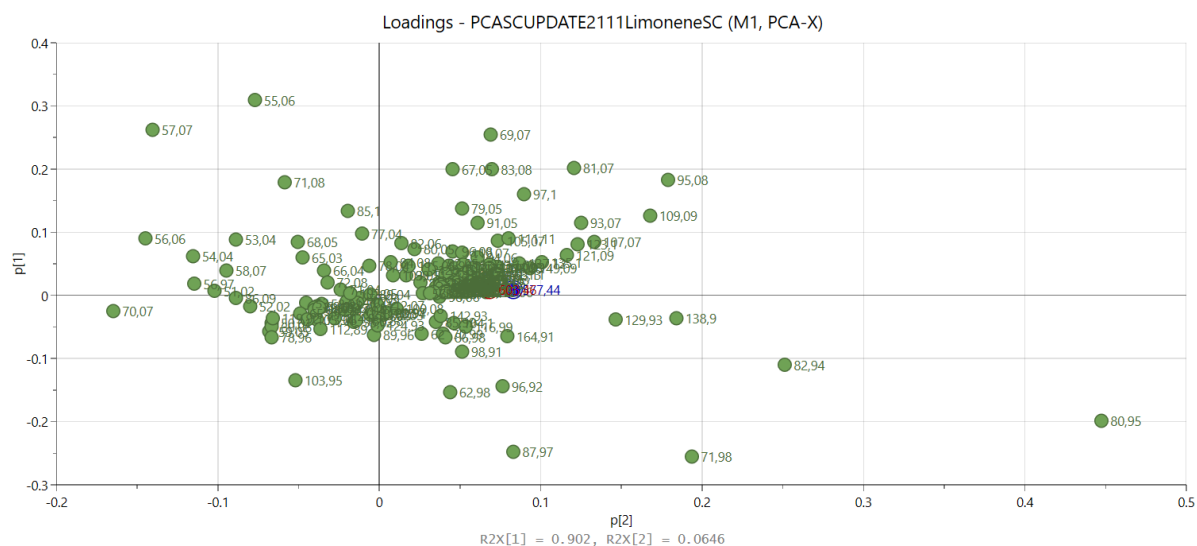


Figure 5.23 2D loadings plot of the SC of hydroperoxides of linalool 1.0% and hydroperoxides of limonene 0.3% treated ex vivo skin. (a) 2D loadings plot of the SC of hydroperoxides of linalool 1.0% treated ex vivo human skin and control (Vaseline treated) ex vivo skin. and (b) 2D loadings plot of the SC of hydroperoxides of limonene 0.3% treated ex vivo human skin and control (Vaseline treated) ex vivo skin. DAGs at m/z 577.44 and 603.46 are highlighted in blue and red respectively. Data was extracted and analyzed as normalized intensities to total ion count.

To investigate the lipid changes in more detail, a selection of ion peaks corresponding to specific lipid species were selected and analysed. The ions were selected based on indicated differences in the PCA loadings plot and additional ions of interest. The lipid species selected included PC headgroup (m/z 86 and 184), cholesterol (m/z 369), MAGs (m/z 311, 313 and 339) and DAGs (m/z 575, 577, 601 and 603). The SC for skin samples from two different patients were analysed (Figure 5.24).

There was no common trend for PCs (m/z 86 and 184) across both patients for either of the hydroperoxides [Figure 5.24(a)]. An increase in the intensity level of the cholesterol ion (m/z 369) was noted for both patients for both fragrance hydroperoxides, with a more pronounced effect for hydroperoxides of linalool-treated skin in patient 2 [Figure 5.24(b)]. The baseline cholesterol levels in Vaseline-exposed skin were notably higher in patient 2 than patient 1. The most notable effects were on MAG and DAG for both patients, particularly patient 1 for both limonene and linalool hydroperoxides, and patient 2 for linalool hydroperoxides [Figure 5.24(c) and 5.24(d)]. In patient 1, LinOOH induced a significant increase in MAG levels (m/z 313 and 339). While no significance was observed, there was an increasing trend in DAG levels in LinOOH treated samples, in comparison to control treated samples. There were significant increases in MAG (m/z 311 and 313), and DAG intensity at m/z 601. Non-significant increases in intensity were observed for all other MAG and DAG fragments. In contrast to patient 1, LinOOH induced a significant increase in PC intensity at m/z 86 and 184 in patient 2.

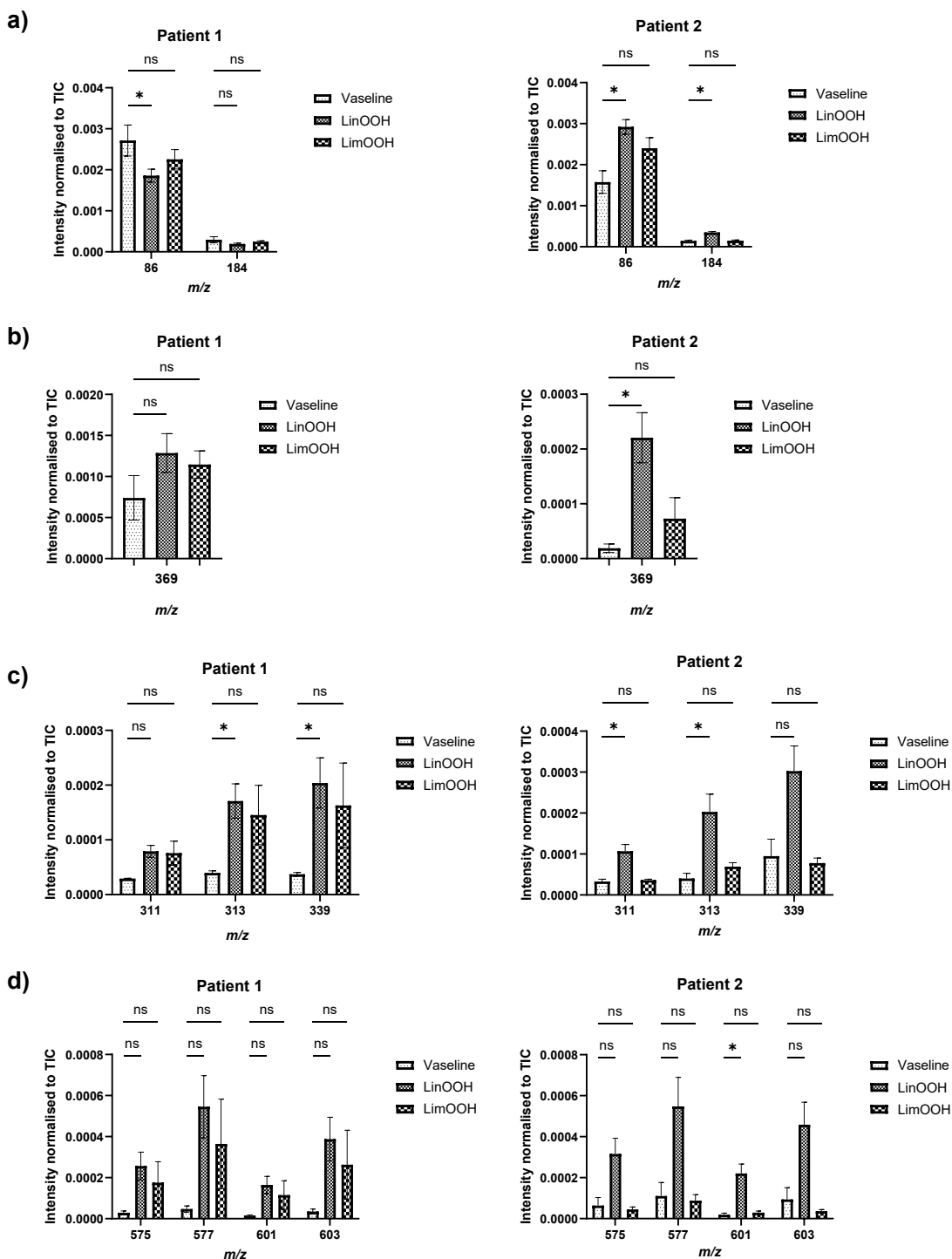


Figure 5.24 Stratum corneum (SC) lipid composition of ex vivo skin treated with hydroperoxides of linalool 1.0%, hydroperoxides of limonene 0.3% and Vaseline (control). Lipid species analysed were a) PC (m/z 86 and 184), b) cholesterol (m/z 369), c) monoacylglycerols (MAG; m/z 311, 313 and 339) and d) diacylglycerols (DAG; m/z 575, 577, 601 and 603). Data analysed by repeated measures (RM) two-way ANOVA with Tukey post-hoc test for comparison of means. Cholesterol data analysed by repeated measures one-way ANOVA with Tukey post-hoc test. * = $p < 0.05$.

