# *Proteomic investigation of the effect of PDE4 inhibitor on Atopic Dermatitis*

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# Declaration

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Petrina Chand

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# Abstract

Atopic Dermatitis (AD) is a common inflammatory skin disease characterized by the development of chronic or relapsing eczematous lesions. A phase III clinical trial study has shown that patients with mild to moderate AD who were treated with Crisaborole ointment experienced rapid and clinically relevant improvement. Crisaborole is a nonsteroidal phosphodiesterase 4 (PDE4) inhibitor. Clinical studies have shown that Crisaborole is effective and has a low incidence of treatment-related and treatment-emergent adverse events in patients with mild-to-moderate AD .

This study used the technique of tape stripping sample collection coupled with proteomics and extensive bioinformatics analysis that allows an understanding of the proteomic differences between TS samples of AD collected before and after treatment with the PDE4 inhibitor, Crisaborole, and control groups. This study aims to discover the effect of Crisaborole on adaptive immunity, inflammation, skin barrier and repair on AD, and potentially investigate and identify alternative or complementary mechanism of actions of Crisaborole.

The skin samples were collected from patients that attended the AD clinics in the Dermatology Department at Westmead Hospital, New South Wales. The samples were collected from three areas on the body, which consisted of normal skin (NS), untreated AD lesion (UAD) and to be treated AD lesion (TAD) on Day 1 for baseline and Day 45 after the treatment, using a scarless, non-invasive technique with adhesive discs. Proteins were extracted from the discs, and the samples underwent analysis through mass spectrometry-based proteomics to identify proteins. The proteomic data obtained was subjected to differential abundance analysis through linear modelling for microarray analysis (LIMMA) to identify protein signatures that significantly distinguish between the different lesions. Subsequently, clustering analysis was conducted utilising principal component analysis (PCA), followed by molecular pathway analysis by using the Ingenuity Pathway Analysis (IPA) bioinformatics tool.

This study showed that tape stripping was able to find the proteomic changes between NS, UAD and TAD after the use of Crisaborole after 45 days. The proteins found has shown that there's a link between AD and the PDE4 pathways with the proteomic changes in the use of Crisaborole on AD skin. The effect of Crisaborole on AD showed that inflammatory pathways, mediators and cytokines were inhibited. Furthermore, there's improvement of the skin barrier that activates the expression of keratinocytes differentiation molecules such as filaggrin, loricrin, and involucrin. Additionally, there's a decrease in disease severity, supresses itch, clinical symptoms of AD and skin lesions.

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# List of abbreviations

**AC**: Adenylyl cyclase

**AD**: Atopic Dermatitis

**ADSI**: Atopic Dermatitis Severity Index

**AMP**: antimicrobial peptides

**ATF-1**: activating transcription factor

**ATP**: Adenosine triphosphate

**Bcl-6**: B-cell lymphoma 6 protein

**BETA**: Betamethasone

**BSA**: Body Surface Area

**cAMP**: cyclic adenosine monophosphate

**CCL4**: C-C chemokine ligand 4

**CCL17**: C-C chemokine ligand 17

**CCL18**: C-C chemokine ligand 18

**CCL22**: C-C chemokine ligand 22

**cGMP**: cyclic guanosine monophosphate

**COPD**: chronic obstructive pulmonary disease

**CREB**: cAMP response element-binding protein

**CRIS**: Crisaborole

**CXCL9**: C-X-C motif ligand 9

**CXCL10**: C-X-C motif ligand 10

**DALY**: Daily-Adjusted Life Year

**DC**: Dendritic cells

**EASI**: Eczema Area and Severity Index

**Epac**: exchange proteins directly activated by cAMP

**GBD**: Global Burden of Disease

**GM-CSF**: granulocyte-macrophage colony-stimulation

**IDEC**: inflammatory dendritic epidermal cells

**IFN-y**: Interferon gamma y

**IgE**: Immunoglobulin E

**IL-1**: Interleukin 1

**IL-1A**: Interleukin 1 alpha

**IL-1B**:Interleukin 1 beta

**IL-2**: Interleukin 2

**IL-4**: Interleukin 4

**IL-4R**: Interleukin 4 receptor

**IL-5**: Interleukin 5

**IL-6**: Interleukin 6

**IL-8**: Interleukin 8

**IL-10**: Interleukin 10

**IL-12**: Interleukin 12

**IL-13**: Interleukin 13

**IL-15**: Interleukin 15

**IL-16**: Interleukin 16

**IL-17**: Interleukin 17

**IL-17A**: Interleukin 17 alpha

**IL-17F**: Interleukin 17F

**IL-22**: Interleukin 22

**IL-23**: Interleukin 23

**IL-25**: Interleukin 25

**IL-31**: Interleukin 31

**ISGA**: Investigator's Static Global Assessment

**JAK**: Janus kinase

**JAK1**: Janus kinase 1

**LC**: langerhans cells

**PCA**: Principle Component Analysis

**PDE**: Phosphodiesterase

**PDE4**: Phosphodiesterase 4

**PKA**: Protein kinase A

**PKA-c/r**: cAMP-dependent protein kinase catalytic subunits c and r

**NFAT**: Nuclear factor of activated T cells

**NF-kB**: Nuclear Factor kappa B

**NS**: Normal skin

**S. aureus**: Staphylococcus aureus

**STAT**: Signal Transducer and Activator of Transcription

**TAD**: Treated Atopic Dermatitis

**TCI**: Topical calcineurin inhibitors

**TCS**: Topical glucocorticosteroids

**T helper 1**: Th1

**T helper 2**: Th2

**T helper 17**: Th17

**T helper 22**: Th22

**TNF**: Tumor necrosis factor

**TNF-α**: Tumor necrosis factor alpha

**UAD**: Untreated Atopic Dermatitis

**YLD**: Years Lived with Disability

**YLL**: Years of Life Lost

# Contents

# Chapter 1: Introduction

## 1.1 Introduction

Atopic Dermatitis (AD) (also known as Atopic Eczema) is a common chronic and relapsing inflammatory skin condition that causes dry, inflamed, itchy skin (Kim et al. 2019; Fortson et al. 2017). The pathogenesis of AD is complex, it's a multifaceted condition that entails genetic mutations and environmental factors among others that lead to a dysfunctional epidermal barrier and immunological response (Einchenfield et al. 2022; Sroka-Tomaszewska and Trzeciak. 2021; Nutten 2015; Tsakok 2019).

The clinical symptoms and characteristics of AD vary on certain factors such as age and the stage of the disease. AD presents with different intensities of redness (erythema), edema, vesiculation, exudation, crusting, scaling and lichenification (skin thickening). Not all signs are present at the same time. Patients with AD have a background of diffusely scaly or "dry" skin and suffer from mild to severe forms or pruritus (itch) (Fortson 2017, Sroka-Tomaszewska and Trzeciak 2021; Tsakok 2019). During the acute stages of AD, the clinical symptoms noticed are erythema, edema, vesiculation and exudation when the vesicles break. In comparison, in the chronic stages of AD, the clinical symptoms are crusting and scaling leading to lichenification due to chronic scratching (Fortson 2017; Nutten et al 2015). AD occurs in infants, older children and adults but the distributions of the lesion are influenced by age. In infants, the AD lesions are more frequent on the head, forehead, scalp, cheeks and extensor surfaces of the knees and elbows (Table 1.1) (Fortson 2017; Nutten et al. 2015). Whilst in older children and adults, the AD lesions are more frequent on the hands, wrists, ankles, and flexural surfaces of the knee and elbows (Fortson 2017; Vakharia and Silverberg 2019; Chan et al. 2021). Clinical images of various AD lesions are shown in figure 1.1.

**Table 1.1** - Location of AD lesions in adults and children (Fortson 2017, Vakharia and Silverberg 2019, Chan et al. 2021).

| Adult | Child |
|---|---|
| Hands | Head |
| Wrists | Forehead |
| Popliteal fossae | Scalp |
| Cubital fossae | Cheeks |
| | Front of Knee |
| | Back of the Elbow |


Figure A. Chronic Atopic Dermatitis on back of the elbow

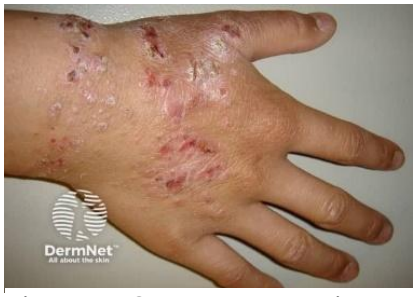


Figure B. Subacute Atopic Dermatitis on the hand

**Figure 1.1** - Clinical images of various Atopic Dermatitis lesions - Images sources from (https://dermnetnz.org/topics/atopic-dermatitis)

## 1.2 AD Subtypes

AD can be classified in multiple ways, such as by age group (paediatric/adult), ethnicity and intrinsic/extrinsic. While some argue that acute and chronic AD should be classified as subtypes, it is more accurate to interpret them as phases or stages that can occur across all AD classifications (Tokura 2022). As this review focuses on the molecular mechanisms/ pathophysiology of AD, we'll focus mainly on the intrinsic and extrinsic subtype.

**Table 1.2** - Comparison of characteristics of intrinsic and extrinsic subtypes of AD *(*Czarnowicki *et al. 2019, Song et al 2022, Yao et al. 2021)*.

| *Characteristics* | *Intrinsic* | *Extrinsic* |
|---|---|---|
| *Prevalence* | 20% | 80% |
| *Age* | Adulthood | Childhood |
| *IgE levels* | <200kU/L | >200kU/L |
| *Sex* | Predominant in Females | Male |
| *Skin barrier* | Normal | Abnormal |
| *Atopic comorbidities* | No association | Associated |

Intrinsic and extrinsic subtypes of AD are characterised by the levels of total serum immunoglobulin E (IgE) and other features (Table 1.2). The intrinsic subtype of AD represents approximately 20% of all AD patients. It is characterised by normal IgE levels that is below 200 kU/L and no allergen specific IgE antibodies (Czarnowicki et al. 2019; Lui et al. 2022; Yao et al. 2021). This results in the lack of association with atopic comorbidities (atopic march) like asthma, allergic rhinitis (hay fever) and food allergies antibodies (Czarnowicki et al. 2019; Lui et al. 2022; Yao et al 2021). Also, the intrinsic subtype typically has a delayed onset that manifest later in life, primarily during adulthood, predominantly affecting females, and is associated with normal skin barrier function antibodies (Czarnowicki et al. 2019; Lui et al. 2022; Yao et al. 2021). Despite the intrinsic subtype having a similar clinical presentation to the extrinsic subtype, they do have important epidemiological and molecular differences ( Czarnowickiet al. 2019; Lui et al.2022; Yao et al. 2021).

The extrinsic subtype of AD appears in 80% of AD patients. It is characterised by high/ increased IgE levels that is over 200kU/L and is associated with asthma, allergic rhinitis (hay fever) and food allergies (Carnowicki et al. 2019; Lui et al. 2022; Yao et al. 2021). Epidemiological studies have shown that the extrinsic subtype develops usually during childhood, in patients with a family history of atopy, has increased filaggrin mutations and an abnormally weakened skin barrier function with increased transepidermal water loss (Carnowicki et al.2019; Lui et al. 2022; Yao et al. 2021).

## 1.3 Epidemiology

### 1.3.1 Incidence and prevalence

Disability-Adjusted Life Year (DALY) are a standard measure used to quantify the overall burden of disease by capturing both mortality and morbidity. One DALY equates to the loss of one year of healthy life, and it is calculated as the sum of years of life lost due to premature mortality (YLL) and years lived with disability (YLD). In the context of non-fatal chronic conditions such as AD, DALYs are predominantly driven by YLD, reflecting the prolonged impact on patients' quality of life.

The reported age-standardised prevalence DALY rates for AD varies globally and is measured per 100,000 persons. According to the 2022 Global Burden of Disease (GBD) data, in 2017, the 5 highest prevalent countries were Sweden (326.91), United Kingdom (284.15), Iceland (276.98), Finland (263.57) and Denmark (254.63). In contrast, the 5 lowest prevalent countries are Uzbekistan (85.14) , Armenia (85.12), Tajikistan (85.11), China (82.10) and Kazakhstan (80.91) (Laughter 2021; Global Atopic Dermatitis Atlas 2022).

Although AD is misconceptulised as a “Western” and industrialised condition, however this is not the case as the data for the world regions were also collated The reported age-standardised prevalence DALY rates is measured per 100,000 persons by each region (Laughter 2021, Global Atopic Dermatitis Atlas 2022). According to the 2022 GBD data, in 2017, the 5 highest age-standardised DALYS regions were Andrea Latin America (220.3), high income North America (211.7), high income Asia Pacific (209.2), Western Europe (205.0) and Southern sub-Sharan Africa (192.9) with Australasia at number 6 with 182.0 and the 5 lowest age-standardised DALYS regions were Central Europe (116.6), Eastern Europe (111.6), South Asia (92.3), Central Asia (84.2) and East Asia (82.6) (Laughter 2021; Global Atopic Dermatitis Atlas 2022).

### 1.3.2 Burden of AD

The estimates indicated from the GBD show that AD is considered as a burden to individuals as the most common, non-communicable, chronic skin condition (Laughter 2021; Global Atopic Dermatitis Atlas 2022). It ranks 1$^{st}$ amongst all skin conditions in terms of DALY and 15$^{th}$ overall with all non-fatal diseases (Laughter 2021; Global Atopic Dermatitis Atlas 2022). AD has a high prevalence that affects approximately 20% of children and 10% of adults. Also, research shows that 2.8 million people in Australian are affected by AD (Zeleke et al 2023).

In 2022, GBD states that approximately 223 million people are suffering/living with AD, of those are approximately 43 million are children between the ages of 1-4 (Laughter 2021; Global Atopic Dermatitis Atlas 2022). The data from GBD exhibits a bimodal curve, this demonstrates that the reported age-standardised prevalence per age group for AD has a high prevalence in younger children, which decreases as an adult and increases later in life (Laughter 2021; Global Atopic Dermatitis Atlas 2022).

The burden of AD has a negative impact on an individual that affects them financially, physically and mentally. Many individuals that suffer from AD are unable to work or contribute fully to society, pay substantial out of pocket expenses for specialised care

and treatment, and frequently uses the healthcare system through doctor visits and hospitalisation. In Australia, patients with AD may incur out of pocket expenses of up to $2,000 annually (Eczema Support Australia 2019).

AD is a crippling condition (Schonmann et al. 2020). Persistent or repeated itchiness and scratching, pain in the skin and disfiguring lesions can lead to psychological anguish, sleeping difficulties, stigmatisation, social shame and a decreased quality of life (Silverberg 2019, Silverberg et al. 2019; Rønnstad et al. 2018; Patel et al. 2019). Due to these variables, individuals who are affected by these circumstances may find it difficult to focus, which could affect their performance in the workplace or education (Ronnstad et al. 2019).

Children who suffer from severe AD are more likely to experience depression and internalising tendencies (acts that stem from negativity directed inside, such as social withdrawal, feeling unwanted and feeling of loneliness or guilt) than other children (Kern et al. 2021). Similar findings were found in a meta-analysis that analysed the effects on both children and adults that revealed a substantial link between depression, anxiety, suicidal thoughts and AD(Ronnstad et al. 2018). These findings were supported by a second meta-analysis, which found that 1 in 6 individuals who suffer from AD had clinical depression, 1 in 4 had depressive symptoms and 1 in 8 had suicidal thoughts (Patel et al. 2019).

Individuals who suffer from AD can be impacted by various areas of one's life, but their families, partners and carer's may also be affected. Sleep, relationships, academic performance, jobs, mental health and socialising may all be affected by the condition (Stander 2021; Wollenberg et al. 2020; Wollenberg et al. 2022). Social stigma and low self-esteem can be a result of prominent skin lesions (Girolomoni et al. 2021). A study that included 602 individuals with AD revealed that 51.3% of them felt as though their lifestyle was restricted due to the skin condition, while 39.1% avoided social situations owing to how they looked and 43.3% reported that the disease has an impact on their everyday activities (Silverberg 2018). Nearly 1 in 2 individuals with self-reported severe AD said that their lifestyle, social interactions and leisure activities was a burden (Silverberg 2018).

## 1.4 Pathophysiology of AD

The pathogenesis of AD is not fully understood but it is evident that the skin condition results in a multifaceted interaction between the environment, a defective epidermal barrier and a dysfunctional immunological system (Guttman-Yassky et al. 2017). Studies have been controversial about either if AD is primarily driven by a dysfunctional immune system (inside-out concept) or a defective epidermal barrier (outside-in concept)(Guttman-Yassky et al. 2017; Elias and Schmuth 2009; Jensen et al. 2004; Elias et al. 2008). According to the "outside-in" concept, immunological sensitisation and activation is caused by antigen permeation, which results in innate abnormalities in barrier function and keratinocyte differentiation. In comparison to the "inside-out" concept, postulates that the AD phenotype is the outcome of T helper (Th2) cell activation and the ensuing immunologic cascade (Guttman-Yassky et al. 2017; Elias and Schmuth 2009; Jensen et al. 2004; Elias et al. 2008).

### 1.4.1 Defective epidermal barrier

The skin is an organ that plays an important function acting as an efficient barrier between an organism's external and internal surroundings. Consequently, the skin protects and sustains the organism it encloses. As a result, serves as a contact between the organism and its external environment (Yang et al. 2020; Lee et al. 2006).

The epidermal barrier of the skin provides three main purposes such as minimising passive water loss, blocking the absorption of chemicals from the environment and preventing microbial infection (Yang et al. 2020; Wickett and Visscher 2006). The epidermal barrier of the skin creates a process called keratinisation which is the development of terminally differentiated keratinocytes (Yang et al. 2020; Pouillot et al. 2008). This results in the epidermal barrier as an external-internal barrier that defends against mechanical, chemical, and microbial harm (Yang et al 2020; Pouillot et al. 2008). During the process of keratinisation, epidermal cells gradually mature from the bottom epidermal layers to create flattened cells of the stratum corneum (Yang et al. 2020; Pouillot et al. 2008). Keratinocytes growth is only found in the basal cell layers of the epidermis and undergo differentiation after mitosis in the basal layer, and they move through the epidermis towards the stratum corneum (Yang et al. 2020; Elsholz et al. 2014). Several keratinocyte layers, including the stratum basal, stratum spinosum, stratum granulosum, and stratum corneum, are produced by the differentiation process within the epidermis (Yang et al. 2020; Elsholz et al. 2014). At each step of development, keratinocytes express several marker genes (Yang et al. 2020; Elsholz et al. 2014).

The stratum corneum, the outermost layer of the skin, plays a crucial role in maintaining the epidermal permeability barrier. With a thickness of 10–20 μm, it serves as the primary regulator of skin permeability and is responsible for more than 90% of the skin's overall function, protecting against environmental threats while preserving moisture balance (Yang et al. 2020; Darlenski and Kazandjieva 2011). Therefore, adequate stratum corneum formation and upkeep are crucial to preserving the stratum corneum amazing capacity to protect the body from both chemical and microbial threats as well as dehydration(Yang et al. 2020; Menon et al. 2012). By limiting the unregulated loss of water, ions, and serum proteins, the skin is able to preserve homoeostasis, which is one of its primary protective roles (Yang et al. 2020; Pouillot et al. 2008). Enzymatic processes, commensal bacterial colonisation, immunological signalling, antimicrobial lipids and peptides, low pH, and naturally occurring moisturising agents are only a few of the various techniques the stratum corneum employs to maintain the health of the epidermis (Yang et al. 2020; Pouillot et al. 2008).

According to Elias (2008), a brick and mortar concept was developed and depicts that the corneocytes are the (bricks) that are embedded in a continuous matrix of specialised intercellular lipids which is the (mortar) (Yang et al. 2020; Pouillot et al. 2008). The corneocytes are in charge of guarding against chemical and mechanical harm, whilst the lipid matrix serves as the crucial element of the water barrier(Yang et al. 2020; Pouillot et al. 2008). Corneocytes are primarily responsible for the mechanical resistance provided by the epidermal barrier (Guttman-Yassky et al. 2017; Yang et al. 2020). The stratum corneum is capsulised in an insoluble cornified envelope that surrounds a continuous neutral lipid layer, which is made up of disulphide and gammaglutamyl-lysine bound, cross-linked structural proteins (such as loricrin,

involucrin, and tiny proline-rich proteins) and encasing keratin proteins (including ceramides, cholesterol, and free fatty acids)(Guttman-Yassky E et al. 2017). The development of the functioning skin barrier depends on the presence of these lipid and protein-rich corneocytes. As a result, the barrier function of the normal epidermis is a result of the calibre of its constituent parts (brick and mortar) (Yang et al. 2020).

The epidermis's stratum granulosum contains an additional protective barrier system (tight junctions) that are located deep within. The tight junction protein claudins, which creates an impermeable barrier and is downregulated in AD patients, is one such example (Guttman-Yassky E et al 2017; Choi 2021). By aggregating keratin filaments to stop water loss and restrict the entry of foreign substances during the terminal differentiation phase, filaggrin contributes to the maintenance of skin cell integrity and barrier function (Guttman-Yassky E et al. 2017; Peng and Novak 2015; Choi 2021). It is clear that filaggrin mutations are associated with AD since a lack of filaggrin breakdown products is directly linked to transepidermal water loss, Staphylococcus aureus (S. aureus) skin colonisation, and allergen penetration (Guttman-Yassky E et al 2017; Peng and Novak 2015; Choi 2021).

### 1.4.2 Environment and AD

Through mechanical damage and the activation of host proteases, scratching-induced skin injury greatly contributes to the degradation of skin barrier function (Patrick et al. 2021; Hachem et al. 2006). Additionally, degradation of intracellular junctions, disruption of the epithelial barrier, and a reduction in lamellar body secretion—all of which are necessary for the recovery of the epidermis—can also be caused by microbial and/or host proteases produced or induced by typical environmental skin exposures to house dust mites, cockroaches, fungi, pollen, and bacteria (Patrick et al. 2021; Jeong et al. 2008; Taka and Ikeda 2011). Furthermore, through a number of factors, such as decreased skin moisture, decreased extensibility, and increased sensation of itching, weather conditions, particularly cold temperatures and low humidity, can also enhance the permeability of the skin (Patrick et al. 2021; Engebretsen et al. 2016).

### 1.4.3 Microbiome and AD

The pathophysiology of AD may be significantly affected by irregularities in the microbial colonization of the skin. The skin hosts billions of diverse commensal bacteria that generate antimicrobial peptides (AMP), which enhance the skin's protective mechanisms against pathogens (Howell et al. 2006). Individuals that suffer from AD have shown a deficiency in AMP such as defensins and cathelicidins in the skin, contributing significantly to the defence against microbial infections via direct mechanisms and their ability to stimulate the immune response (Guttman-Yassky E et al. 2017; Howell et al, 2006; Ong et al. 2002). Additionally, cutaneous microbial diversity of commensal skin bacteria was found to be reduced in AD lesional skin and is gradually restored by topical anti-inflammatory therapy (Howell et al, 2006). The clinical findings suggest that the skin microbiota could play a role in determining the severity of AD. Additionally, the absence of specific commensal skin bacteria, including Staphylococcus epidermidis and other coagulase-negative staphylococci, may lead to an abnormal proliferation of S. aureus (Niebuhr 2010). The consistent use of topical antibiotics in managing AD may lead to a decrease in commensal microorganisms,

thereby promoting the colonization of S. aureus (Niebuhr 2010; Niebuhr et al. 2011). The exotoxins generated by this colonization can exacerbate symptoms in individuals with AD by stimulating the proliferation of T cells and the release of immunomodulatory and inflammatory cytokines, specifically Interleukin-31 (IL-31) and Interleukin-22 (IL-22) (Niebuhr 2010; Niebuhret al. 2011).

Regular application of topical antibiotics in the treatment of AD may also reduce commensal microorganisms, facilitating S. aureus colonisation (Niebuhr 2010; Niebuhr 2011). Exotoxins produced by S. aureus colonisation and their subsequent effects in AD patients may exacerbate symptoms by promoting the growth of T cells and immunomodulatory and inflammatory cytokines (IL-31 and IL-22) (Niebuhr 2010; Niebuhr et al. 2011). The increased synthesis of serine proteases by S. aureus, which are known to compromise the epidermal barrier, alters barrier function in another way (Schlievert et al. 2010). Additionally, it has been demonstrated that the promotion of S. aureus colonisation encourages Interleukin-4 (IL-4), Interleukin-13 (IL-13) and IL-22 (Sroka-Tomaszewska and Trzeciak 2021). The elevated production of serine proteases by S. aureus, recognised for their detrimental effects on the epidermal barrier, modifies barrier function in a distinct manner (Schlievert et al. 2010).

## 1.4.4 Dysfunctional Immunological System

The defective epidermal barrier of AD allows allergens, microbes and mechanical injury to penetrate through the skin that triggers the skin's innate immune system inciting increased expression of inflammatory mediators such as Thymic Stromal Lipoproteins (TLSP), Dendritic cells, Innate Lymphoid Cells, Macrophages, Keratinocytes, T helper cells such as T-helper 1 (Th1) , Th2, T-helper 17 (Th17), T-helper 22 (Th22), cytokines such as IL-4, IL-13, Interleukin 5 (IL-5), IL-31 and chemokines such as C-C chemokine ligand 17 (CCL17), C-C chemokine ligand 18 (CCL18) and C-C chemokine ligand 22 (CCL22) (Guttman-Yassky E et al. 2017; Fujii 2021; Facheris et al. 2023).

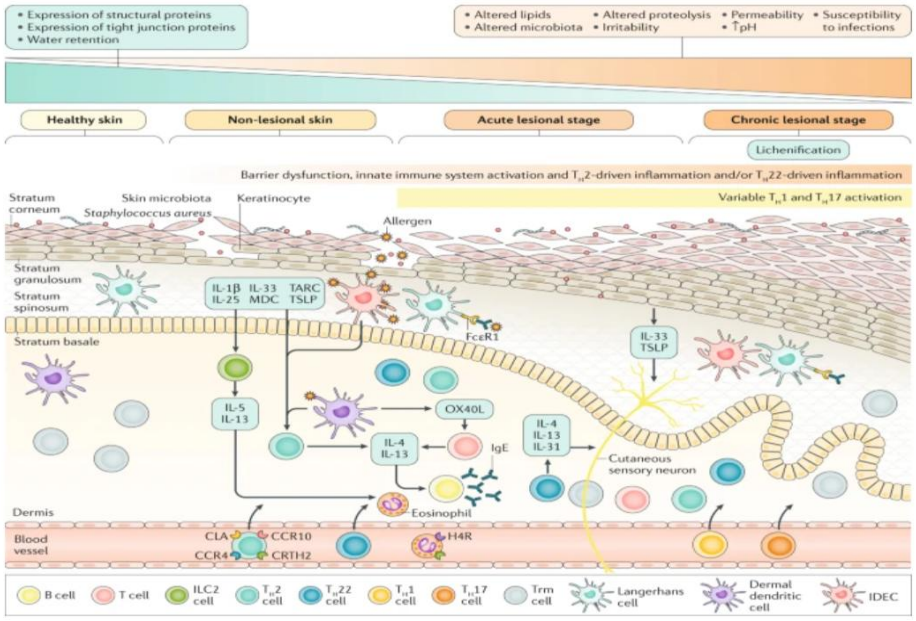


**Figure 1.2** – pathogenesis mechanism of AD (image adapted from Weindinger et al 2018)

Thymic stromal lipoproteins are an epidermal keratinocyte cytokine that stimulates dendritic cells (Salimian et al. 2022). These dendritic cells are inflammatory dendritic epidermal cells (IDEC) and langerhans cells (LC) (Fujii 2021).

These dendritic cells express a high-affinity IgE receptor FcεRI and have a role the development of sensitisation to protein antigens (Figure 1.2). The external antigens are presented by the dendritic epidermal cells and langerhans cells to naive T-helper 0 cells and mature them to activate a Th2 cell response. Th2 cell activation is associated with an upregulation of the cytokines IL-4, IL-13, IL-5, IL-31 (Fujii 2020, Facheris et al. 2023).

Proinflammatory chemokines such CCLI7, CCL18, and CCL22 are secreted by LC, IDEC and DC which recruits additional Th2 cells and amplify the response (Guttman-Yassky et al 2017; Facheris et al. 2023). The CCLI7, CCL18, and CCL22 chemokines increase the production of Th2 and T22 cytokines such as IL-4, IL-13, IL-31, and IL-22 (Guttman-Yassky E et al 2017; Facheris et al. 2023). These cytokines downregulate tight junction proteins in the epidermal barrier such filaggrin, loricrin, involucrin , periplakin, claudin and keratins (Kim et al 2019).

The activation and survival of Th2 T cells and subsequent molecules depend on the interleukins IL-4 and IL-13. The IL-4 and IL-13 cytokines influence the Interleukin 4 receptor (IL-4R), which is expressed on B cells, T cells, macrophages, and other immune cells(Guttman-Yassky et al. 2017). This also affects IgE level class switching in B cells, which leads to the development of eosinophils. Along with Th2 polarisation, which makes it easier and promotes S. aureus to bind. This is done by preventing the formation of AMPs in the cutaneous surface, which results in individuals with AD to be more susceptible to a S. aureus infection (Guttman-Yassky et al. 2017).

IL-4 and IL-13 in AD play significant roles in the production of chemokines, the maintenance of barrier function, inducing pruritis and facilitating the synthesis of AMP (Yamamura et al. 2024). Additionally, with barrier function, the presence of IL-4 and IL-13 leads to a decrease in the expression of barrier-related molecules such as filaggrin, loricrin, and involucrin that compromising skin barrier function for AD individuals (Yamamura et al. 2024).

IL-5 in AD lesion skin, plays a crucial role in development, survival, and proliferation of eosinophils (Yamamura et al. 2024). This cytokine activates eosinophils directly, resulting in their degranulation and the release of both eosinophils and their associated granules (Guttman-Yassky et al. 2017; Facheris et al. 2023). IL-31 and other crucial mediators including histamine, TSLP, tryptase, and neuropeptides, it plays a role in the itch-scratch cycle in AD individuals (Guttman-Yassky et al. 2017; Hashimoto et al. 2023).

Additionally, Th17/ Interleukin-17 (IL-17) and Interleukin-23 (IL-23) have been found to contribute to the immune response in AD (Guttman-Yassky et al. 2017). IL-17 and IL-22 are produced by Th17 cells which play a role in keratinocytes in inflammatory skin disease (Yamamura et al. 2024). In addition, IL-17 stimulates the release of multiple inflammatory mediators, leading to an increase in neutrophils, T cells, and dendritic cells. Meanwhile, IL-23 serves as an important modulation cytokine that promote the production and differentiation Th17 cells (Guttman-Yassky et al. 2017; Yamamura et al. 2024).

The initial phase of AD is predominantly influenced by Th2 and Th22 immune responses, whilst the chronic phase of AD lesions is significantly associated with Th1 immune responses (Fania et al. 2022). Th1 induces a number of cytokines including interferon gamma and Interleukin-12 (IL-12). Interferon gamma is recognised as the primary cytokine within the Th1 pathway, driving keratinocyte apoptosis and amplifying the inflammatory response in the skin, which leads to IL-12 enhancing the process and fostering the production of T cells, NK cells. (Guttman-Yassky et al 2017; Fania et al. 2022).

## 1.5 Treatments for AD

When treating moderate to severe AD – you can use either topical anti-inflammatory or systemic therapy (immunosuppressant or immunomodulatory).

**Table 1.3**– Types of treatment for AD

| **Topical anti-inflammatory treatment** | **Systemic therapy (immunosuppressant or immunomodulatory)** | **Other treatment** |
|---|---|---|
| Topical glucocorticosteroids (TCS) | Cyclosporine | Phototherapy |
| Topical calcineurin inhibitors (TCI) | Azathioprine | |
| Topical phosphodiesterase inhibitors | Methotrexate | |
| | Mycophenolate mofetil | |
| | Dupilumab | |
| | Upadacitinib | |

### 1.5.1 Topical Anti-Inflammatory Therapy

Topical anti-inflammatory therapy for AD has necessary principles such as adequate strength, adequate dosage and proper application. There are 3 different classes of topical anti-inflammatory therapies for AD. The topical anti-inflammatory therapies consist of topical glucocorticosteroids, calcineurin inhibitors and a phosphodiesterase 4- inhibitor (PDE4 inhibitor) (Kumar et al. 2020). Topical anti-inflammatory therapy is used for flare management and for proactive therapy for long-term control (Torres et al. 2019). Typically, the treatment of topical anti-inflammatory is used on AD lesions and decreases with the frequency of flares. This type of treatment is now more used and is referred to as proactive therapy. Proactive therapy is used as long-term anti-inflammatory management after AD lesions have healed from systemic therapy (Kumar et al. 2020; Torres et al. 2019).