PCA analysis was performed to assess the effect of hydroperoxides of limonene (0.3%)[Appendix, Figure A2.2(a)] and hydroperoxides of linalool (1.0%) [[Appendix, Figure A2.2(b)] on skin lipids in the VE. A scores plot showed an overlap of samples treated with hydroperoxides of limonene (0.3%) and samples treated with Vaseline, with no clustering of samples away from each other, which indicated that there were no differences in the chemical composition of skin samples treated with hydroperoxides of limonene (0.3%) and Vaseline treated samples. There did appear to be some clustering of hydroperoxides of linalool (1%) treated skin samples away from the Vaseline treated samples, indicating that there are differences in the chemical composition of the hydroperoxides of linalool (1%) treated samples in comparison to the Vaseline treated control skin samples.

Loadings plots showed the m/z values at which there was the most variation (Figure 5.25). Samples spread out from the cluster of samples in the centre indicated that there was variance at these m/z values. The loadings plot did not indicate that there was any variance in any of the lipid ions between the hydroperoxides of limonene (0.3%) treated samples and the Vaseline control samples, as none of the lipid ions of interest were located outside the centre cluster. However, Figure 5.25(b) indicated that there was potentially some variance in the PC headgroup fragment between the hydroperoxides of linalool (1.0%) treated skin samples and the Vaseline treated control samples with the ion at m/z 86.09 positioned outside the cluster of m/z values in the centre of the graph.

As before with SC, a selection of ion peaks corresponding to specific lipid species were selected and analysed (Figure 5.28).

The levels of phospholipids in the SC are negligible⁴⁰⁰, so a better indication of the effect of fragrance hydroperoxides on PC was obtained by looking at the VE. There was no consistent trend in PC headgroup intensity across the two patients. In patient 1, there was a decrease in the intensity of the ions at m/z 184 and m/z 86 in both hydroperoxides of linalool (1.0%), and hydroperoxides of limonene treated skin samples (0.3%). However, in patient 2 there was a significant increase in the intensity of the PC headgroup ion at m/z 184 in hydroperoxides of linalool (1.0%) treated skin in comparison to the Vaseline control treated skin [Figure 5.28(a)]. There were non-significant increases in the intensity of the PC headgroup ion at m/z 86 in both hydroperoxides of limonene (0.3%) and hydroperoxides of linalool (1.0%) treated skin in comparison to Vaseline treated skin for patient 2. Neither hydroperoxides of linalool nor limonene had a significant effect on the intensity of cholesterol ions at m/z 369 in human ex vivo skin samples, however, there did appear to be an increasing trend in cholesterol ion intensity [Figure 5.28(b)]. The lack of an effect on cholesterol was unsurprising, as cholesterol is primarily found in the SC and not present in high abundance in the VE. There appeared to be a consistent trend in MAG and DAG ion intensity across both patients, in which the intensity of MAG ions at m/z 311, 313, and 339 appeared to increase in skin samples treated with hydroperoxides of linalool (1.0%) and hydroperoxides of limonene (0.3%) in comparison to Vaseline treated samples [Figure 5.28(c)]. There were also increases in the intensity of DAG ions at m/z 575, 577, 601, and 603 in both hydroperoxide treated samples in comparison to samples treated with Vaseline [Figure 5.28(d)].

Overall, there were a lack of significant changes in the intensity the ions of different lipid species in the VE indicating that fragrance hydroperoxides did not have a significant effect on the skin lipidome in the VE. This correlates to the distribution of linalool hydroperoxide and limonene hydroperoxide ions within the skin, which showed that the ions were mainly confined to the SC of the skin, and present in a lower intensity in the upper layers of the VE.

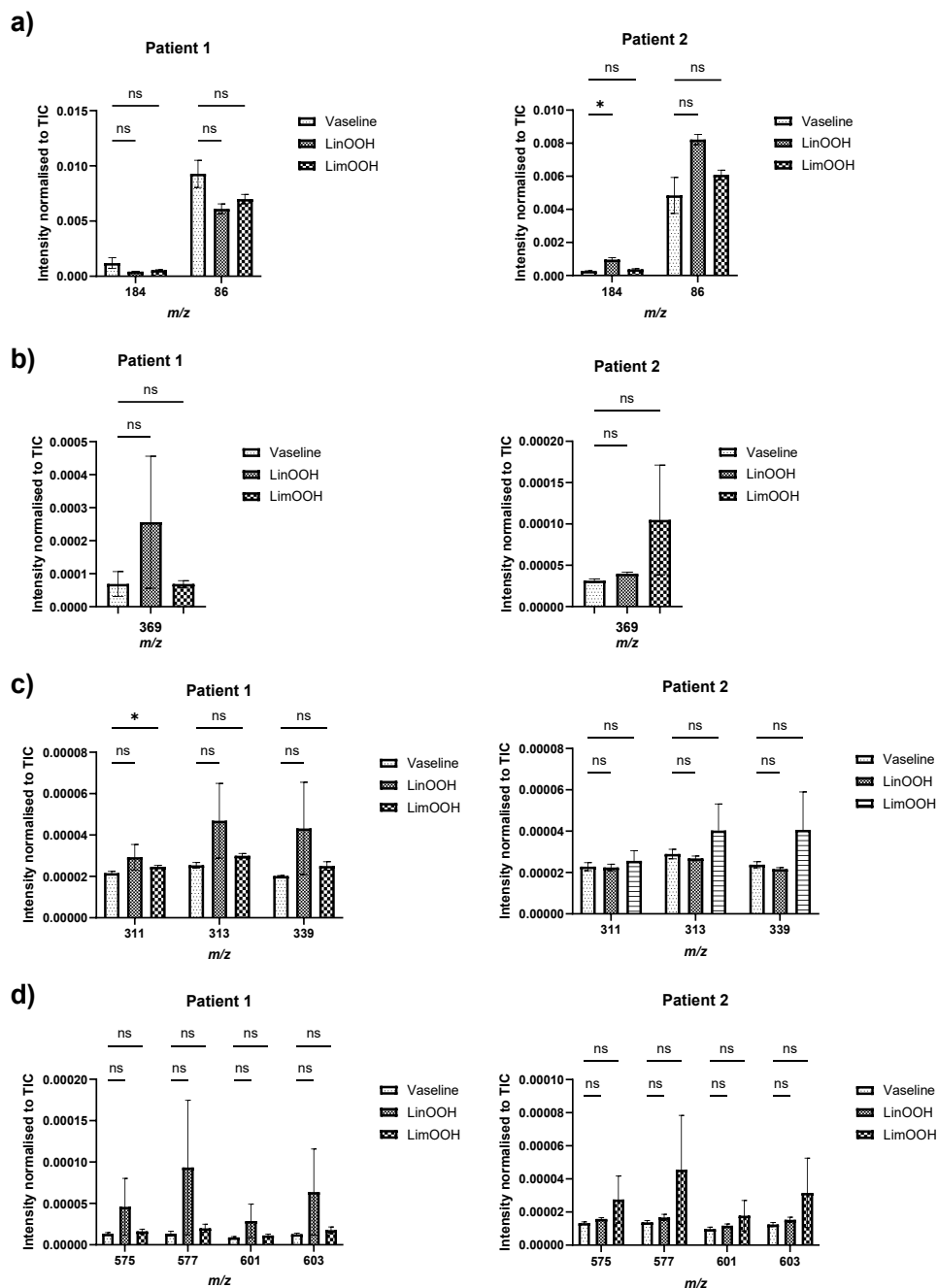


Figure 5.26 Viable Epidermis (VE) lipid composition of ex vivo skin from two persons treated with hydroperoxides of linalool 1.0%, hydroperoxides of limonene 0.3% and Vaseline (control). Lipid species analysed were a) PC (m/z 86 and 184), b) cholesterol (m/z 369), c) monoacylglycerols (MAG; m/z 311, 313 and 339) and d) diacylglycerols (DAG; m/z 575, 577, 601 and 603). Four sections of the skin were analysed via ToF-SIMS for each patient. Data analysed by repeated measures (RM) two-way ANOVA with Tukey post-hoc test for comparison of means. Cholesterol data analysed by repeated measures one-way ANOVA with Tukey post-hoc test. * = $p < 0.05$.