Topical glucocorticosteroids (TCS) are a first line management for AD, that is used to prevent the occurrence of AD and applied occasionally to individuals with an established condition. The anti-inflammatory potency of topical glucocorticosteroids is classified into different groups. When choosing a topical glucocorticoid, the following should be taken into consideration such as dosage, individuals age, location of lesion,

extent and nature of lesions – acute or chronic, and the severity of the skin condition (Kumar et al. 2020; Torres et al. 2019; Li et al 2021). The potency of topical glucocorticosteroids is classified into four classes such as mild (class I), moderate or mid strength (class II), potent (class III) and very potent or ultra/super potent (class IV). Mild (class I) topical glucocorticosteroids include Hydrocortisone 0.5–1% and Hydrocortisone acetate 0.5–1%. Moderate or mid strength (class II) topical glucocorticosteroids include Clobetasone butyrate 0.05%, Hydrocortisone butyrate 0.1%, Betamethasone valerate 0.02–0.05%, Triamcinolone acetonide 0.02–0.05% and Methylprednisolone aceponate 0.1%.Potent (class III) topical glucocorticosteroids include Betamethasone dipropionate 0.05%, Betamethasone valerate 0.05–0.1% and Mometasone furoate 0.1%. Very potent or ultra/super potent (class IV) topical glucocorticosteroids include Clobetasol propionate 0.05% and Betamethasone dipropionate 0.05% (Li et al. 2021).

A study had compared the potency of a strong topical glucocorticoid vs weak topical glucocorticoid. The study consisted of moderate vs mild, potent vs mild, potent very moderate and potent vs very potent. When comparing moderate vs mild, moderate-potency topical glucocorticosteroids resulted in participants achieving treatment success than mild potency topical glucocorticosteroids (52% vs. 34%). When comparing potent vs mild, potent topical glucocorticosteroids resulted in an increased number achieving treatment success than mild potency topical glucocorticosteroids (70% vs. 39). However, evidence was insufficient for differences between potent and moderate, and uncertain between potent and very potent glucocorticoids (Banerjee and El Rhermoul 2022). When topical glucocorticosteroids are used correctly, the adverse effects of the drug are rare. Adverse effects are more frequently reported in countries where higher potency topical glucocorticosteroids are easily available over the counter or with the misuse of steroid in unregulated products (Banerjee and El Rhermoul 2022).

Topical calcineurin inhibitors (TCI) are a second line management for AD for different stages but can be considered a first-line treatment for some sensitive locations such as the face or neck (Li et al. 2021). Topical calcineurin inhibitors suppress T cell activation and downregulate the secretion of pro-inflammatory mediators (Goh et al. 2022). There are two types of topical calcineurin inhibitors such as Pimecrolimus 1% cream and Tacrolimus 0.03% or 0.1% cream, which are non-steroidal anti-inflammatories. The Pimecrolimus 1% potency is equivalent to mild potency topical corticosteroids and Tacrolimus 0.03% or 0.1% potency is equivalent to moderate to potent topical corticosteroids (Goh et al. 2022). In comparison to the Tacrolimus 0.03% and 0.1%, a study has found Pimecrolimus 1% has the same overall effectiveness (Li et al. 2021; Goh et al. 2022). Also, in a meta-analysis of randomized clinical trials, when topical glucocorticosteroids and topical calcineurin inhibitors were compared both drugs have shown that the drugs were equivalently effective. The disadvantage of topical calcineurin inhibitors is expense and adverse effect of temporary burning and itching of lesion (Li et al. 2021; Goh et al. 2022).

Topical phosphodiesterase inhibitors are a non-steroidal alternative for people individuals with mild-to-moderate AD and treat sensitive areas (Torres et al. 2019). Crisaborole 2% ointment is a new topical anti-inflammatory that regulates the production of cytokines and chemokines in the inflammation in AD and inhibits the intracellular enzyme cyclic adenosine monophosphate (cAMP)-specific 3',5'-cyclic

PDE4 (Torres et al. 2019; Li et al. 2021). It also reduces the production of anti-inflammatory factors. By blocking PDE4, it could reduce the expression of inflammatory mediators and relieve the symptoms of AD (Torres et al. 2019; Li et al. 2021; Goh et al. 2022). Studies have shown that the PDE4 inhibitor has shown to be effective. The adverse effects of Crisaborole are the temporary burning sensation (Torres et al. 2019; Li et al. 2021; Goh et al. 2022).

### 1.5.2 Systemic therapy (Immunosuppressants or immunomodulatory)

Systemic therapy is used when individuals with moderate or severe AD fail to response or becomes impractical using topical therapy or phototherapy, which controls skin inflammation, reduces symptoms, prevents flares, and improves quality of life (Torres et al. 2019; Li et al. 2021; Goh et al. 2022). The non-biological therapies which are either know as immunosuppressive or immunomodulatory agents were common options used before biological agents were found (Torres et al. 2019; Li et al. 2021; Goh et al. 2022). Those immunosuppressive or immunomodulatory agents are Cyclosporine, Azathioprine, Methotrexate and Mycophenolate mofetil. The biological agent currently being utilised as a new systemic therapy is Dupilumab.(Torres et al. 2019; Li et al. 2021; Goh et al. 2022).

#### 1.5.2.1 Cyclosporine

Cyclosporine is a potent oral calcineurin inhibitor that is an immunosuppressive systemic therapy that is frequently used to treat AD (Kumar et al. 2020; Goh et al. 2022; Davari et al. 2021; Akhavan and Rudikoff 2008). Cyclosporine forms a complex between cyclophilin by binding to the intracellular receptor of cyclophilin that inhibits calcineurin. This leads to the activation of nuclear factor of activated T cells being inhibited (Kumar et al. 2020; Goh et al. 2022; Davari et al. 2021; Akhavan and Rudikoff 2008). This results in a decrease of T-lymphocyte activation and transcription of Interleukin 2 (IL-2) and other cytokines in the epidermis. Numerous studies have shown that cyclosporine was effective when treating individuals with AD (Kumar et al. 2020; Goh et al. 2022; Davari et al. 2021; Akhavan and Rudikoff 2008). The disadvantage with using cyclosporine as a treatment is only restricted to 1 year of use as it's risk of adverse effects. The disadvantage of Cyclosporine is the adverse side effects that includes nephrotoxicity, hyperlipidemia, hypertension, hypertrichosis, gingival hyperplasia, adverse drug interactions, and occasional rebound flare after discontinuation of treatment (Kumar et al. 2020; Goh et al. 2022; Davari et al. 2021; Akhavan and Rudikoff 2008).

#### 1.5.2.2 Azathioprine

Azathioprine is a purine antagonist that is an immunosuppressive systemic therapy that has been used for 5 decades now (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). The purine antagonist of Azathioprine is cleaved to 6-mercaptopurine which inhibits the purine metabolism and cell division when incorporated into DNA and RNA (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). This results in the synthesis and function of T-cell, B-cell, and antigen-presenting cells. The effectiveness of azathioprine for treating AD has been shown in numerous studies. In one study, 69% of patients who were treated by

azathioprine showed an improvement of the symptoms for AD (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). Another study has showed that the treatment was effective in 80% of patients that also had a decrease in IgE levels (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). The disadvantage of Azathioprine is the adverse side effects that includes bone marrow suppression, hepatotoxicity, and increased risk of malignancy, including non-Hodgkin's lymphoma and squamous cell skin cancer (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008).

#### 1.5.2.3 Methotrexate

Methotrexate is a folic acid antagonist that is an immunosuppressive systemic therapy(Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). The folic acid antagonist of Methotrexate inhibits dihydrofolate reductase that prevents dihydrofolate converting into tetrahydrofolate (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). This results in affecting the synthesis of DNA and RNA, and the T-cells. A study has found that the use of Methotrexate to treat AD had improved by 52% after 24 weeks. The disadvantage of Methotrexate is the adverse side effects that includes hepatotoxicity, bone marrow suppression, and pneumonitis (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008).

#### 1.5.2.4 Mycophenolate mofetil

Mycophenolate mofetil is an antimetabolite that is an immunosuppressive systemic therapy. Mycophenolate mofetil inhibits a key enzyme called inosine monophosphate dehydrogenase, that is part of the de novo purine synthesis pathway (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). By inhibiting the enzyme, this decreases the guanine nucleotides for RNA-primed DNA synthesis and proliferation in T and B lymphocytes. Mycophenolate mofetil has been a more favourable drug for safety and numerous studies have shown the effectiveness as a treatment for AD (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). It has been shown that the use of Mycophenolate mofetil in adult's patients with AD has cleared the lesions that were resistant to other treatments such as topical glucocorticosteroids or phototherapy using UVA (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). Another study has shown that use of Mycophenolate mofetil after 4 weeks of treatment had completely cleared the moderate to severe AD in patients. The disadvantage of Mycophenolate mofetil is the adverse side effects that includes gastrointestinal side effects include nausea, diarrhoea, soft stools, anorexia, abdominal cramps, vomiting and peptic ulcer disease. Other adverse side effects have been reported are urinary, infectious and neurological but are rarely severe (Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008).

#### 1.5.2.5 Dupilumab

Dupilumab is a fully humanized monoclonal antibody that is used as a first available targeted systemic therapy (Kumar et al. 2020; Li et al. 2021; Goh et al. 2022; Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). Dupilumab's monoclonal antibody inhibits the alpha subunit of the IL-4 receptor that blocks the signalling of IL-4 and IL-13. This leads to the suppresses the expression of mRNA that is responsible for stimulating T cells, inflammatory cascade, dendritic cells, eosinophils, and Th2

cytokines (Kumar et al. 2020; Li et al. 2021; Goh et al. 2022; Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). It has also been found that by inhibiting IL-4 and IL-13, may decrease the severity of AD and re-establish barrier function of skin due to increase in loricrin, claudin, filaggrin and lipid product levels (Kumar et al. 2020; Li et al. 2021; Goh et al. 2022; Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). It has been found that use of Dupilumab of AD as treatment has a greater effectiveness and safety compared with existing non-targeted immunosuppressive therapies (Kumar et al. 2020; Li et al. 2021; Goh et al. 2022; Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008). In two phase 3 studies, 51.3% and 44.2% of adult patients that used Dupilumab achieved a EASI score of 75 by week 16 compared with 14.7% and 25.0% of patients in the placebo groups, respectively. The disadvantage of Dupilumab is the adverse side effects that includes conjunctivitis (Kumar et al. 2020; Li et al. 2021; Goh et al. 2022; Rademaker et al. 2020; Davari et al. 2021; Akhavan and Rudikoff 2008).

#### 1.5.2.6 Upadacitinib JAK inhibitor

Upadacitinib is an oral, second-generation selective Janus kinase (JAK) inhibitor approved for the treatment of AD. It selectively inhibits Janus kinase 1 (JAK1), thereby modulating the JAK-STAT signaling pathway, which plays a central role in the transduction of pro-inflammatory cytokines implicated in the pathogenesis of AD. By targeting JAK1, Upadacitinib downregulates cytokines such as IL-4, IL-13, IL-22, which are critical mediators of inflammation, pruritus, and barrier dysfunction in atopic skin (Blauvelt et al. 2021).

Its clinical efficacy has been demonstrated in multiple Phase III trials, including the Measure Up 1 and Measure Up 2 studies, as well as the AD Up trial. In Measure Up 1 and 2, Upadacitinib (15 mg and 30 mg once daily) significantly improved Eczema Area and Severity Index (EASI-75) scores and reduced pruritus compared to placebo at week 16 (Guttman-Yassky et al. 2021).

### 1.5.3 Other treatment

#### 1.5.3.1 Phototherapy

Phototherapy is used when AD shows no improvement with topical therapy (Li et al 2021). There are 2 types of ultra- violet light used for phototherapy, that includes narrow-band ultraviolet B (NB-UVB/UVB 311-313 nm) and medium-dose ultraviolet A1 (UVA1 340-400 nm) light (Kumar et al. 2020; Torres et al. 2019; Li et al. 2021; Goh et al. 2022). Phototherapy used as a treatment for AD can be beneficial as it inhibits the apoptosis of antigen-presenting cells, Langerhans cells and keratinocyte cytokine production. It had been found that UVA1 phototherapy suppresses IL-5, IL-13 and IL-31(Kumar et al. 2020; Torres et al. 2019; Li et al. 2021; Goh et al. 2022).

## 1.6 PDE4 and AD

Phosphodiesterase's (PDE's) are cyclic nucleotide enzymes that are responsible in the process of hydrolysing cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) into 5'-AMP and 5'-GMP(Peng et al. 2020; Li et al. 2023). cAMP and cGMP have various essential functions throughout the mammalian intracellular

signalling pathway that includes functioning as “second messengers” of signals sent by G protein-coupled receptors, controlling the entry and exit of ligand-gated ion channels, activating guanine-nucleotide exchange factors, and activating kinase signalling cascades (Peng et al. 2020; Li et al. 2023). The signalling system of cAMP and cGMP controls the function of numerous physiological systems such as immunological, cardiovascular, central nervous and inflammatory conditions (Peng et al. 2020; Li et al. 2023).

The rates of cyclic nucleotide production and degradation dictate the intracellular levels of cAMP and cGMP under physiologically normal circumstances, as a result this is maintained in a dynamic balance between synthesis and hydrolysis (Peng et al. 2020; Li et al. 2023). The concentration levels of intracellular cAMP and cGMP can be increased by either: increasing cAMP/cGMP synthesis by stimulating adenylyl/guanylyl cyclases through external stimuli such as hormones and neurotransmitters and preventing cAMP/cGMP hydrolysis by inhibiting PDE’s (Peng et al. 2020; Li et al. 2023). The inhibition of PDE’s would be an ideal treatment for low intracellular levels of cAMP / cGMP, as they are linked to numerous diseases. The processes that PDE regulate are numerous physiological and metabolic that include inflammation, ion channel signalling, cell differentiation and apoptosis, muscle contraction, lipogenesis, glycogen synthesis, and gluconeogenesis (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).

The PDE’s are a superfamily of 21 multigenes in the human genome. Those genes use mRNA splicing to express over 100 PDE isoforms in human tissues, which those isoforms of the PDE genes are divided into 11 families which are defined by sequence, biological and pharmacological properties (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015). The function of these 11 PDE’s are either cAMP-specific, specifically hydrolyses cGMP or hydrolyses cAMP and cGMP. cAMP specific PDE’s are PDE4, PDE7 and PDE8, specifically hydrolyses cGMP are PDE5, PDE6 and PDE9, and hydrolysis of cAMP and cGMP are PDE1, PDE2, PDE3, PDE10 and PDE11 (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).

### 1.6.1 PDE4

The PDE4 subfamily is the most diverse of the phosphodiesterase’s (PDEs) and is extensively expressed in the majority of cells. It is primarily responsible for the hydrolysis of cAMP(Peng et al. 2020; Li et al. 2023). The protein PDE4 family is classified into four distinct subtypes which consisted of PDE4A, PDE4B, PDE4C and PDE4D (Peng et al, 2020; Li et al. 2023). The PDE4 genes are found on chromosomes 19p13.2, 1p31, 19p13.11, and 5q12, respectively. Each type of PDE4 gene is capable of expressing 3 – 11 proteins, this involves alternative promoters/start sites as well as variable mRNA splicing allow PDE4 to be transcribed from the 4 PDE4 genes, resulting in more than 25 different PDE4 isoforms (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).

Proteins belonging to the PDE4 family are distributed throughout the cell compartment and are controlled by kinases. These proteins play a fundamental role in the functioning of the cell. The molecules of PDE4 can be divided into long, short and supershorts, depending on their molecular size. Each of the isoforms has an individual N-terminus region that coordinates its subcellular site of localization through interactions mediated by scaffolding proteins (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).The

classification of the isoforms is further subdivided into long-, short-, and supershorts (Figure 1.3) depending on whether or not two previously conserved regions are present (UCR 1 and UCR 2). PDE4 in the long form has two conserved regions at its N-terminus (UCR 1 and UCR 2) and the short form only contains UCR2, while the super short form contains a truncated version of UCR2 (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015). UCR1 is approximately 60 amino acids in length, whereas UCR2 is approximately 80 amino acids in length. Each PDE4 molecule has a high-conservation catalytic domain in its C terminus region, with an amino acid content of 300–350 amino acids (Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).