LimOOH and LinOOH were both found mainly confined to the SC of ex vivo human skin following ToF-SIMS analysis. The localisation in the SC and poor penetration in the VE correlates to previous findings that documented poor skin absorption and low penetration potential of the related fragrance terpenes geraniol and citronellol⁴⁰¹. An additional study investigating the penetration of terpenes from matrix-type transdermal systems concluded that terpenes including limonene have a high affinity to the SC⁴⁰². It is also possible that LimOOH and LinOOH could bind to endogenous biomolecules, e.g. proteins, in the ex vivo skin model^{378, 403}. This would form a conjugate with a higher molecular weight that we have not searched for. Additionally, fragrance hydroperoxides could be metabolised upon absorption into the skin, as metabolic enzymes may still be functioning, leading to metabolites that were not detected. The site of the skin sample, from the abdomen, and interpatient variability also need to be considered as factors that contribute to absorption and skin lipid profile. Previous studies have concluded that the function and composition of the SC differs with location⁴⁰⁴, for example the SC is thicker in facial skin than in the limbs. The lipid composition of the SC also differs with age⁴⁰⁵, with ToF-SIMS analysis identifying an increase in sterol cholesterol sulphate levels in the SC of elderly women in comparison to younger women.

Changes in skin lipids were observed after treatment with hydroperoxides of linalool 1.0% and hydroperoxides of limonene 0.3%. The upregulation of DAG and MAG levels, and decrease in PC levels observed in hydroperoxides of linalool 1.0% treated skin in comparison to control treated skin are consistent with previously reported findings, which used a lipidomic approach to investigate the changes in the keratinocyte lipidome upon treatment with fragrance allergens including linalool-6/7-hydroperoxide⁴⁰⁶. Decreases in phospholipid intensity indicates degradation, potentially through hydroperoxide induced oxidative stress. Triacylglycerols (TAG) are crucial for the maintenance of the skin permeability barrier function⁴⁰⁷, so increases in MAG and DAG intensity could be a response to fragrance hydroperoxide induced SC disruption, and an attempt to restore skin homeostasis. Notably in ToF-SIMS, DAG ions may represent TAG, as TAG is effectively fragmented in ToF-SIMS yielding mainly DAG ions³⁹³. LimOOH did not induce any significant

differences in lipid composition of the SC, but there were decreasing trends in PC intensity seen at both m/z values, and an increasing trend in the intensity of cholesterol, MAG and DAG in comparison to the control treated skin samples. Increases in DAG were previously noted in the SC of chromium-treated human skin samples (from breast tissue that had been previously frozen), whilst MAG were increased in the SC of both chromium- and cobalt-treated skin³⁹¹. While lipid changes were observed in preservative and fragrance hydroperoxide treated skin, it is important to note that ToF-SIMS is only a semi quantitative technique, and quantitative analysis has been previously reported to be difficult using this technique⁴⁰⁸.

This finding has implications for the pathophysiology of LimOOH and LinOOH induced ACD and suggests that the penetration of only minimal amounts of the allergen is required to cause sensitization. Alternatively, it raises the possibility of changes taking place within the SC that contribute to the pathophysiology of ACD. These mechanisms have been documented in other skin diseases like atopic dermatitis, in which there is an increase in T_H2 inflammation and the secretion of IL-4 which affects the integrity of the SC barrier⁴⁰⁹. Additionally, the compromise of the SC in psoriasis leads to activation of innate immune responses which includes the release of cytokines like TNF and IL-1⁴¹⁰. The role of the SC in the pathophysiology of ACD is still to be fully established, however it has been hypothesised that skin barrier damage may promote activation of innate immunity signals crucial for the development of sensitization⁴¹¹.

These findings are clinically relevant, as the study applied patch test preparations of hydroperoxides to viable ex vivo skin samples in a manner similar to how patch tests are performed on patients during ACD diagnosis. However, since patch test preparations also contain additional chemicals that were not analysed in this study, their potential contribution to the lipid changes observed in the skin samples cannot be excluded.

5.5 Conclusions of Chapter

ToF-SIMS was found to be effective at analysing the penetration of contact allergens within the skin. Metal allergens have been previously analysed using ToF-SIMS; however, this study is the first use of ToF-SIMS to analyse small organic allergens. While MI and fragrance allergen treated skin samples were chosen for analysis using ToF-SIMS, the use of more classical, direct acting haptens like DNCB would have made the analysis simpler as they provide higher predictability of the nature and site of protein binding, in comparison to fragrance hydroperoxides which first require oxidation and radical formation to form protein adducts. The same concept could potentially be applied to skin lipid analysis. This would simplify interpretation of the mass spectra obtained as the m/z ratios of expected fragments are better known.

ToF-SIMS system 2, which included sputtering with an Ar-GCIB, removed the top layer of the sample and resulted in higher clarity images, with clear skin structures observed. MI was seen to penetrate through the SC and was localized in both the SC and VE. Both limonene hydroperoxide and linalool hydroperoxide appeared to be confined to the SC, but comparison to a linescan of the PC headgroup ion at m/z 86 (a marker for the VE) showed that there was some penetration from both fragrance allergens into the upper layers of the VE.

The effect of preservative and fragrance allergen treatment on the skin lipidome was also investigated. PCA analysis showed that there were differences in the chemical composition of both the SC and VE of MI treated skin samples in comparison to Vaseline treated samples. There were significant decreases in the intensity of cholesterol, MAG and DAG ions in the SC of MI treated skin samples, and an increase in the intensity of the PC headgroup ion at m/z 86 in the VE.

PCA analysis indicated differences in the chemical composition of the SC in both hydroperoxides of linalool (1.0%) and hydroperoxides of limonene (0.3%) treated skin samples in comparison to the Vaseline treated samples, however there were no changes in the chemical composition of the VE indicated. Significant increases in the intensity of PC

headgroup, cholesterol, MAG and DAG ions were observed in the SC of skin samples treated with hydroperoxides of linalool (1.0%). There were no significant changes observed in the lipid ion fragments in the SC of hydroperoxides of limonene (0.3%) treated skin samples.

The lipid changes seen here after treatment of skin samples with fragrance hydroperoxides correlate with other previously published research into the lipid changes in keratinocytes treated with the same fragrance hydroperoxides⁴⁰⁶, however more research is needed to fully quantify the effects of MI, LimOOH and LinOOH treatment on skin lipids. Additionally, changes in lipid composition have been previously implicated in the pathophysiology of multiple skin conditions including acne, atopic dermatitis, and psoriasis⁴¹²⁻⁴¹⁴.

Chapter 6: Conclusions and Future Work

6.1 Concluding Remarks

ACD is a hypersensitivity reaction affecting up to 20% of the general population with the potential of significant impact on quality of life. 5% –10% of people experience clinical symptoms at least once a year. Over 4000 contact allergens have been identified, including nickel which is the most common contact allergen. Nickel allergy affects approximately 17.5% of the general population. While the role of proteins in the pathophysiology of ACD has been well characterized, the role of lipids remains poorly understood. Given the abundance of lipids in the skin, their chemical reactivity, and their involvement in other pro-inflammatory skin conditions, they are likely to play a critical role in ACD.