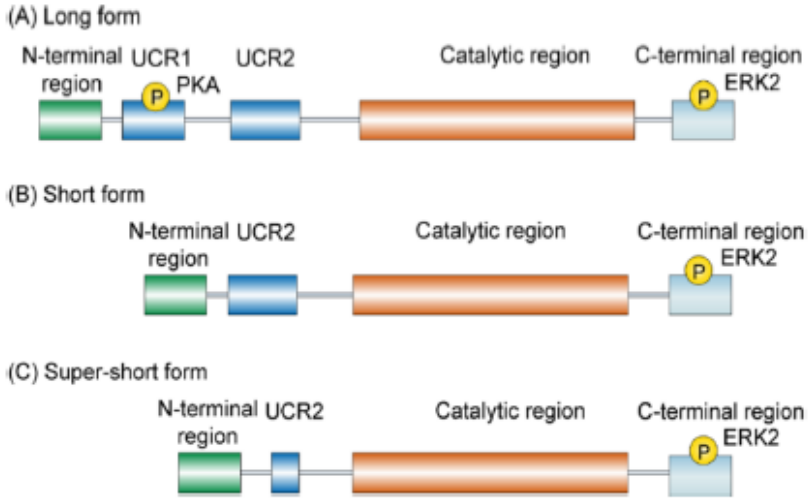


**Figure 1.3** – PDE4 molecules in long, short and super-short form (Peng et al. 2020)

PDE4 inhibitors can be characterized by the presence of three pockets in their active site, as demonstrated by X-ray structures (Figure 1.4 ). These pockets are 2 hydrogen bonds which interacts with inhibitors (Q pocket) and metal binding pockets (M pocket) which are associated with hydrophobic and phosphate moiety cAMP interactions, as well as solvent filled side pockets (S pocket) (Peng et al. 2020; Li et al. 2023). This active site is occupied by PDE4 inhibitors, which engage in a range of interactions, such as hydrophobic interaction with the inhibited phenylalanine, isoleucine, and hydrogen bonding with the conserved glutamine. The fact that PDE4 catalytic domains are highly conserved, as well as their structural homology, makes it difficult to identify PDE4 substrates for selective inhibitors. The UCR's are the key players in a unique role in regulating PDE4 activity, and are phosphorylated by PKA (Protein Kinase A) and ERK (Extracellular Signal-Regulatory Kinase). UCR1 contains a conserved phosphorylation site of a PKA in the subfamilies of PDE4, which allows PDE4 to be dimerised and catalysed. PDE4 activity can be regulated by either long-term transcriptional regulation or short-term posttranslational modification. In long term transcriptional regulation, an increase in cAMP induced by PDE inhibition is conjugated with activation of adenylyl cyclase (AC) by hormonal stimulation, which results in an increased gene expression(Peng et al. 2020; Li et al. 2023). In short-term posttranslational modification, an increase in cAMP stimulates PKA phosphorylates particular serine residues in the long form of PDE4 in UCR1, which as a result leads to a return to equilibrium in cAMP levels due to hydrolysis of the cAMP. Additionally, PDE4 action is regulated by a variety

of other proteins such as arrestin, Src family tyrosine protein kinases, A-kinase anchoring proteins and receptor for activated C kinase 1 (RACK1(Peng et al. 2020; Li et al. 2023; Cedervall et al. 2015).

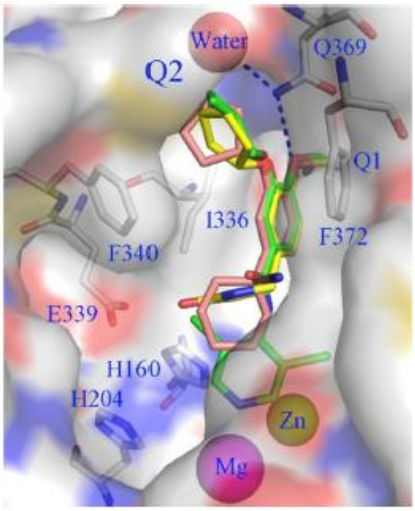


**Figure 1.4** - X-ray structures of PDE4 molecules (Peng et al. 2020)

## 1.6.2 The involvement of PDE4 in AD and other diseases

PDE4 inhibitors have been observed to be a major contributor for targeting a variety of diseases such as inflammatory conditions, including AD, psoriasis, asthma, chronic obstructive pulmonary disease (COPD), allergic rhinitis/rhinosinusitis, inflammatory bowel diseases, rheumatoid arthritis and central nervous system conditions such as major depressive disorder (clinical depression), anxiety disorders, schizophrenia, Parkinson's disease, Alzheimer's disease, multiple sclerosis, attention deficit-hyperactivity disorder, Huntington's disease, stroke, autism. In addition, PDE4 has been implicated to play a significant role in the progression of cancer(Peng et al. 2020; Li et al. 2023).

Lungs

In the lungs, cAMP and cGMP play a significant role in the regulation of cell proliferation and migration in the lungs, as well as in the differentiation and remodelling of cells, the secretion of cytokines associated with inflammation, the toning of smooth muscle cells, and the stabilization of the endothelium and epithelium (Schick and Schlegal 2022. In lung tissues, cAMP and cGMP are further mediated by Cyclic nucleotide-gated ion channels, PKA and CGMP-dependent Protein Kinase enzymes, as well as by the exchange of proteins directly activated by the cAMP (Epac) molecule and via the Popeye domain (Schick and Schlegal 2022, Kolb et al. 2023).

The subtypes found in the lungs are PDE4A and PDE4B. PDE4A along with the corresponding PDE4A1 has been observed to be present in the lung in the form of inflammatory cells, fibroblasts as well as in the form of pulmonary arterial smooth muscles. Whereas PDE4B is also seen in the lungs but exhibits a high level of expression in inflammatory cells (Schick and Schlegal 2022; Kolb et al. 2023).

PDE4 inhibitors, was one of the first focus for clinical use and the development of PDE-I for asthma and COPD (Schick and Schlegal 2022; Kolb et al. 2023).The bronchodilation

effect is predominantly driven via PDE4D, while the anti-inflammatory effect could potentially be associated PDE4A in humans. (Schick and Schlegal 2022; Kolb et al. 2023). Airway obstruction is a characterisation in asthma and COPD. In COPD, using PDE4 can decrease inflammation in neutrophils, macrophages and CD8+ T-lymphocytes. In asthma, using PDE4 can decrease inflammation in mast cells, eosinophils and CD4+ T-lymphocytes (Schick and Schlegal 2022; Kolb et al. 2023).

Skin

In the skin, Psoriasis and AD are chronic inflammatory skin diseases that not only affect the skin but immunologically. The inflammation in both skin conditions sees an increase in proinflammatory cytokines, leucocytes and lymphocytes. It is noteworthy that the pathways of Th1 and Th17 play a significant role in psoriasis, whilst the pathway Th2 plays a significant role in AD (Schick and Schlegal 2022; Milakovic and Gooderham 2021).

In psoriatic skin, different isoforms of PDE4 are overexpressed. The subtypes found are PDE4A, PDE4B and PDE4D. In the majority of all skin cell types of psoriasis skin, the expression of the PDE4A isoform elevated. PDE4B is present in vessels and in immune cells, whereas PDE4D is expressed in fibroblasts and endothelial cell (Schick and Schlegal 2022; Li et al. 2018). In the dermis and epidermis of psoriasis patients, PDE4-I can decrease the amount of T-cells, NK-cells, and CD11c myeloid dendritic cells. Tumor necrosis factor alpha (TNF-a), Interleukin 17F (IL-17F), Interleukin 17A (IL-17A), and 22 plasma levels were downregulated by PDE4 (Schick and Schlegal 2022).

In AD, the skin triggers inflammation that elevates the amount of Th2, Th22 and Th17 in skin lesions. AD is recognised to be characterised by a TH1/TH2 imbalance and an elevated levels of IL-4, IL-5, IL-13, IL-25, IL-31, IL-33, and other cytokines (Schnick and Schlegal 2022).

Lymphocytes of AD patients demonstrated an increase in PDE4 activity compared to healthy individuals. Additionally, fibroblasts from AD skin lesions all demonstrated an increase in the PDE4 subtypes PDE4A, PDE4B, PDE4C and PDE4D (Schick and Schlegal 2022). Using Crisaborole (PDE4 inhibitor) as a treatment for AD showed that in human peripheral blood mononuclear cells, human monocytes and monocyte-derived dendritic cells, the release of IL-4, IL-5, IL-13, IL-17 and IL-23, TNF-a and INF-y were decreased. Therefore, PDE4-I is modulated the Th2/Th17/Th22 pathway, resulting in improvements in lesion and symptomatology, skin barrier function, hyperplasia and epidermal proliferation (Schick and Schlegal 2022).

### 1.6.3 PDE4 Inhibitors

PDE4 inhibitors have emerged as a promising class of drugs for treating various inflammatory and immune-related conditions. Several PDE4 inhibitors have been approved for different diseases, including Crisaborole, a topical treatment AD; Roflumilast, which is used for COPD; Apremilast, approved for psoriatic arthritis; and Ibudilast, investigated for neurological disorders such as Krabbe's disease.

Roflumilast: COPD

Roflumilast is a selective PDE4 inhibitor indicated to decrease the risk of exacerbations in patients with severe COPD (Drug and Therapy Perspectives 2011). This structure involves belonging to the class of benzamide, which is produced by the formal condensation of the carboxy group of 3-(cyclopropylmethoxy)-4-(difluoromethoxy)benzoic acid and the amino group of 3,5-dichloropyridin-4-amine (National Center for Biotechnology Information 2024).

PDE-4 inhibitors are primarily responsible for the inhibition of cytokine production, cellular proliferation, chemotaxis, inflammatory mediator release, and oxidative activity (NADPH oxidase) through an increase in endocellular concentration (Zhang et al. 2018). CAMP has been extensively studied in recent years, and has been identified as a critical regulator of cellular functions and plays a fundamental role in the regulation of cell metabolism and the manifestation of physiological effects. Inflammation in COPD is characterized by airway inflammation and structural damage to the lungs due to the action of CD8+ T cells mediated neutrophil-mediated chronic inflammation, which is accompanied by Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 16 (IL-16), and TNF-alpha (Zhang et al. 2018).

Roflumilast is a new PDE4 inhibitor, that not only works on either directly or indirectly on inflammatory cells including eosinophils and neutrophils, but it also helps reduce airway inflammation caused by COPD. It helps clear inflammatory mediators from the airways or other pathways, and it also affects the levels of specific inflammatory mediators (Zhang et al. 2018).

Apremilast: Psoriatic arthritis

Apremilast is a highly selective PDE4 inhibitor non-steroidal medication used for the treatment of inflammatory conditions such as psoriatic arthritis. (National Center for Biotechnology Information 2024). This structure involves belonging to the isoindole class, specifically isoindole-1,3-dione, which is modified at the 4-position with an acetamido group and at the 1-position with a 1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl group (National Center for Biotechnology Information 2024).

When Apremilast is used as a treatment, this causes the PDE4 to be inhibited, this results in preventing degradation of cAMP. This leads to an increase of cAMP levels in cells that express PDE4, which hinders the production of numerous of pro-inflammatory markers such TNF-a, IFN-g, interleukins, and chemokines (e.g. C-X-C motif ligand 9 (CXCL9), C-X-C motif ligand 10 (CXCL10) and C-C motif chemokine ligand 4 (CCL4) (Abdulrahim et al. 2015). The increase of cAMP through the application of a PDE4 inhibitor activates the PKA signalling pathway and reinstates the Csk/Lck inhibitory axis (Abdulrahim et al 2015). This activation process leads to the phosphorylation of the cAMP response element-binding protein (CREB) transcription factor, which in turn facilitates the transcription of genes such as Interleukin 10 (IL-10 and IL-6. Conversely, the expression of TNF-a is predominantly governed by the NF-kB Nuclear Factor kappa B) transcription factor (Abdulrahim et al 2015). The increase in cAMP interferes with this mechanism due to the competitive interaction between CREB

and the NF-kB p65 subunit for the CREB-binding protein, which binds directly interacts with the TATA box located in the promoter region of the respective gene, initiating transcription processes (Abdulrahim et al 2015). Consequently, the increase in cAMP inhibits NF-kB-dependent gene transcription while enhancing CREB-dependent gene expression (Abdulrahim et al 2015). In summary, the inhibition of PDE4 is mediated through cAMP, the PKA pathway, and NF-kB-dependent mechanisms (Abdulrahim et al. 2015).

Ibudilast: Krabbe's disease

Ibudilast is a non-selective PDE4 inhibitor that is used for the treatment such as Krabbe's disease (globoid cell leukodystrophy) (Collongues et al. 2022). Ibudilast has been shown to reduce the incidence of Krabbe's disease in a genetic model by inhibiting the production of TNF-labelled cells, reducing the number of oligodendrocytes that undergo apoptosis, and inhibiting demyelination, among other neuroprotective mechanisms(Collongues et al. 2022). Furthermore, PDE inhibition has been demonstrated to reduce inflammatory activities of a variety of non-neuronic cell types, including the production of reactive oxygen species (ROS), inhibition of TNF-alpha release from astrocytes, microglia, and neurotoxic mediators such as IL-6 and nitric oxide (NO), which can cause damage to both neurons and oligodendrocytes (Collongues et al. 2022).

Crisaborole : AD

Crisaborole is a PDE4 inhibitor non-steroidal topical medication used for the treatment of mild-moderate AD. This will be described in more detail in the next section.

## 1.7 Crisaborole

Crisaborole is novel oxaborole 2% topical ointment that is an anti-inflammatory, non-steroidal phosphodiesterase inhibitor that is used for the treatment of mild to moderate AD (ChEBI 2017). This structure involves belonging to the class of benzoxaboroles and is identified as 5-hydroxy-1,3-dihydro-2,1-benzoxaborole, characterised by the substitution of the phenolic hydrogen with a 4-cyanophenyl group (Chebi 2017). This led to the formation of Crisaborole, 5-(4-cyanophenoy)-1,3-dihydro-1- hydroxy-[2,1]-benzoxaborole (ChEBI 2017).

Crisaborole is a nonsteroidal, small molecule containing a boron atom that selectively inhibits phosphodiesterase thereby modulating multiple immune and inflammatory pathways. The boron moiety is critical to its inhibitory activity, acting as a phosphate isostere within the PDE4 active site (Zebda and Paller. 2018; Jackson et al. 2020; Parker and Jacob 2017). Notably, substituting the boron with a carbon atom bonded to either an oxygen or hydroxyl group eliminates its ability to effectively inhibit the enzyme, leading to a diminished reduction in cytokine levels in peripheral blood mononuclear cells. In addition, the incorporation of boron into Crisaborole structure allows for a low molecular weight (251 Daltons) and thus easier penetration into the skin (Zebda and Paller 2018; Jackson et al. 2020; Parker and Jacob 2017). The presence of boron in this benzoxaborole structure confers the ability to inhibit PDE4 by acting as a mimic of the phosphate of cAMP. Its unique geometry enables it to target and inhibit PDE4, which

degrades cAMP. Crisaborole is suitable for topical formulation because of its physicochemical properties and low molecular weight, which allow it to penetrate the epidermis and dermis to reach the site of inflammation. Once it reaches the systemic circulation, Crisaborole is rapidly metabolized to inactive metabolites, resulting in limited systemic exposure (Zebda and Paller 2018; Jackson et al. 2020; Parker et al 2017).

In summary, intracellular cyclic adenosine monophosphate (cAMP) levels depend on the relative rate of cAMP production and degradation. PDE 4 degrades cAMP (Zebda and Paller. 2018) By inhibiting PDE4, cris increases intracellular cAMP( Zane et al. 2016). When PDE4 is inhibited by Crisaborole, levels of protein kinase A increase, which creates a different cascade resulting in anti-inflammatory effects and the suppression of proinflammatory cytokines and inflammatory mediators(Parker and Jacob 2017).