The overarching aim of this thesis was to investigate the contribution of lipids to the pathogenesis of metal-induced ACD. Specifically, the work sought to determine whether metals can induce lipid peroxidation in a keratinocyte model, to assess whether this process contributes to the extensive cytotoxicity observed at clinically relevant doses, and to further elucidate the roles of CD1a and CD1d in metal allergy. In addition, this thesis explored the ability of small organic molecules to penetrate the skin and examined their effects on the skin lipidome.

The ability of NiSO_4 , CoCl_2 , and $\text{K}_2\text{Cr}_2\text{O}_7$ to induce lipid peroxidation was examined using two complementary models: a single-lipid linoleic acid system and a keratinocyte cell culture model assessed with the C11-BODIPY assay. In the linoleic acid model, all three metals induced lipid peroxidation at their respective patch test concentrations. In keratinocytes, $\text{K}_2\text{Cr}_2\text{O}_7$ caused significant lipid peroxidation and reactive oxygen species (ROS) generation, as measured by the H_2DCFDA assay. CoCl_2 induced lipid peroxidation only at the highest concentrations tested but promoted ROS production across all concentrations. In contrast, NiSO_4 did not induce either lipid peroxidation or ROS production in the keratinocyte model. Notably, exposure to these metals also led to reduced cell viability, raising the important question of whether lipid peroxidation contributes directly to cell death or instead represents a downstream consequence of it.

To investigate the mechanism of cell death induced by metal allergens, Annexin V/PI staining was performed. This revealed that metal-induced cell death bypassed the early apoptotic stage and progressed directly to late apoptosis/necrosis, suggesting a predominantly necrotic mechanism at the concentrations tested. Consistently, cell cycle analysis using PI staining showed no sub-G₁ peak, which is typically associated with apoptotic DNA fragmentation. To assess potential ferroptosis involvement, the ferroptosis inhibitors DFO and Fer-1 were tested. Neither rescued HaCaT cells from metal-induced death; however, Fer-1 also failed to protect cells from *t*-BuOOH, a known ferroptosis inducer, limiting interpretation. The lack of rescue by DFO suggests that metals did not trigger ferroptosis, although further studies—such as assessing GPX4 and GSH levels—are required to confirm this definitively.

The role of CD1a and CD1d lipid antigen presentation in metal allergy was next investigated using tetramer binding and functional assays with both metals and lipid peroxidation products. NiSO₄-loaded CD1a and CD1d tetramers recognized significantly more T cells in PBMCs from nickel-allergic patients compared with unloaded controls. NiSO₄ increased the frequency of IFN- γ , IL-2, and TNF-positive T cells, indicating that CD1a- and CD1d-mediated pathways contribute to the pathophysiology of nickel allergy. In contrast, CoCl₂ and K₂Cr₂O₇ did not increase T cell recognition or cytokine responses in a CD1a- or CD1d-dependent manner. A limitation of this study, however, is that PBMCs were only obtained from nickel-allergic patients, leaving open the possibility that CoCl₂ or K₂Cr₂O₇ might activate CD1 pathways in patients with those respective allergies. Lipid peroxidation products generally did not enhance CD1a or CD1d recognition, although MDA did increase IFN- γ and IL-2 responses in a CD1a-dependent manner. Overall, these findings suggest that CD1a and CD1d contribute to nickel sensitization and that lipid peroxidation products, such as MDA, may also serve as CD1-presented antigens.

Finally, the penetration of low-molecular-weight organic allergens and their impact on skin lipids were examined, extending previous work on metals. ToF-SIMS analysis of ex vivo skin revealed that MI penetrated through the SC into the VE, accompanied by significant reductions in cholesterol, MAG, and DAG levels within the SC. In contrast, LimOOH and

LinOOH primarily accumulated in the SC, with limited penetration into the upper VE. Both fragrance allergens altered SC composition compared with Vaseline-treated controls. Specifically, LinOOH significantly increased cholesterol, MAG, and DAG levels in the SC, and elevated phosphatidylcholine (PC) levels in the VE. While ToF-SIMS effectively tracked allergen penetration and lipid alterations, its semi-quantitative nature limits the strength of conclusions drawn.

Overall, this thesis demonstrated that metals impact skin lipids through lipid peroxidation and that lipid antigen presentation molecules contribute to T cell recognition and activation in metal allergy. Together, these findings provide a foundation for future studies into the role of lipids in the pathophysiology of metal-induced ACD. A summary of the key elements and findings is presented in Figure 6.1.

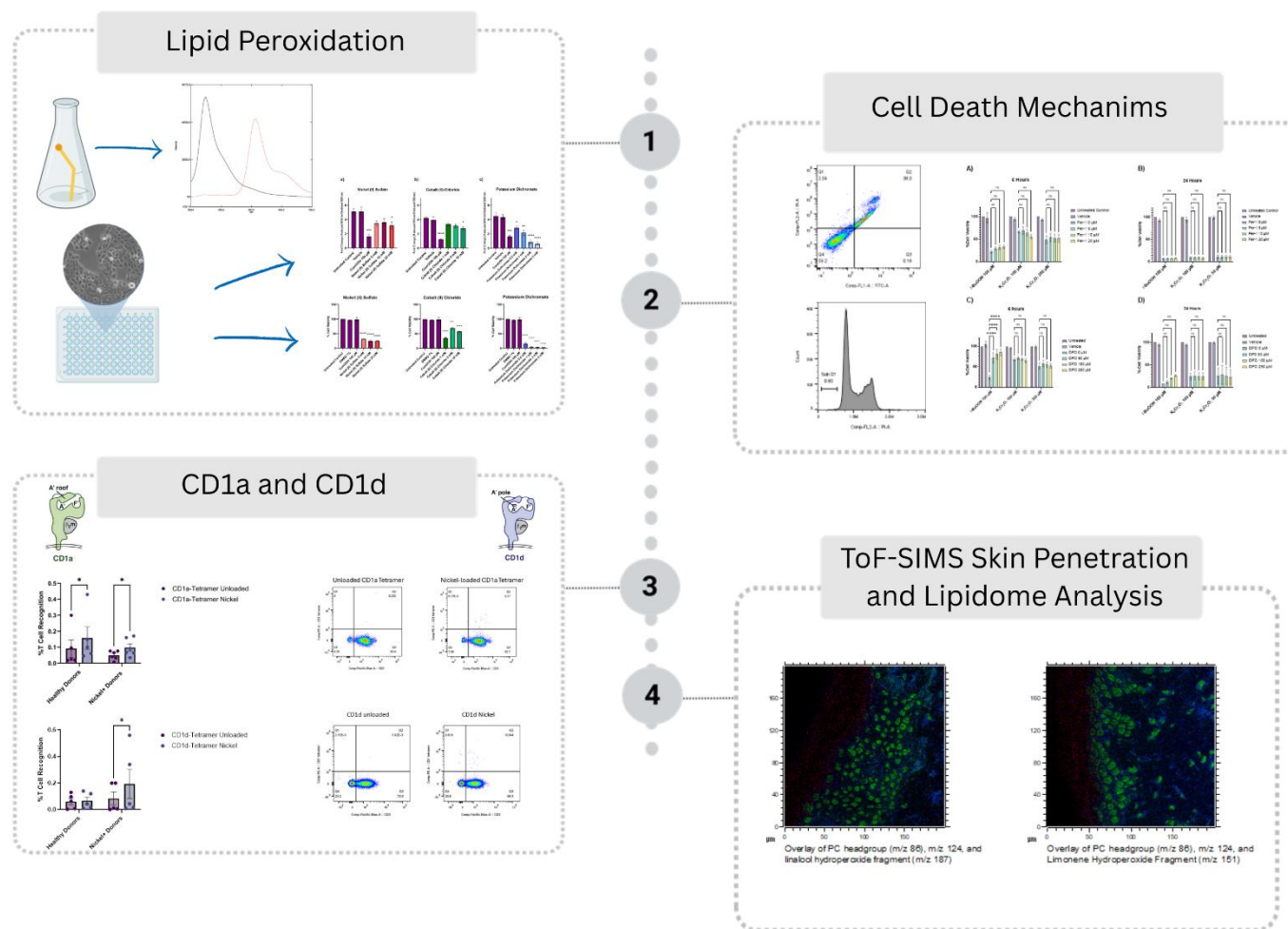


Figure 6.1 Concept Diagram of Main Processes and Findings in the Thesis. The first stage of the thesis found that metals induced lipid peroxidation but also had a significant effect on cell viability. Studies to characterise the mechanism of cell death identified that the cell death was necrotic in nature, but could not be confirmed as lipid peroxidation driven ferroptosis (stage 2). Immunological studies found that nickel recognizes and activates T cells from nickel allergic patients in a CD1a and CD1d dependent manner (stage 3). Finally, ToF-SIMS analysis produced high quality images which showed that MI and fragrance allergens accumulate in the SC and can penetrate into the VE, and that they have effects on the lipid composition of the SC (stage 4).