The effectiveness of Crisaborole used as a treatment for AD has demonstrated an improvement of Investigator's Static Global Assessment (ISGA) score, improvement in AD Severity Index (ADSI), improvement in pruritus and other findings.

A review has found that the use of Investigator's Static Global Assessment (ISGA) scores showed that the use of Crisaborole on AD had a grade 2 improvement with scores of either 0 (clear) or 1 (almost clear) in 47.1% of patients after 29 days. Other data has also been shown that the use of Crisaborole on AD had a grade 2 improvement with scores of either 0 (clear) or 1 (almost clear) in 38.1% of patients (Zane et al 2016).

A review has found that the use of in AD Severity Index (ADSI) scores showed that the use of Crisaborole on AD has a greater improvement when compared with the vehicle in 68% of patients after 28 days and improvement from baseline after 29 days in 70% of patients (Zane et al 2016, Parker and Jacob 2017). Another review has shown improvement from baseline in 71% of patients (Parker and Jacob 2017; Moustafa and Feldman 2014).

A review has revealed that the application of Crisaborole for AD has led to a 68.4% and 75.6% improvement in moderate to severe pruritus amongst patients after 8 days of treatment (Moustafa and Feldman 2014, Zane et al 2016). Another review has shown a reduction in pruritis in patients after 28 days (Parker and Jacob 2017; Moustafa and Feldman 2014)

In a review, other findings have shown that the use of Crisaborole on AD has marked reduction in severity of symptoms or signs, Early and sustained improvement in lesion-specific disease severity. Another review has shown in other findings that the use of Crisaborole on AD has cleared or partially cleared 62% of lesions (Parker and Jacob 2017; Moustafa and Feldman 2014).

### 1.7.1 Mechanism in PDE4 inhibitors change AD

The mechanism of action of Crisaborole to change AD involves G- protein coupled receptor, Adenylyl cyclase (AC), Adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), Phosphodiesterase 4 (PDE4), cAMP-dependent protein kinase catalytic subunits c and r (PKA-c/r) ,Nuclear factor of activated T cells (NFAT), Nuclear factor κB (NF-κB), Interferon gamma( IFN-γ), Tumor necrosis factor alpha (TNF-α), Interleukin (IL) (Zebda and Paller. 2018; Schick and Schlegal 2022).

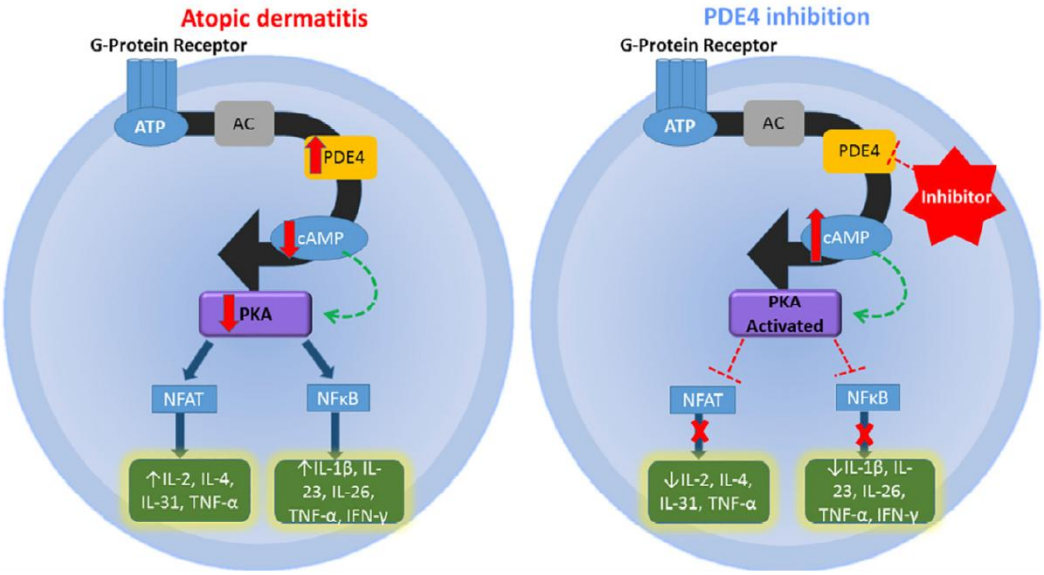


**Figure 1.5** – Mechanism of PDE4 (Zebda and Paller. 2018)

G-protein-coupled receptors use additional cellular signals to regulate the levels of cyclic adenosine monophosphate (cAMP) in the cell. G proteins are active by the signals, once G proteins are activated, this in turn stimulates adenylyl cyclase to utilise adenosine triphosphate to produce cAMP (figure1.5) (Schick and Schlegal 2022).

Following the activation of cAMP, the two primary signalling pathways are the activation of PKA and the exchange of cAMP-activated proteins (epac) (Li et al. 2023; Schick and Schlegal 2022). These subsequent pathways contribute to the production of cytokines that are pro-inflammatory or anti-inflammatory release. Furthermore, cAMP is also activated and interacts downstream by cyclic nucleotide-mediated ion channels (Li et al. 2023; Schick and Schlegal 2022).

Cyclic cAMP has the ability to activate cyclic nucleotide-gated ion channels, as a result leads to their opening and cAMP-induced calcium influx in cells. Consequently, cAMP exerts pleiotropic action via these ion channels in various cell types. Increased cAMP levels trigger the activation of PKA (protein kinase A), Epac (1/2) and cyclic nucleotide-gated ion channels (Schick and Schlegal 2022).

When cAMP attaches to PKA, it triggers PKA to release the catalytic subunits of PKA, which as a result leads to the phosphorylation of the subunits that target a variety of substrates. These targets include the cyclic AMP response element-binding protein (CREB), which functions as a transcription factor for the cAMP response element (CRE). When CREB becomes active and the CREB binding protein is produced, this results in the start of the transcription in the target gene. Additionally, the cAMP and pKA regulate the cAMP dependent activating transcription factor (ATF-1) (Schick and Schlegal 2022).

PKA has another target which is the nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells and NFAT which stimulates inflammatory gene expression and causes cells to proliferate and produce inflammatory cytokines (Zebda and Paller 2018). Since NF-KB and NFAT is inhibited by the cyclic AMP/PKA cascade in the majority of cell types,

proinflammatory cytokines are decreased as a result. B-cell lymphoma 6 protein (Bcl-6) can stimulate cell proliferation and the production of pro-inflammatory cytokines. PKA can supress Bcl-6 through ERK ½ (Zebda and Paller. 2018). The decrease of inflammatory gene transcription can also be regulated with cAMP by Epac 1/2, which activates the transcription protein Rap 1 (Ras-related protein 1) (Scnick 2022). Activation of PKA inhibits NFAT which downstreams IL-2, IL-4, IL-31, TNF-a and inhibited NF-KB which downstreams IL-1B, IL-23, TNF-a, IFN-y (Zebda and Paller. 2018).

Crisaborole downregulates the production of proinflammatory cytokines and inflammatory mediators. These involve Th1, Th2, Th17, Th22, monocytes, DC or macrophages and neutrophils (Kim et al 2023). When PDE4 inhibitors affect Th1 cells, there's a decrease in interferon gamma (IFNy), IL-2, TNF-a, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kim et al 2023).

PDE4 inhibitors have been shown to decrease the production of IL-4, IL-5, IL-6, IL-10, and IL13 in Th2 cells. Similarly, Th17 cells exhibit a reduction in IL-17 and IL-22 levels when subjected to these inhibitors (Kim et al, 2023). Th22 cells also demonstrate a decrease in IL-22 in response to PDE4 inhibitors. In monocytes, there is a notable decline in IL-6 and IL-10 levels due to the action of these inhibitors. Furthermore, the effect of PDE4 inhibitors on dendritic cells or macrophages is characterised by a decrease in IL-12 and IL-23 levels. Finally, neutrophils exhibit a reduction in IL-8 levels when influenced by PDE4 inhibitors (Kim et al 2023; Guttman-Yassky et al. 2017).

## 1.8 Betamethasone

Betamethasone valerate is a topical treatment that is an anti-inflammatory and immunosuppressive glucocorticoid that is used for the treatment of mild to moderate AD. The structure of Betamethasone valerate is a steroid ester that is essentially betamethasone with the hydroxy group at position 17alpha changed to the equivalent pentanoate ester. It constitutes of a 20-oxo, 21-hydroxy, 11 beta-hydroxy, a 3-oxo-Delta(1), Delta(4)-steroid, a fluorinated steroid and a primary alpha-hydroxy ketone steroid, both of the compounds share a similar function (Vardanyan and Hruby 2006).

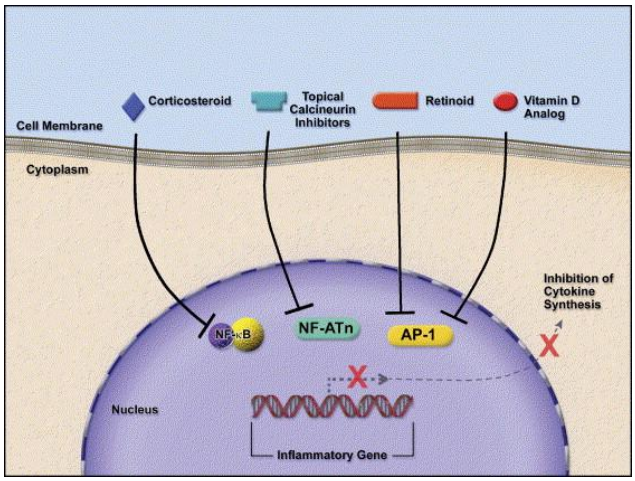


**Figure 1.6** – Mechanism of Action of Corticosteroids (Image adapted from Norris 2005)

Betamethasone (topical corticosteroid) enters the cell, which then binds to (Figure1.6) the cytoplasmic glucocorticoids receptor and is translocated into the nucleus of the cell (Norris 2005). The corticosteroid-glucocorticoid receptor is involved in the binding of glucocorticoids to certain elements in the promoter region of multiple genes and helps to control the transcription of certain genes by stimulating or inhibiting the transcription of certain mRNA and production (Norris 2003; Yasir et al. 2023). Betamethasone can also inhibit the activity of other transcription factors, such as activator protein 1, nuclear factor-kappa B (NFkB) and NFAT (Yasir et al. 2023).

A number of local cellular interactions result in these events that includes, inhibition of inflammatory cytokine release; inhibition of T cell activation; inhibition of synthesis and secretion of prostaglandins and other inflammation mediators; decrease in an anti-inflammatory protein synthesis; interferes mitotic activity inhibits epidermal cell function and dermal fibroblast function; and alter the functionality of endothelial cells, granulocytes, mast cells and Langerhans cells (Norris 2005; Yasir et al. 2023). Strong inflammatory mediators, including prostaglandins and leukotrienes, are prevented from forming by lipocortin's' inhibition of phospholipase A2 and blocking of arachidonic acid and platelet-activating factor release from cell membranes (Yasir et al. 2023).

## 1.9 Known gaps in research

Although I've highlighted the known effects of PDE4 inhibitors and betamethasone on AD lesional skin above, there are multiple gaps in the literature. One of the main ones is to understand the effect at the molecular level of PDE4 inhibitors and betamethasone in patients with AD.

Addressing this problem involves the collection of skin biopsies for genomic, transcriptomic and proteomic analysis, making the collection of samples a very slow process as many patients will not consent to have biopsies that create permanent scars only for research purposes.

The research group of the Department of Dermatology at Westmead has developed a "scarless biopsy" workflow to study skin diseases by applying tapes on the skin. These tapes collect the external surface of the skin (stratum corneum of the epidermis) and allows biomarker analysis. This will allow the identification of the proteome profile in patients with AD and the changes induced by topical treatments .

## 1.10 Proteomics Investigation

Proteomics, the large-scale study of proteins and their functions, represents a critical frontier in molecular biology by enabling comprehensive understanding of cellular processes, disease mechanisms, and potential therapeutic targets, ultimately transforming modern biomedical research and personalized medicine (Aebersold and Mann. 2016; Yates et al. 2009). The word 'proteome' refers to the collection of proteins that are produced based on the genetic information encoded within the genome (Adams 2008). The word 'proteomics' is derived from the term proteome which is protein and genome combined.

Proteomics encompasses the integration of experimental techniques with data analysis related to the proteome, investigates the overall composition, structure, modification status, and the interrelations of proteins (Cui et al. 2022; Wilhem et al. 2014). This field provides valuable insights that complements genomics and transcriptomics. Additionally, proteomics plays a crucial role in constructing a detailed map of the intricate pathways, networks, and molecular systems that govern vital biological processes, including cell proliferation, differentiation, senescence, and apoptosis (Wilhem et al. 2014).

Proteomics uses a wide range of techniques that includes conventional techniques, advanced techniques, quantitative techniques and high-throughput techniques. High throughput techniques include and label-free quantification of high mass resolution liquid chromatography (LC)-tandem mass spectrometry (MS) (Aslam et al. 2017; Al-Amrani et al. 2021).

The field of proteomics can be categorised into either protein expression mapping or protein interaction mapping (Yoithapprabhunath et al. 2015; Al-Amrani et al. 2021). The analysis of PTM's of expressed proteins in diverse environmental conditions or diseases sates can be achieved through protein expression mapping (Yoithapprabhunath et al. 2015; Al-Amrani et al. 2021). Utilising proteomics allows for the examination of protein expression across various levels, thereby facilitating an understanding of how specific cells react to that protein in both quantitative and qualitative terms. The qualitative and quantitative aspects of proteomes are assessed at the post-transcriptional, transcriptomic, and genomic levels (Yoithapprabhunath et al. 2015; Al-Amrani et al. 2021). Qualitative proteomics utilises a diverse of techniques to analyse the composition of protein mixtures and identify changes in protein expression that occurs under various conditions (Al-Amrani et al. 2021). It can also provide insight into the molecular mechanisms that contribute to disease and enables comparison between two population, such as patients and healthy controls. On the other hand, quantitative proteomics also provide comprehensive information on disease pathways, cellular function, and the discovery of biomarkers (Schubert et al. 2017; Al-Amrani et al. 2021).

Proteomics has three main types: expression proteomics, functional proteomics, and structural proteomics (Ravi 2014). Expression proteomics is a new way of looking at how proteins are expressed, both quantitatively and qualitatively. The purpose of expression proteomics is to determine the differential expression of a protein in two conditions, such as a patient and a control. Additionally, it can be used to identify disease-specific proteins and novel proteins in the context of signal transduction. Typically, expression proteomics experiments are used to investigate the mechanisms of protein expression in various cells (Ravi 2014). Functional proteomics analyses the functions and molecular mechanisms of proteins in the cells and the interactions of the protein partner. Specifically, it looks at how an unknown protein interacts with partners from a particular protein complex that is involved in a specific process. This may provide insight into the protein's biological function. Furthermore, in vivo protein-protein interactions can be elucidated to provide a detailed description of cellular signalling pathways (Ravi 2014). Structural proteomics utilises nuclear magnetic resonance spectroscopy and x-ray crystallography to identify the three dimensional arrangement and structural complexity of functional proteins. This method quantifies all protein

interactions in the mixture, including those involving membranes, cellular organelles and ribosomes (Ravi 2014).

### 1.10.1 Scarless Proteomics

Tape stripping is the method of removing gradual layers of the stratum corneum successively through the application of pressure on the skin surface using an adhesive base. The sample obtained as a result of this process can then be subjected to various techniques for further research (figure 1.7) (Barber and Boiko 2022).

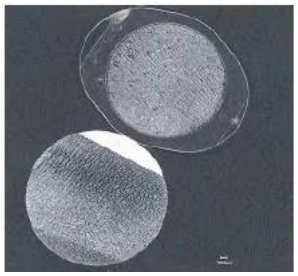

**Figure 1.7**- Image of adhesive tap stripping discs. D-Squame adhesive discs with skin cells (Ostojic et al 2021).

The use of tape stripping in dermatology research has recently been reinvigorated due to advances in epidermal sample analysis. Furthermore, tape stripping of the stratum corneum provides additional information on lamellar body secretions, epithelial remodelling, wound healing, pH of stratum corneum, lipid composition, epithelial permeability and kinetic processes, epidermal microbiomes, neoplastic and inflammatory processes (Barber and Boiko 2022).