6.2 Future Directions

The research findings in this thesis have led to the identification of several promising directions for future studies.

This thesis demonstrated that metals can induce lipid peroxidation in a keratinocyte model. Building on these findings, future studies should employ lipidomics-based mass spectrometry to identify the specific lipid peroxidation products generated by different metals. Characterizing these products would enable more targeted immunological experiments and help to establish a direct link between metal-induced lipid peroxidation and the immune mechanisms underlying ACD. Additionally, assays such as C11-BODIPY or Liperfluo could be applied to ex vivo skin samples to assess metal-induced lipid peroxidation in a more clinically relevant setting.

Further studies are required to clarify the mechanisms of cell death induced by metal allergens. In particular, the potential involvement of ferroptosis warrants closer investigation. This could be addressed by measuring GPX4, GSH, and FSP1 levels using techniques such as western blotting or ELISA, as decreases in GPX4 and GSH are considered key indicators of ferroptosis by the Nomenclature Committee on Cell Death. In addition, the efficacy of ferroptosis inhibitors should be explored in greater depth. For example, re-examining the protective capacity of Fer-1 in HaCaT cells, alongside testing alternative inhibitors such as Lip-1, may help determine whether ferroptosis contributes to the observed cell death and provide stronger mechanistic insights.

While the findings of this thesis suggest that nickel can act as a CD1a- and CD1d-presented antigen, further work is required to confirm CD1a and CD1d dependency. One approach would be the isolation and expansion of CD1a- and CD1d-restricted T cells using cell sorting based on recognition of T cells by nickel-loaded tetramers, thereby generating defined T cell lines for subsequent functional studies. These cell lines could then be employed in ELISA-based cytokine assays to comprehensively examine the capacity of nickel to induce cytokine production in a CD1a- or CD1d-dependent manner, with the inclusion of blocking

antibodies serving to confirm pathway specificity. Future studies should also investigate the molecular basis of nickel's interaction with CD1 proteins. For example, nickel may directly complex with CD1 molecules or alternatively modulate endogenous lipid presentation in a manner that indirectly enables TCR engagement. Mass spectrometry could be used to determine whether nickel treatment promotes lipid displacement from CD1 binding grooves, supporting a mechanism akin to the "absence of interference" model in which nickel buries within the pocket and exposes previously occluded epitopes. Complementary structural approaches, such as crystallography, would provide direct insight into the binding mode of nickel with CD1a and CD1d. Once the mechanism of interaction is defined, surface plasmon resonance (SPR) could be employed to quantify the binding affinity between nickel-loaded CD1a/CD1d and the TCR. Together, these studies would not only confirm the molecular basis of nickel recognition but also help establish whether CD1a and CD1d represent viable therapeutic targets in ACD.

A lipidomics study using liquid chromatography–mass spectrometry (LC–MS) could be undertaken to more precisely quantify the effects of MI and fragrance hydroperoxides on *ex vivo* skin tissue. This approach could involve tape stripping of treated skin samples to isolate keratinocytes from the SC, or the use of skin biopsies, followed by microdissection to obtain keratinocytes from the VE. Lipids extracted from these compartments could then be subjected to LC–MS–based lipidomic profiling, which offers superior quantitative power compared to ToF-SIMS. This analysis would provide a more definitive assessment of the lipid changes observed in this thesis and clarify their biological significance.

Appendix I

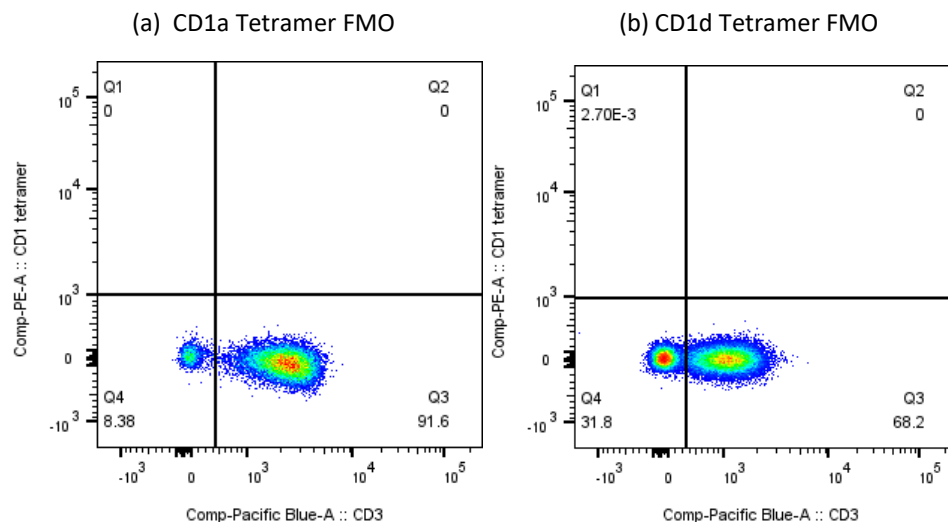


Figure A.1 FMO Control for CD1 tetramers 5×10^6 PBMC were stained with PBMC are also stained with Pacific Blue conjugated anti-CD3 antibodies to identify T cells, but cells were not stained with PE-conjugated tetramer. (a) CD1a tetramer FMO control, (b) CD1d tetramer FMO control

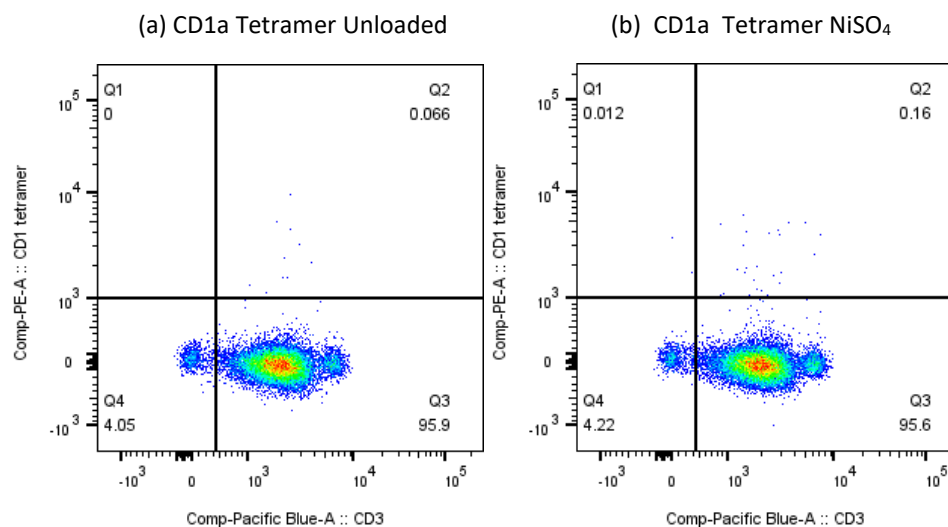


Figure A.2 Additional Flow Cytometry Plot of T cell recognition by NiSO₄ loaded CD1a tetramer in a Nickel+ Patient. (a) Cells stained with unloaded CD1a tetramer, (b) cells stained with nickel loaded CD1a tetramer