The tape stripping technique has not changed significantly over the years; however, the way in which data is processed and the methods of analysing the data continues to change. The stratum corneum of tape stripped cells can be examined for cell morphology, structures and integrity. The majority of the epidermis in the human body comprises of keratinocytes, accounting for 90% of the total epidermis, with the remaining composed of Merkel cells, lymphocytes and melanocytes (Barber and Boiko 2022). The types of information that can be gained or assessed by tap stripping are variations in skin cell populations such as organisms like bacteria, fungi, and mites, cellular like keratinocytes, RNA, Pagetoid cells, corneocytes, melanocytes, Langerhans cells, Neutrophils, red blood cells, lymphocytes, Merkel cells, and cell mediators like chemokines, cytokines (Interleukins, Tumor necrosis factor (TNF)), genetic composition structural proteins (keratins, proteases), melanin, hormones, and lipids (Barber and Boiko 2022).

The application of tape stripping involves applying the tape with gentle and even pressure with either the pressure applying equipment or the investigator's fingers to ensure consistent contact with the skin surface in the region (Keurenties et al. 2021). Subsequently, the tape is rapidly removed with shearing force and places into a test tube or glass slide.

Tape stripping is a straightforward, cost-effective and non-invasive approach for sampling patient's skin. In contrast to biopsies, minimal non-invasive tape-stripping are preferred in order to facilitate more frequent and consistent skin sampling over time due

to treatment, environmental conditions and disease flares (Alikhan and Maibach 2010; Méhul et al. 2017; Sakabe et al. 2014; Azimi et al. 2019). Tape stripping has not been associated with any reported complications such as it does not influence inflammatory components, decreases discomfort, risk of infection and scarring that is caused by skin biopsies(Alikhan and Maibach 2010; Méhul et al. 2017; Sakabe et al. 2014; Azimi et al. 2019). Tape stripping has become a more popular technique and has proven useful in stratum corneum research due to its exclusive access to the stratum corneum, thus decreasing the potential for the extraction of substances from deeper layers of the skin such as the dermis. Skin samples obtained through TS have been extensively used for proteomic investigation of a variety of skin disorders, such as AD etc (Alikhan and Maibach 2010; Méhul et al. 2017; Sakabe et al. 2014; Azimi et al. 2019).

By using the application of tape stripping, this can collect samples of scarless biopsy skin lesions for subsequent proteomics analysis using mass spectrometry. This is done by the extraction of proteins from the stratum corneum layer of the skin and delivers molecular markers for the diagnosis of skin conditions in a safe manner (Alikhan and Maibach 2010; Méhul et al. 2017; Sakabe et al. 2014; Azimi et al. 2019). This technique can provide protein sampling of AD lesions to help understand the changes in the skin proteome.

# Hypothesis

The proteomic analysis of scarless biopsy samples will identify proteomic differences of a PDE4 topical treatment (Crisaborole) applied on patients with AD and will help understand the mechanism of action of these treatments.

The aims of the are:

1. To identify the proteomic of atopic dermatitis lesions and compare it to normal skin.

2. To identify  proteomic changes in AD of after treatment with Crisaborole or betamethasone.

3. To compare the mechanisms of action of crisaborole and betamethasone.

# Chapter 2: Materials and Methods

# 2 Methods

## 2.1 Population and Sample size

The study was carried out at the Department of Dermatology, Westmead Hospital, NSW, Australia. Patients 18 or over diagnosed with mild or moderate AD with at least 2% Body Surface Area were offered to participate. After being informed of the study, subjects provided written consent. This study was approved by the Western Sydney Local Health District WSLHD) ethics committee (2019/ETH12950).

The samples size was determined to be 20 patients in each cohort for both treatments in this study as that number has been shown to produce the optimal protein output and statistical significance in differentiating between lesions (Azimi et al, 2019).

## 2.2 Sample Collection

The samples were collected from 3 areas on the body: normal skin (NS), an untreated AD lesion (UAD) and a treated AD lesion (TAD). Samples were collected on Day 1 (before initiation of any therapy) and at 45 days after the treatment. Samples were collected using a scarless, non-invasive technique. This was performed by the application of adhesive discs (D104, D-squame, Clinical&Derm, Dallas TX, USA) to the skin. The discs was applied to lesion with a gentle pressure for 5 seconds and then lightly peeled off. A total of 9 discs were collected from normal skin, untreated AD and treated AD lesion. The 1st three discs (D 1-3) were placed into a 2 mL cryotube (Greiner Bio-One, Frickenhausen, Germany) with the adhesive side facing inwards. The next three discs were applied consecutively to the same area and placed into the second cryotube (D 4-6), this was repeated three more times (D 7-9) followed by storing them in the -80oC freezer immediately after collection until sample preparation.

## 2.3 Sample Preparation

The large D-squame adhesive discs (D104 – D-squame, CuDerm,Dallas Tx,USA) were cut into half and placed into 2mL cryotubes (Greiner Bio-One, Frickenhausen, Germany; Ref 122263). 300uL of protein extraction buffer 5% (1x) SDS in 50 mM triethylammonium bicarbonate, Sigma-Aldrich, MO, USA) was added into each tube and vortexed. Tubes rotated on the Ratek roller mixer (Ratek, VIC, Australia) overnight at speed 4 in a cool room.

Tubes were placed on a water bath sonicator (Unisonics FXP10, Brookvale, NSW, Australia) for 10 minutes, at 4C in iced water. Vortexed then transfer scarless discs into labelled elution tube. Centrifuge the original tube and elution tube for 2 minutes at 2000 rpm. Transfer the supernatant and liquid from both tubes into labelled boil proof tubes (1.5mL) (Axygen Scientific, Union City, CA, USA).

In brief, the homogenate in the boil-proof tube was sonicated Digital Sonifer 450 (Branson Ultrasonics, Danbury, CT,USA). Parameters were set for 1 minute with pulse at 59 seconds, pulse off at 0.1 seconds and 20% amplitude. Following sonication, samples were placed on a heat block at 95°C for 10 minutes. Samples were then centrifuged for 8 minutes at 4000rpm at 4oC to clarify debris in samples and supernatant transferred into new boil proof labelled tubes (1.5mL).

Reduction was performed by adding 8.7ul of 5mM tris(2-carboxytheyl) phosphine) (TCEP, Sigma Aldrich) for to 200uL aliquots, vortexed then incubated for another 15 minutes at 55oC. Alkylation of 20mM iodoacetamide, of 8.7ul was added to the samples, vortexed then further incubated at room temperature in the dark for 10 minutes. 21.7uL acidifier, 27.5% phosphoric acid was added to each sample to bring pH down to <1. Quantification of proteins yield was performed using Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

Samples were added into 1435 uL of binding/wash buffer in 2ml protein LoBind tubes (Eppendorf, Hamburg, Germany) and vortexed. All samples were then transferred to the S -Trap by placing the S-Trap micro column into a 2ml protein LoBind tubes (Eppendorf, Hamburg, Germany) for waste flow. This was done by pipetting samples into the S-Trap column(Protifi, Fairport, NY, USA) and centrifuged at 7000rpm for 30 second and checked to ensure that all the sample had passed through the S-Trap column and discard any flow as necessary. As the volume was more than it could fit into the S-Trap column, the process was repeated until all protein was trapped and all flow was discarded.

The protein was cleaned by adding 150 ul of binding/ wash buffer to each S-Trap column and centrifuge at 7000 rpm for 30 seconds, then repeat for another 2 more times and discard flow as necessary. Followed by adding 200ul of 50% chloroform/50% methanol, centrifuged at 7000rpm for 30 seconds twice, then washed with 200ul of binding/ wash buffer twice and centrifuged at 7000rpm for 30 seconds. The S-Trap column was centrifuged at 7000rpm for 1 minute to fully remove wash/binding buffer. The S-Trap mini column was transferred to a new labelled 1.5ml boil proof tubes. Samples were digested using sequencing grade modified trypsin (1:30 trypsin: protein ratio; Promega, Madison, WI, USA). Trypsin amount was calculated by converting the protein yield (ug/ul) to ug in total volume, divided by 30, following overnight digestion and incubation at 37oC.

Following incubation, S-Trap columns were placed on new properly labelled 1.5ml boil proof tubes. Peptides were eluted by 40uL of 50mM TEAB to the S-Trap column and centrifuged at 7000rpm for 1 minute, 40uL of 0.2% formic acid to the S-trap and centrifuged at 7000rpm for 1 minute and 100uL of 60% Acetonitrile (ACN) to the S-trap and centrifuge 7000rpm for 1 minute. Quantitation and final protein yield was performed on the Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Aliquots of 2ug per sample were prepared into 500ul LoBind tubes followed by drying at 35oC using a Savant Speedvac SPED140DDA (ThermoFisher Scientific), and then stored in -20oC until analysis.

## 2.4 LC-MS/MS analysis

The stratum corneum samples of 600ng, 800ng, 1ug, 1,5ug and 2ug were resuspended in loading buffer of 3.5ul, 4ul, 5ul, 7.5 ul and 10 ul respectively ((3% ACN, Thermo OPTIMA LC/MS grade, Cat # 34851-4; 96% Optima LC/MS grade Water, Thermo Fisher Scientific; 0.1% formic acid, FA, LC/MS grade, Thermo Fisher Scientific, Cat # W6-4), vortexed, centrifuged, sonicated in a water bath for 3 minutes, followed by centrifugation. Samples were separated using a nanoflow HPLC system (Ultimate 3000, Dionex, Idstein, Germany) coupled to a Thermo Scientific Q Exactive HF-X Quadrupole-

Orbitrap MS (Thermo Fisher Scientific). Samples were loaded at a flow rate of 300nL/min using an in-house C18 reversed-phase column over a 90 min linear gradient with mobile phase buffer A (0.1% LC/MS grade FA in Optima LC/MS grade water) and B (80% LC/MS grade ACN, 0.1% FA). The Mass Spectrometry machine MS was operated in a DIA fashion with 23 dynamic isolation windows (21 to 580 m/z depending on the peptide precursor density), covering the precursor mass range from 200 to 3000 m/z. The ion source was set to nanospray ionisation (NSI) with static spray voltage and the ion transfer tube temperature was set at 300◦C. Considering the full MS setting, the expected LC peak width was 15s and the default charge state was 3. The Orbitrap resolution for the MS1 scan was 120,000 with auto maximum injection time, 30% RF Lens and standard automatic gain control (AGC) target. The DIA scans were acquired at a resolution of 30,000 after fragmentation in the higher-energy collisional dissociation (HCD) cell (stepped collision energy: true; max injection time: auto; AGC target: standard; collision energy: 30%). The scan range was set to 200–2000 m/z. All data were acquired in profile mode used positive polarity. The samples were run in a MS in randomised order, and one pooled sample prepared by adding equal aliquots from each sample and Bovine serum albumin protein standards were run across the experiment to monitor the MS's performance.

## 2.5 Data analysis

### 2.5.1 Database search

The DIA-MS data from the raw files was processed for protein identification and label-free quantitation and searched in Spectronaut Pulsar X software (version 19.0, Biognosys AG, Schlieren, Switzerland) using spectral library free directDIA Hybrid with the Human Uniprot FASTA database (23/12/2023) using Spectronaut Pulsar's default parameters. The parameters for the search specified a fixed modification for Carbamidomethylation (C) alongside variable modifications for oxidation (M) and acetylation at the N-terminus of the protein. The allowed peptide length ranged from a minimum of 7 to a maximum of 52, with allowances for up to two missed cleavages and five variable modifications. Quantification was conducted at the MS2 level, and the data underwent normalisation across samples using the cross-run normalisation feature inherent to Spectronaut Pulsar. The final protein list, which adhered to a maximum false discovery rate (FDR) of 1%, was then exported into a Microsoft Excel file for subsequent statistical analysis. The Human UniProt FASTA database consists of a FASTA formatted file that includes the protein sequences corresponding of the human proteome, which involves protein isoforms.

### 2.5.2 Statistical analysis

For data filtering, the missing values were replaced with the minimum observable number of 1, followed by log-2 transformation in Excel. The data was uploaded into Multi Experiment Viewer (Web- MeV, v2.0, TM4) software, where differentially abundant analyses were performed between the lesion groups using Linear Models for Microarray data (LIMMA). The *p*-values were adjusted to control for multiple testing. Differentially abundant proteins with an adjusted $p < 0.05$ and a fold change $\leq -2$ or $\geq +2$ were considered statistically significant.

### 2.5.4 Pathway analysis

For bioinformatic analysis, the data was processed using the QIAGEN-Ingenuity Pathway Analysis (IPA) software tool (Ingenuity Systems USA). The LIMMA results of differential abundant proteins with a p-vale of <0.05 and absolute fold change of <-2 and >2 was uploaded into the IPA software and was used to perform core analysis, including molecular/ canonical pathway analysis and biological function analysis. IPA analyses were performed against the general Ingenuity Knowledgebase restricted to humans and using a stringent filter. Pathway and biofunctions with a p-value <0.05 and absolute z-score ≥2 was considered statistically significant.

### 2.5.5 Classification analysis

In classification analysis, the proteomic data was uploaded into the Perseus software where principal component analysis (PCA) was performed.

### 2.5.6 Calculation of Treatment Improvement

For the calculation of treatment improvement, the BSA of a patient was recorded on day 1 and after the use of treatment for 45 days. The data was calculated by the number of patient's BSA that improved after 45 days divided by the total number of patients in each treatment group.

# Chapter 3: Results

# 3 Results

## 3.1 Study Population

The study included a total of 18 participants with AD, who were assigned to receive either Crisaborole (treatment) or Betamethasone (comparator), with 9 patients in each group. Initially, the study aimed to enrol 40 participants—20 in each group. However, this target was not achieved due to several challenges in sample collection. These included the limitation of having only one study site, which restricted recruitment as the clinic primarily served a consistent patient population. Most of these patients were already effectively managing their AD and did not meet the criteria for study participation. Additionally, participant withdrawals further contributed the  reduced the final sample size.

The baseline conditions of all participants consisted of the age range of  21 – 84, specifically 21 – 84 for crisaborole and 21-76 for betamethasone. For gender, the baseline data showed that were more males than females in each group such as crisaborole had 6 males and 3 females, similarly betamethasone had 5 males and 4 females. Samples were taken from 3 location sites for normal skin, untreated AD and treated AD. Location sites of normal skin were arms (Inner left, inner right forearm, right forearm, left upper and left forearm), back, left hand, inner right wrist and right side of neck. Location sites of untreated AD were left ear, back (lower, upper), wrist (right, left), leg (left calf underneath knee, right knee, right back knee, right ankle, right lower leg), neck (left lateral side, right side), left shoulder and left upper arm near elbow. Location sites of treated AD were neck, right wrist, face (right lower eyelid, upper lip, left cheek, right side underneath nose, arm (right inner elbow, left armpit, left forearm, left upper arm near elbow), left shoulder, hand (left hand near pinky finger, left hand, right hand near thumb, leg (left knee, right knee) and right lower back.

The day 45 conditions of the treated AD was determined by identifying the amount of body surface area of the treated AD. The data showed that only 33% of patients responded to the treatment of crisaborole whilst 55% of patients responded to the treatment of betamethasone.

**Table 3.1** - AD samples included in this study treated with Crisaborole and Betamethasone, with individual study code, age, sex, and site of lesion location (NS, UAD and TAD), body surface area % at day 1 and 45

| Study Code | Age | Sex | Location site of Normal Skin | Location site of Untreated AD | Location site of Treaded AD | Body Surface Area % at Day 1 | Body Surface Area % at Day 45 |
|---|---|---|---|---|---|---|---|
| CRIS03 | 32 | M | Left Inner Forearm | Left Ear | Neck | 2.2 (EASI) | 5.5 (EASI) |
| CRIS04 | 42 | M | Right Inner Arm | Lower Back | Right Wrist | 4.25 (EASI) | 6 (EASI) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| CRIS05 | 25 | F | Back | Right Wrist | Right Lower Eyelid | 4.05 (EASI) | 13.75 (EASI) |
| BETA05 | 25 | M | Left Hand | Left Calf underneath Knee | Right inner Elbow | 2.2 (EASI) | 2.2 (EASI) |
| CRIS06 | 32 | F | Right Inner Wrist | Left Lateral Neck | Upper Lip | 3.7 (EASI) | 1 (EASI) |
| BETA07 | 22 | M | Right Forearm | Right Knee | Left Shoulder | 70 | 6 |
| BETA08 | 22 | F | Right Forearm | Right Back Knee | Left Hand near Pinky finger | 3 | 3 |
| CRIS08 | 32 | M | Right Forearm | Lower Back | Left Hand | 5 | 30 |
| BETA09 | 21 | M | Left Hand | Left Shoulder | Left Knee | 60 | 90 |
| BETA10 | 58 | F | Left Upper Arm | Left Wrist | Right Wrist | 2 | 1 |
| BETA11 | 22 | M | Left Upper Arn | Left upper arm near elbow | Right hand near thumb | 5 | 9 |
| BETA12 | 76 | M | Left forearm | Upper Back | Right Knee | 30 | 10 |
| CRIS10 | 21 | F | Right Forearm | Right Ankle | Right Lower Back | 7 | 3.5 |
| CRIS11 | 31 | M | Left Forearm | Upper Back | Left Armpit | 60 | 80 |
| CRIS12 | 84 | M | Right side of Neck | Back | Left Forearm | 30 | 20 |
| BETA15 | 29 | F | Left Forearm | Right side of Neck | Left Cheek | 9 | 1 |
| BETA16 | 27 | F | Left Forearm | Right Lower Leg | Right side underneath Nose | 3 | 1 |
| CRIS13 | 24 | M | Left Forearm | Left Ring Finger | Left Upper Arm near Elbow | 70 | 85 |

## 3.2 Proteomic analysis

For proteomic analysis, 108 samples were processed with each participant having 6 samples each that consisted of day 1 NS, day 1 untreated AD, day 1 treated AD, day 45 NS, day 45 untreated AD and day 45 treated AD. During sample preparation, the protein extraction pre-digestion was in a range of 7.05 – 121 ug/ml and the protein extraction post-digestion after adding trypsin had a range of 6.6 – 155 ug/ml.

There was an issue with 6 samples that had a low amount of protein (7.05, 7.15, 7.2, 7.5, 7.5 and 7.6 ug/ml), this may be due to the amount of pressure used during sample collection or the inadequate amounts of skin cells.

The data obtained from mass spectrometry showed that 4,929 protein groups and 68,032 peptides were the total number found from all the sample in the analysis

## 3.3 Proteomic data at baseline

The baseline conditions of  normal skin, untreated AD and treated AD from the proteomic data found a total of 4,929 proteins.

## 3.4 Proteomic data at day 45

The proteomic data conditions after 45 days of treatment with either Crisaborole or Betamethasone for normal skin, untreated AD and treated AD. For Crisaborole, there were no proteins changed in normal skin and untreated skin groups in Day 45 compared to their baseline whilst 483 proteins were found to be differentially abundant in treated AD. In comparison to Betamethasone, 3 proteins were found to be differentially abundant in normal skin and untreated AD whilst 358 proteins were found in treated AD.

## 3.5 AD lesions post-treatments compared to the normal skin after 45 days

To investigate whether changes in the proteome profile of AD lesions receiving treatment were approaching the normal state compared to lesions that had not received treatment. I ran LIMMA Analysis and PCA comparisons to see if treatment with CRIS and BETA at day 45 to bring the lesions (AD) back or closer to the normal skin state. This was done by seeing the amount of proteins that were significantly changed and to see if there was comparisons with PCA. The amount of proteins were found by the different abundant proteins that were significantly different (FC <-2 and >2, adjusted p-value >0.05).

Table 3.2  shows the amount of abundant protein found that were that were significant different. In Betamethasone, 7 proteins were found in BETA DAY 45 NS vs BETA DAY 45 UAD and 18 proteins in BETA DAY 45 NS vs BETA DAY 45 TAD. In comparison to Crisaborole, 11 proteins were in CRIS DAY 45 NS vs CRIS DAY 45 UAD and 171 proteins in CRIS DAY 45 NS vs CRIS DAY 45 TAD.

**Table 3.2 -** Amount of different abundant proteins that were significantly different (FC <-2 and >2, adjusted p-value >0.05) with treatment with crisaborole and betamethasone at day 45 is bringing the lesions (AD) back or closer to the normal skin state

| | |
|---|---|
| BETA DAY 45 NS vs BETA DAY 45 UAD | 7 proteins |
| CRIS DAY 45 NS vs CRIS DAY 45 UAD | 11 proteins |
| BETA DAY 45 NS vs BETA DAY 45 TAD | 18 proteins |
| CRIS DAY 45 NS vs CRIS DAY 45 TAD | 171 proteins |

I used PCA comparisons to see if treatment with CRIS and BETA at day 45 to bring the lesions (AD) back or closer to the normal skin state. PCA is a technique that visualises extensive datasets and identifying patterns or characteristics that may distinguish between different groups. All day 45 normal skin groups for crisaborole and betamethasone are clustered in the left, in comparison all day 45 untreated and treated AD are clustered to the right with some overlapping (Figures 3.1, 3.2, 3.3 and 3.4). Clearly separated groups indicate that there's a difference in the measured variables between groups whilst large overlaps indicate that there are none or only small differences in the measured variables between groups.

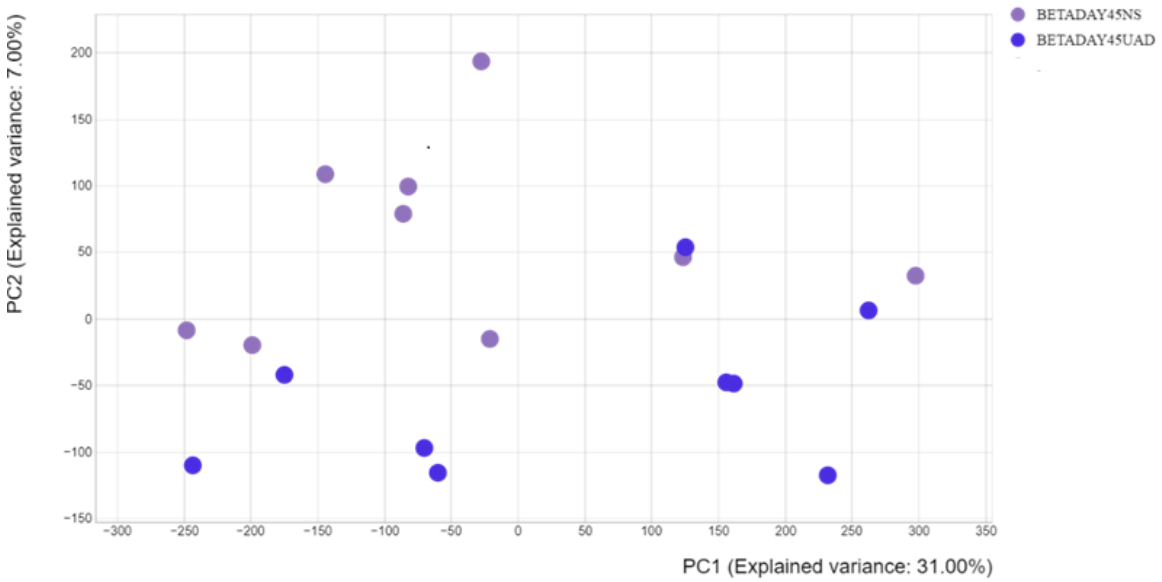


**Figure 3.1** - BETA DAY 45 NS vs BETA DAY 45 UAD .The PCA plots were created using all data with 4929 proteins for BETA samples. Legend on the right indicates colour with associated with each lesion group - purple = BETA Day 45 NS, dark blue = BETA Day 45 UAD; NS = Normal Skin; UAD = Untreated AD.

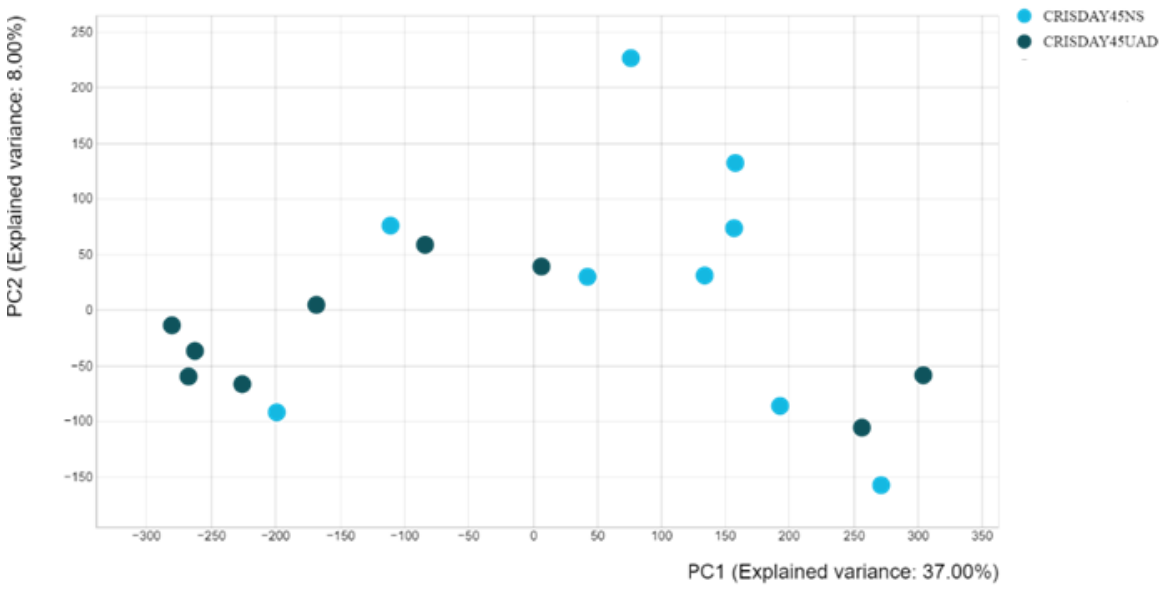


**Figure 3.2** - CRIS DAY 45 NS vs CRIS DAY 45 UAD. The PCA plots were created using all data with 4929 proteins for CRIS samples. Legend on the right indicates colour with associated with each lesion group – sky blue = CRIS Day 45 NS, dark blue = BETA Day 45 UAD; NS = Normal Skin; UAD = Untreated AD.

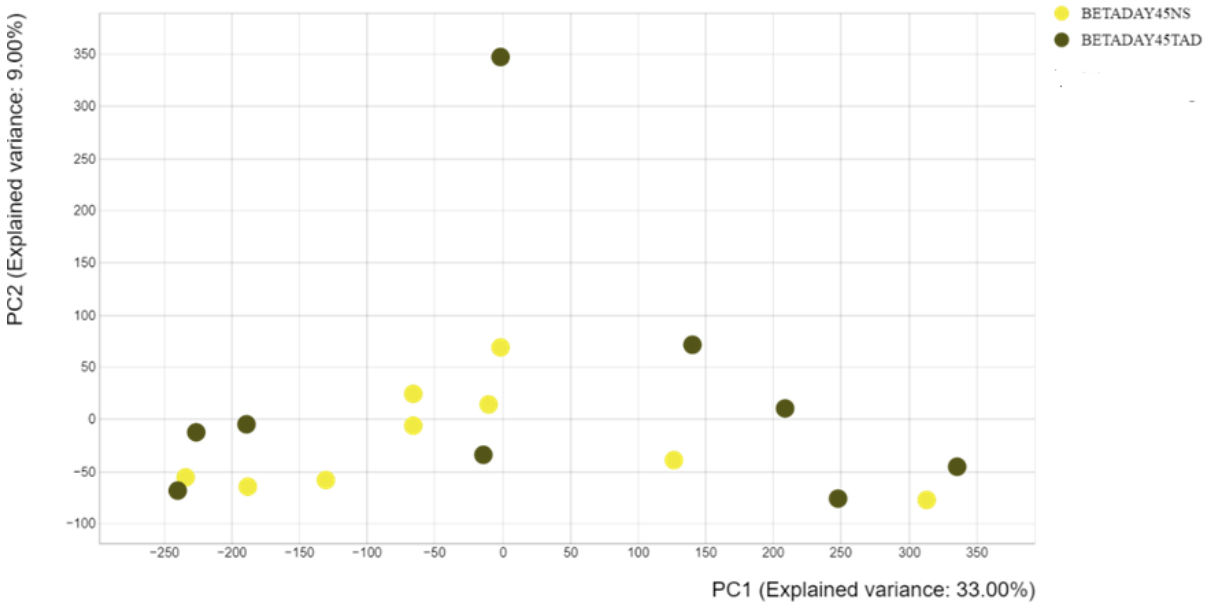


**Figure 3.3** - BETA DAY 45 NS vs BETA DAY 45 TAD. The PCA plots were created using all data with 4929 proteins for BETA samples. Legend on the right indicates colour with associated with each lesion group - yellow = BETA Day 45 NS, grey = BETA Day 45 TAD; NS = Normal Skin; TAD = Treated AD.

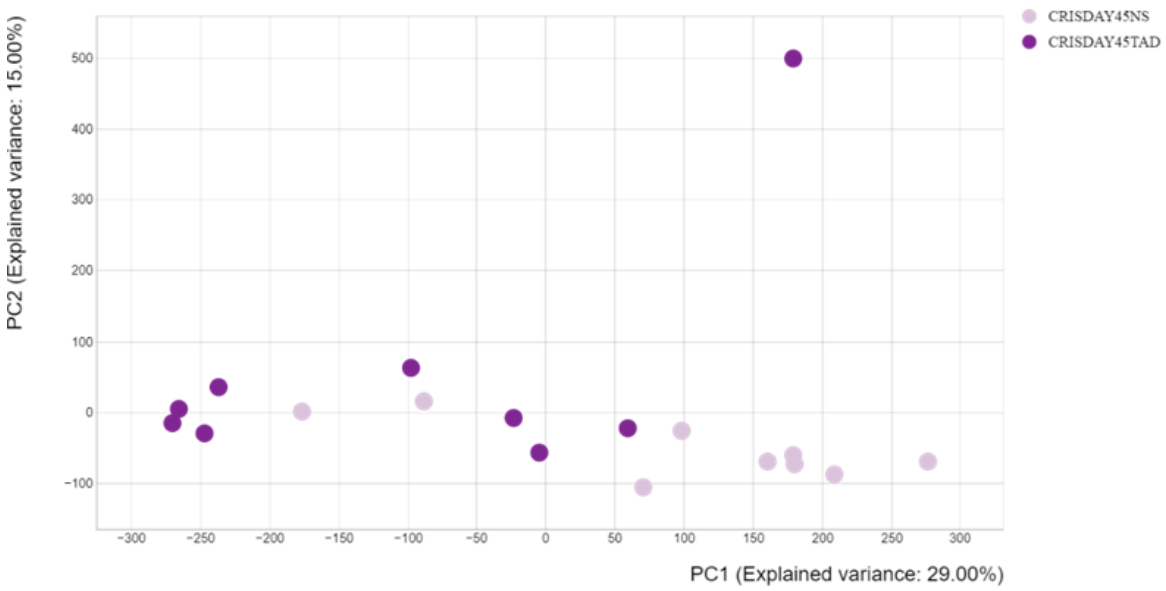


**Figure 3.4** - CRIS DAY 45 NS vs CRIS DAY 45 TAD. The PCA plots were created using all data with 4929 proteins for CRIS samples. Legend on the right indicates colour with associated with each lesion group – light purple = CRIS Day 45 NS, dark purple = CRIS Day 45 TAD; NS = Normal Skin; UAD = Treated AD.

## 3.6 Differential Abundant Proteins

### 3.6.1 Crisaborole

Differential abundance analysis was performed using LIMMA analysis software. From the total of 4929 proteins identified, 483 proteins in CRIS DAY 45 vs CRIS DAY 1 TAD were found to be differentially abundant. Of those 483 proteins, all proteins were found to be significantly down regulated. The top 20 proteins were identified using adjusted p-value <0.05 and fold change -2 < and >2 (Table 3.3). In comparison there was no differential abundance proteins found in Cris Day 45 NS vs Cris Day 1 NS and Cris Day 45 UAD vs Cris Day 1 UAD.