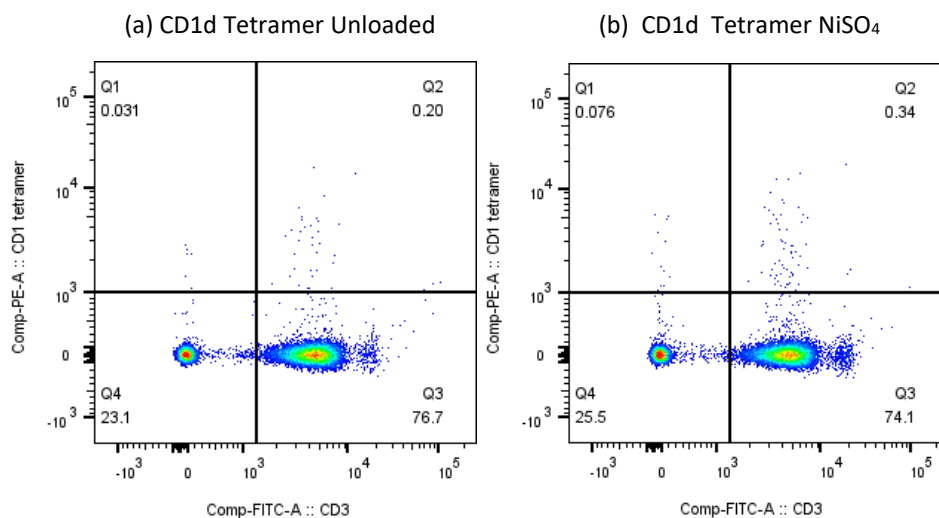


Figure A.3 Additional Flow Cytometry Plot of T cell recognition by NiSO₄ loaded CD1d tetramer in a Nickel+ Patient. (a) Cells stained with unloaded CD1d tetramer, (b) cells stained with nickel loaded CD1d tetramer

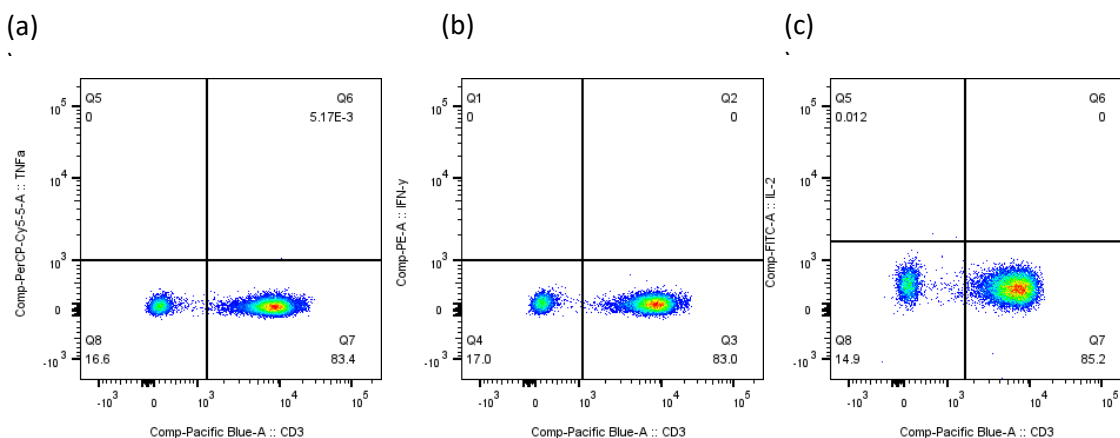


Figure A.4 FMO controls for functional assays. (a) TNF FMO control, (b) IFN- γ FMO control, (c) IL-2 FMO control

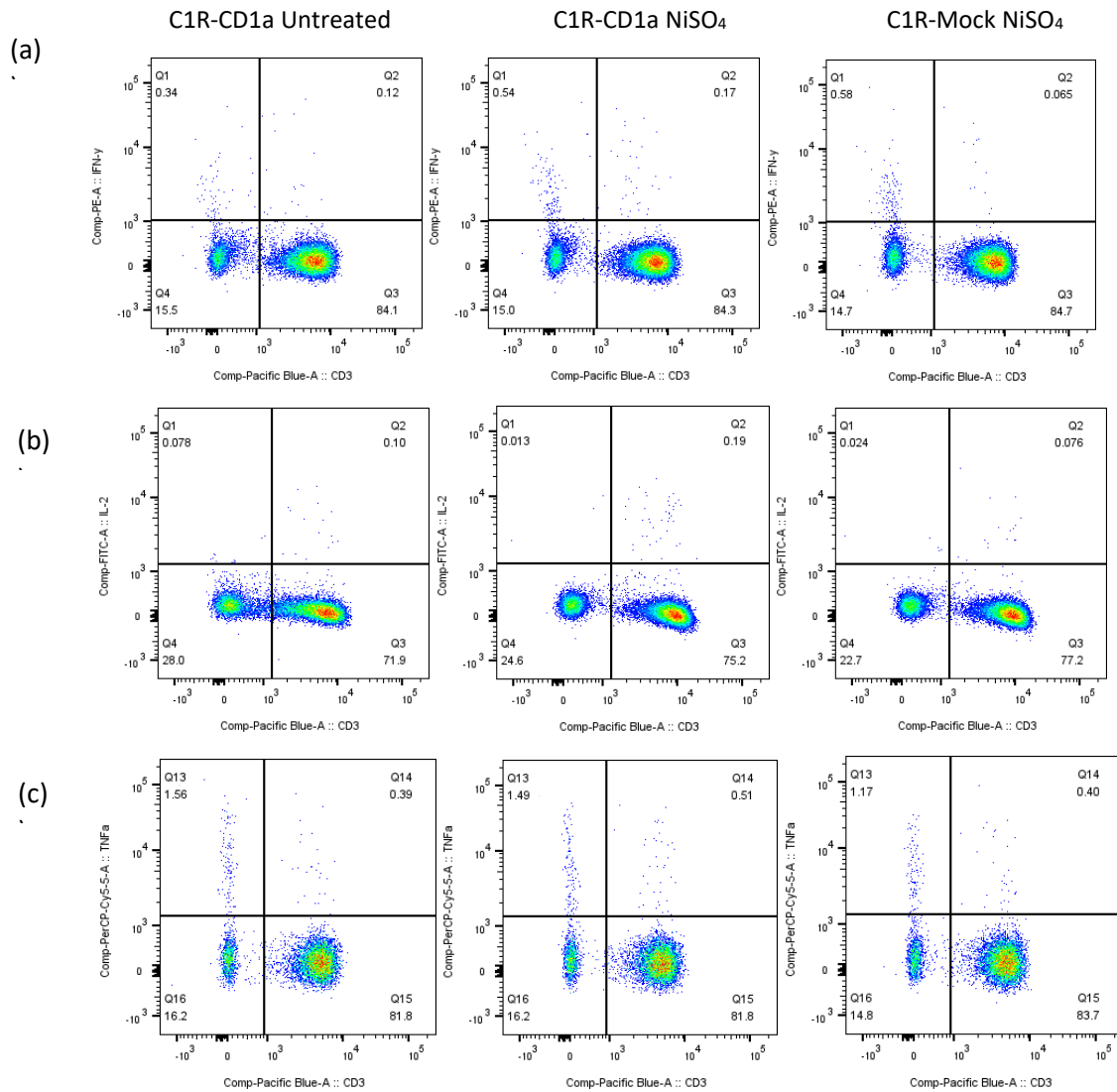


Figure A.5 Additional Replicate of Flow Cytometry Plots Showing Cytokine Production by T cells co-cultured with NiSO₄ pulsed C1R-CD1a APCs. (a) IFN-γ, (b) IL-2, (c) TNF

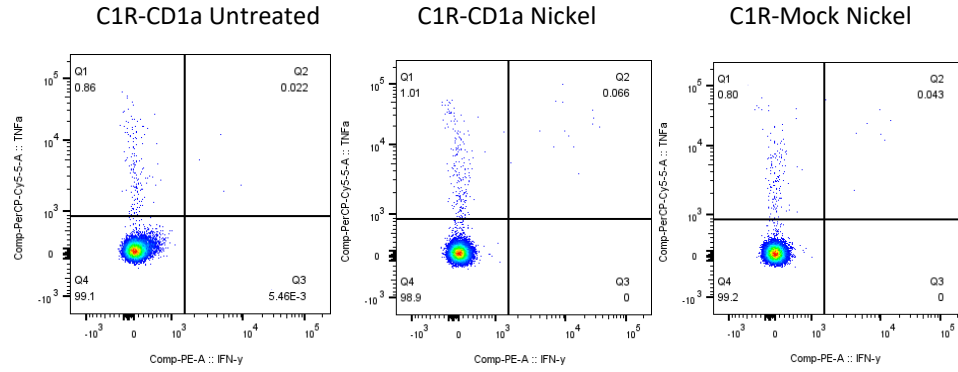


Figure A.6 Additional Replicate of IFN- γ +/TNF+ multi-functional T cells in PBMC co-cultured with NiSO₄ pulsed C1R-CD1a APCs.

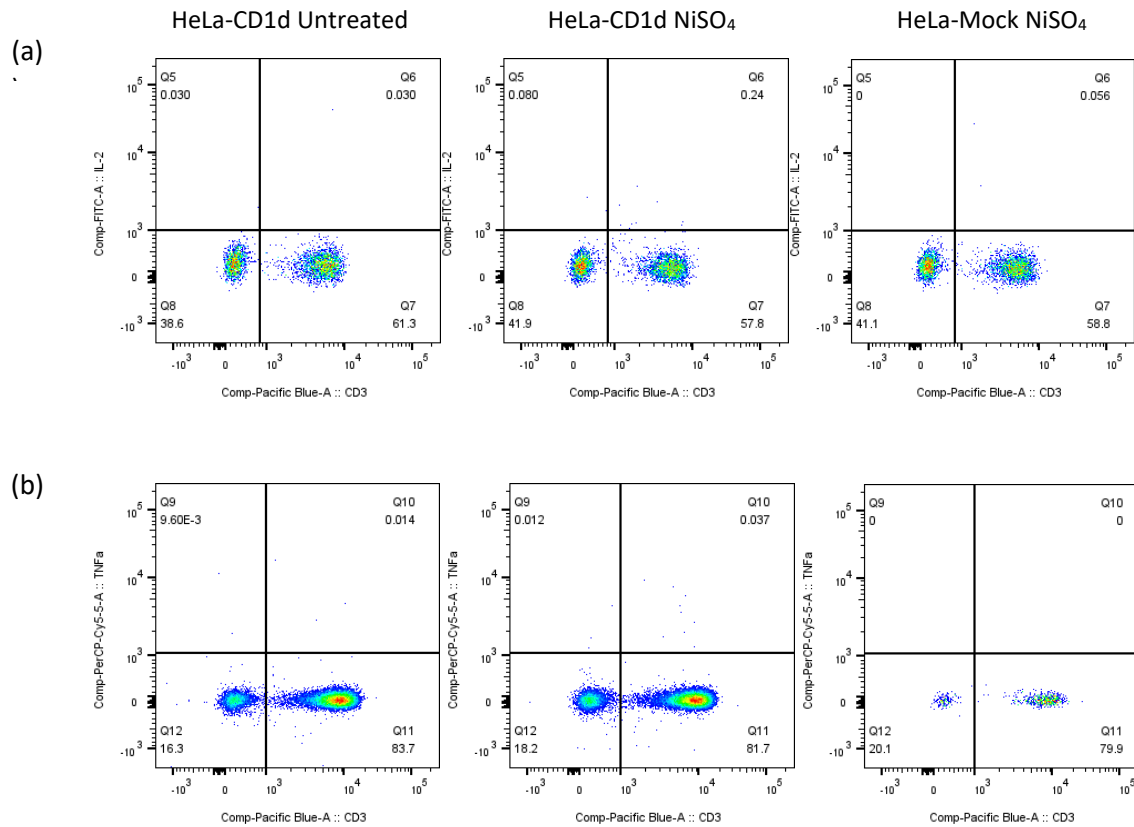


Figure A.7 Additional Replicate of Flow Cytometry Plots Showing Cytokine Production by T cells co-cultured with NiSO₄ pulsed HeLa-CD1d APCs. (a) IL-2, (b) TNF

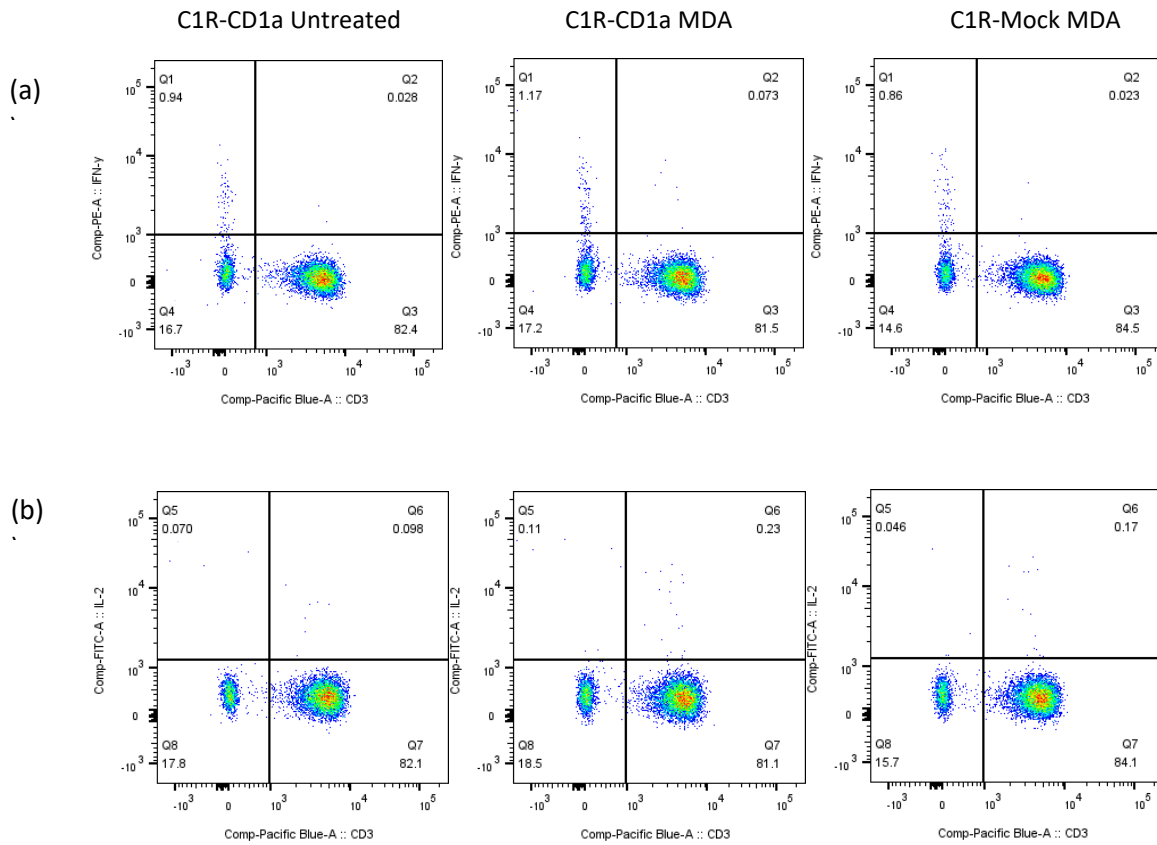
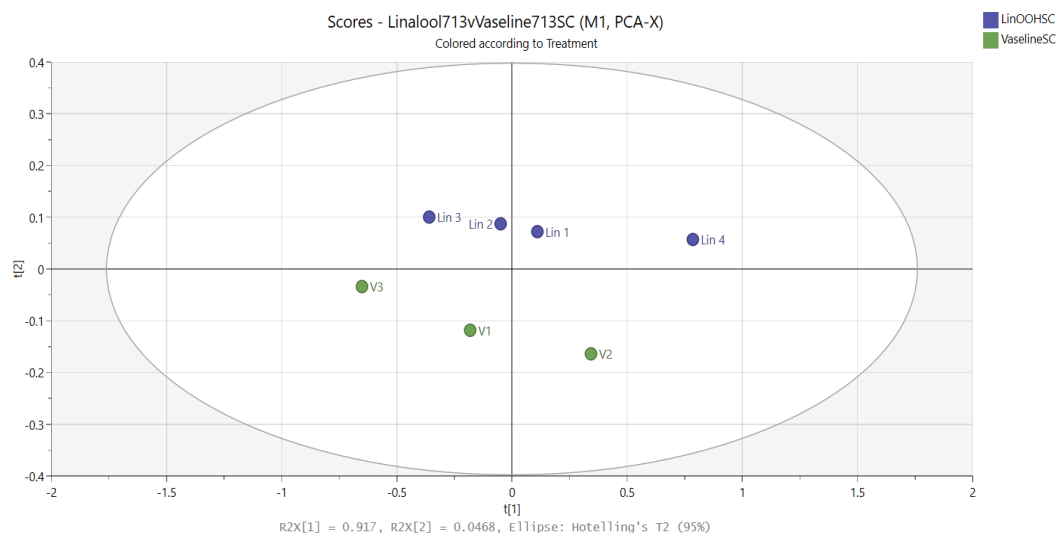


Figure A.8 Flow Cytometry Plots Showing Cytokine Production by T cells co-cultured with MDA pulsed C1R-CD1a APCs. (a) IFN- γ , (b) IL-2

Appendix II

a)



b)

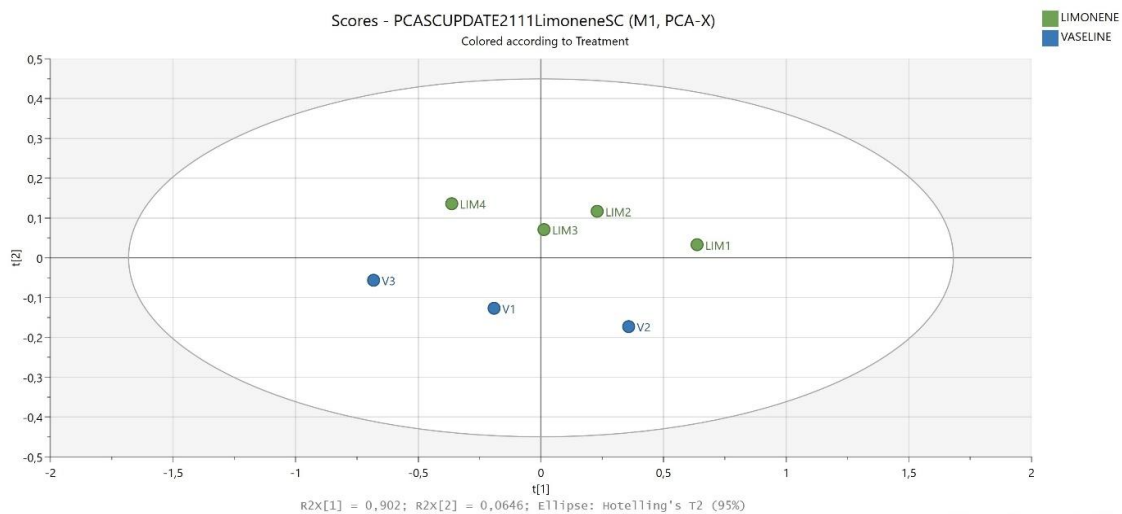
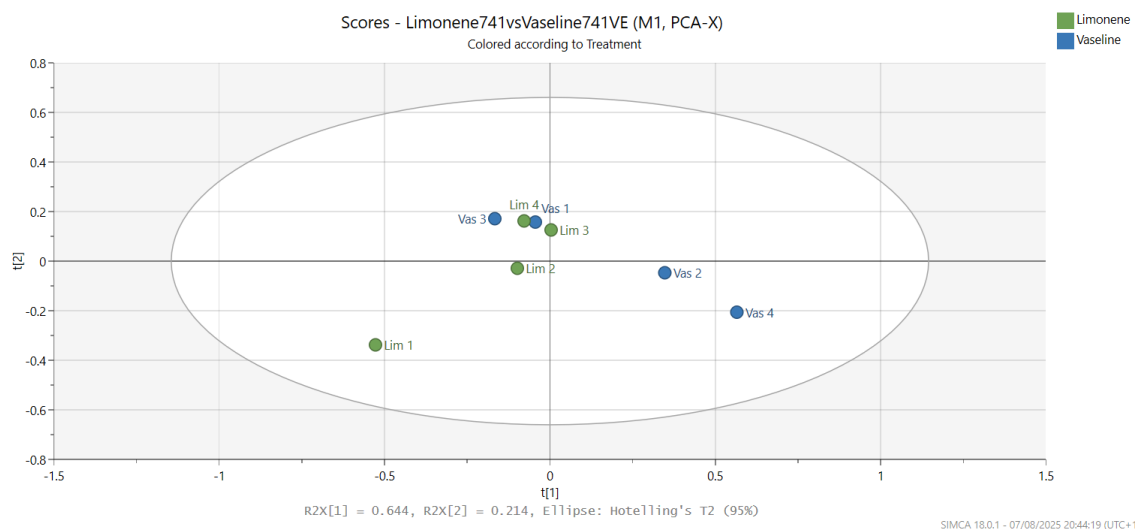


Figure A2.1: 2D Score plot of the SC of fragrance allergen treated skin. . (a) 2D score plot of the SC of hydroperoxides of linalool 1.0% treated ex vivo human skin (blue) and control (Vaseline treated) ex vivo skin (green). and (b) 2D score plot of the SC of hydroperoxides of limonene 0.3% treated ex vivo human skin (green) and control (Vaseline treated) ex vivo skin (blue). Data was extracted and analyzed as normalized intensities to total ion count.

A)



B)

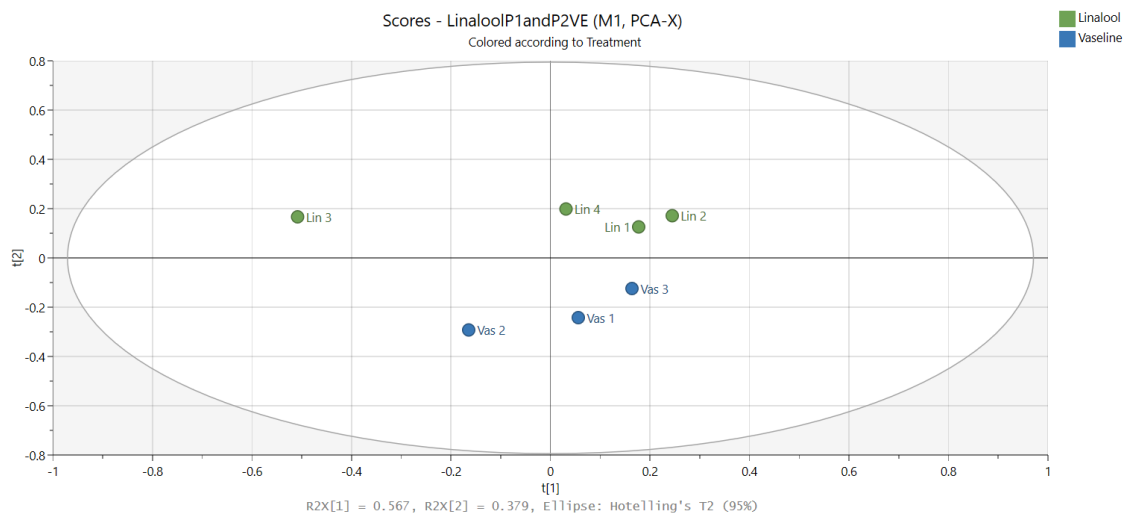


Figure A2.2 2D scores plot of the VE of fragrance allergen treated ex vivo skin samples. (a) 2D score plot of the VE of hydroperoxides of limonene (0.3%) treated ex vivo human skin (green) and control (Vaseline treated) ex vivo skin (blue). (b) 2D score plot of the VE of hydroperoxides of linalool (1%) treated ex vivo human skin (green) and control (Vaseline treated) ex vivo skin (blue). Data was extracted and analyzed as normalized intensities to total ion count.

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