**Table 3.3** - Top 20 differential abundant proteins in CRIS Day 45 against day 1 TAD (adjusted p-value <0.05 and fold change -2 < and >2.

| Protein name | Gene ID | P-adjusted value | Fold change |
|---|---|---|---|
| **Intersectin-2** | ITSN2 | $1.98e^{-05}$ | -30.12 |
| **Trypsin-2;Putative trypsin-6** | PRSS2;PRSS3P2 | $2.09e^{-05}$ | -38.68 |
| **Ribonuclease 7** | RNASE7 | $2.09e^{-05}$ | -26.47 |
| **Cathepsin B** | CTSB | $2.09e^{-05}$ | -26.70 |

| | | | |
|---|---|---|---|
| **Actin, alpha cardiac muscle 1** | ACTC1 | $2.09e^{-05}$ | -26.86 |
| **Protein S100-A14** | S100A14 | $2.10e^{-05}$ | -26.11 |
| **Tubulin alpha-4A chain;Isoform 2 of Tubulin alpha-4A chain** | TUBA4A | $4.62e^{-05}$ | -24.38 |
| **Prostaglandin E synthase 3** | PTGES | $5.49e^{-05}$ | -24.83 |
| **Protein S100-A7A** | S100A7 | $5.49e^{-05}$ | -23.36 |
| **p53 apoptosis effector related to PMP-22** | PERP | $5.49e^{-05}$ | -23.99 |
| **Microsomal glutathione S-transferase 2** | MGST2 | $5.75e^{-05}$ | -22.58 |
| **Translocon-associated protein subunit delta** | SSR4 | $5.75e^{-05}$ | -22.27 |
| **Nicotinamide phosphoribosyltransferase** | NAMPT | $5.75e^{-05}$ | -22.80 |
| **Acyl-protein thioesterase 1;Isoform 2 of Acyl-protein thioesterase 1** | LYPLA1 | $6.85e-^{05}$ | -21.81 |
| **Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2** | ARAP2 | $6.85e-^{05}$ | -21.88 |
| **Proteasome subunit beta type-4** | PSMB4 | $8.32^{e-05}$ | -20.98 |
| **Translocon-associated protein subunit alpha** | SSR1 | $8.34e^{-05}$ | -20.86 |
| **F-box only protein 50** | NCCRP1 | $1.02\ e^{-04}$ | -20.32 |
| **Synaptic vesicle membrane protein VAT-1 homolog** | VAT1 | $1.15\ e^{-04}$ | -19.96 |
| **Aspartate aminotransferase, cytoplasmic** | GOT1 | $1.15\ e^{-04}$ | -19.79 |

### 3.6.2 Betamethasone

In comparison for Betamethasone, out of the total proteins identified only 358 proteins in BETA DAY 45 vs BETA DAY 1 TAD were found to be differentially abundant. Of those 358 proteins, all proteins were found to be significantly down regulated. The top 20 proteins were identified using adjusted p-value <0.05 and fold change -2 < and >2. The top 20 proteins were identified using adjusted p-value <0.05 and fold change -2 < and >2.

**Table 3.4** - Top 20 differential abundant proteins in BETA Day 45 again day 1 TAD (adjusted p-value <0.05 and fold change -2 < and >2.

| Protein name | Gene ID | P-adjusted value | Fold change |
|---|---|---|---|
| **Small glutamine-rich tetratricopeptide repeat-containing protein alpha** | SGTA | $1.03e^{-04}$ | -16.72 |
| **Serine/threonine-protein kinase PAK 2** | PAK4 | $4.18e^{-04}$ | -12.30 |
| **MICOS complex subunit MIC25** | CHCHD6 | $2.577e^{-3}$ | -11.81 |
| **Microfibrillar-associated protein 1** | MFAP1 | $2.577e^{-3}$ | -12.71 |
| **F-actin-uncapping protein LRRC16A** | CARMIL1 | $2.592\,e^{-3}$ | -12.51 |
| **Chitobiosyldiphosphodolichol beta-mannosyltransferase** | ALG1 | $2.592e^{-3}$ | -12.73 |
| **SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1;Isoform B of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1** | SMARCB1 | $2.624e^{-3}$ | -13.60 |
| **Protein Hook homolog 1** | HOOK1 | $2.756\,e^{-3}$ | -9.42 |
| **Ras-related protein Rab-4A** | RAB4A | $2.778\,e^{-3}$ | -9.23 |
| **PAT complex subunit CCDC47** | CCDC47 | $2.778\,e^{-3}$ | -9.26 |
| **Nuclear factor NF-kappa-B p105 subunit;Isoform 2 of Nuclear factor NF-kappa-B p105 subunit** | NFKB1 | $3.126\,e^{-3}$ | -8.85 |
| **N-acetylglucosamine-6-phosphate deacetylase** | AMDHD2 | $3.14\,e^{-4}$ | -8.86 |
| **Bridging integrator 3** | BIN3 | $3.32\,e^{-4}$ | -8.95 |
| **15-hydroxyprostaglandin dehydrogenase [NAD(+)]** | HPGD | $3.333\,e^{-3}$ | -9.18 |
| **Isoform 2 of Nebulette** | NEBL | $3.333\,e^{-3}$ | -8.85 |
| **Serine/arginine repetitive matrix protein 2** | SRRM2 | $3.53\,e^{-4}$ | -8.75 |
| **Exportin-7** | XPO7 | $3.53\,e^{-4}$ | -8.59 |
| **Protocadherin-1** | PCDH1 | $4.777\,e^{-3}$ | -9.68 |
| **eIF-2-alpha kinase GCN2** | EIF2AK4 | $4.777\,e^{-3}$ | -8.60 |
| **Spermatogenesis-defective protein 39 homolog** | VIPAS39 | $5.545\,e^{-3}$ | -12.41 |

### 3.6.3 Differentially Abundant Proteins found in Betamethasone for NS and UAD

When LIMMA statistical analysis was conducted to find differentially abundant proteins using adjusted p-value 0.05 and fold change -2< and >2, there were 3 proteins found in Beta Day 45 NS vs Beta Day 1 NS and 3 proteins found in Beta Day 45 UAD vs Beta Day 1 NS UAD. Although there should not be any observed changes in NS and UAD after 45 days from baseline, these proteins found may be due to other factors. The following 3 proteins were found in Beta Day 45 NS vs Beta Day 1 NS are SKIC2, PSMD4, SLC17A5 and the following 3 proteins were found in Beta Day 45 UAD vs Beta Day 1 UAD are GRN, PNPT1, STRIP1.

**Table 3.5-** Differential abundant proteins found in Beta Day 45 NS vs Beta Day 1 NS and Beta Day 45 UAD vs Beta Day 1 UAD (adjusted p-value <0.05 and fold change -2 < and >2.

| Beta Day 45 NS vs Beta Day 1 NS | Beta Day 45 UAD vs Beta Day 1 UAD |
|---|---|
| SKIC2 | GRN |
| PSMD4 | PNPT1 |
| SLC17A5 | STRIP1 |

### 3.6.4 Shared proteins between treatments of Crisaborole and Betamethasone treatments

After LIMMA analysis, when comparing the proteins between the treatments of Crisaborole and Betamethasone, there was 1 shared protein found between Beta Day 45 NS vs Beta Day 1 NS and Beta Day 45 TAD vs Beta Day 1 TAD. Also, there was 3 shared proteins found between Beta Day 45 TAD vs Beta Day 1 TAD shown in table 3.6.

**Table 3.6** - Shared proteins between treatments of Crisaborole and Betamethasone

| Beta Day 45 NS<br>Vs<br>Beta Day 1 NS | Beta Day 45 TAD<br>Vs<br>Beta Day 1 TAD | Cris Day 45 TAD<br>Vs<br>Cris Day 1TAD |
|---|---|---|
| SKIC2 | SKIC2 | |
| | AP3D1 | AP3D1 |
| | ARHGEF10L | ARHGEF10L |
| | MINK1 | MINK1 |

## 3.7 Canonical pathways

### 3.7.1 Crisaborole

211 canonical pathways were predicted to be significantly changed in CRIS Day 45 TAD lesions (p-value <0.05 and absolute z-score ≥2) samples. Of these, 5 pathways, including RHOGDI signalling, mitochondrial dysfunction, antioxidant action of vitamin C, SPINK1 pancreatic cancer pathway, Sleep NREM signalling pathway were upregulated. The remaining 206 included neutrophil degranulation, SRP-dependent cotranslational protein targeting to membrane, EIF2 were downregulated. The top 10 canonical biological pathways are illustrated in Figure 3.1.

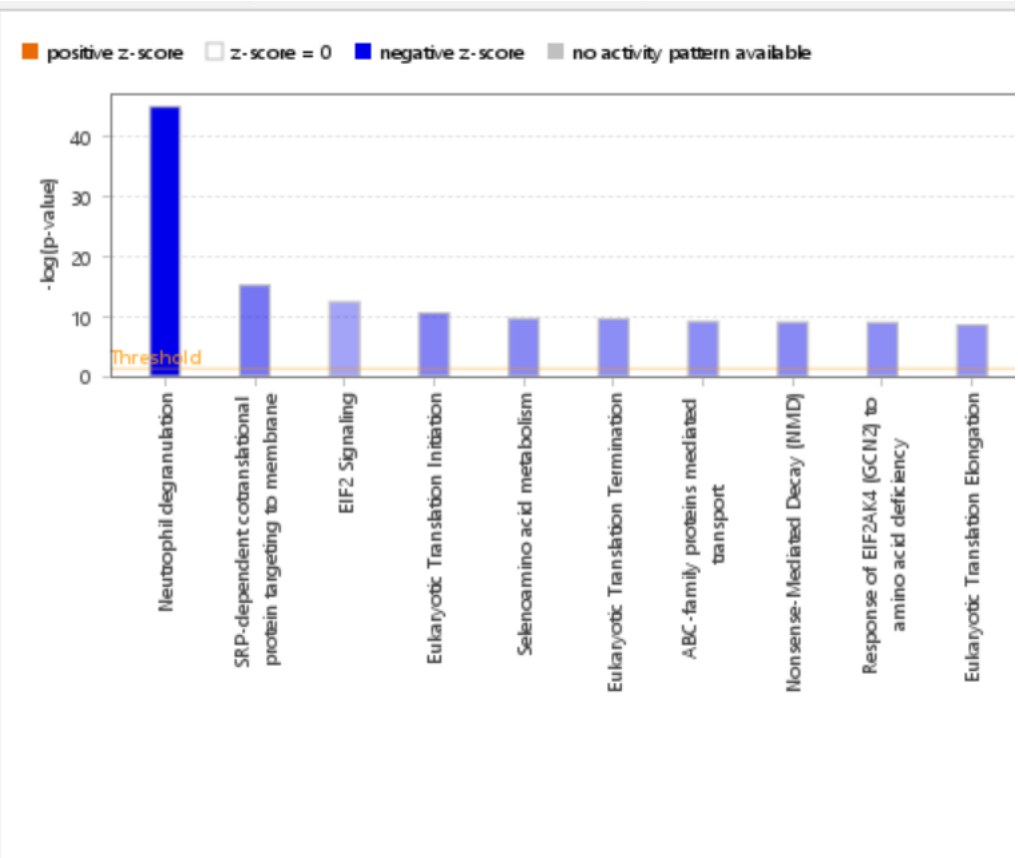


**Figure 3.5** – Canonical pathway of CRIS Day 45 TAD specific biological processes conducted in IPA. The top 10 most significantly disrupted pathways were identified by a log p-value <0.05 (the threshold) and absolute z score ≥2. The blue colour indicates inhibited pathways, and the shade indicates the significance, the darker the colour the higher the z-score.

### 3.7.2 Betamethasone

64 canonical pathways were predicted to be significantly changed in BETA DAY 45 TAD AD lesions (p-value <0.05 and absolute z-score ≥2) samples. Of these, one pathway, including granzyme A signalling were upregulated. The remaining 63 included were processing of capped Intron-Containing Pre-mRNA, ISG15 antiviral mechanism,

SUMOylation of DNA replication proteins were downregulated. The top 10 canonical biological pathways are illustrated in Figure 3.2.

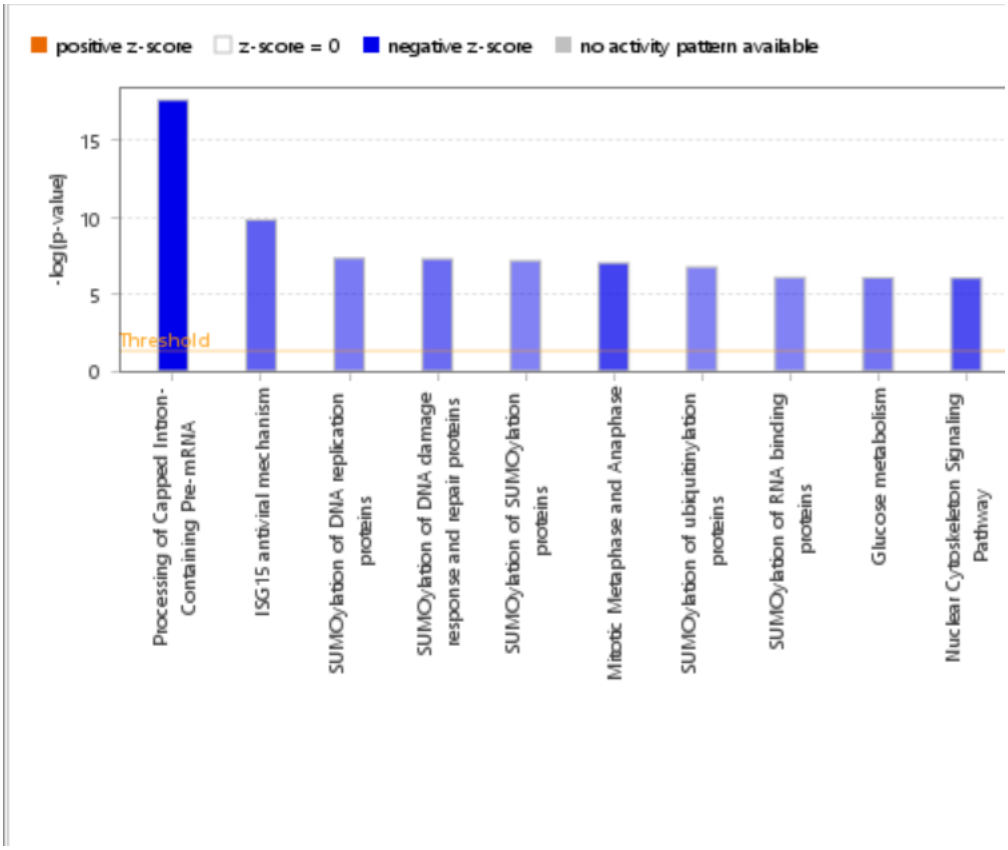


**Figure 3.6** – Canonical pathway of BETA Day 45 TAD specific biological processes conducted in IPA. The top 10 most significantly disrupted pathways were identified by a log p-value <0.05 (the threshold) and absolute zscore ≥2. The blue colour indicates inhibited pathways, and the shade indicates the significance, the darker the colour the higher the z-score.

## 3.8 Upstream regulator analysis

### 3.8.1 Crisaborole

Upstream regulator analysis was conducted in IPA analysis to find inhibited and active proteins. The inhibited regulators proteins for crisaborole treated AD lesions after 45 days consists of molecule types such as complexes, cytokines, family groups, growth factors, kinases, transcription regulators and transmembrane receptors (Table 3.7).

#### 3.8.1.1 Inhibited proteins

**Table 3.7** - Upstream analysis of inhibited proteins of CRIS Day 45 TAD conducted in IPA.

| Inhibited Upstream Regulator | Molecule Type |
|---|---|
| IMMUNOGLOBULIN; CD3; NFKB | complex |
| TNF; IL13; IL-1B; IL-6; IFNG; IL-22; IL-17A; IL-4; NAMPT; IL-1A; CD40LG; IL-5; IL-2 | cytokine |
| IFN; VEGF | Group |
| TGFB1; HBEGF; EGF; IGF1 | Growth Factor |
| EGFR; RAF1 | Kinase |
| TFEB; BHLHE40; ESRRA; STAT5B; PPARGC1A; STAT4; EPAS1; NFKB1; STAT3 | Transcription regulator |
| SIGLEC8; IGF1R; CD28; LRP1 | transmembrane receptor |

#### 3.8.1.2 Activated proteins

The activated regulators proteins for crisaborole treated AD lesions after 45 days consists of molecule types such as complexes, cytokines, family groups, growth factors, kinases, ligand-dependent nuclear receptors, others, phosphatases transcription regulators and transporters (Table 3.8).

**Table 3.8** - Upstream analysis of activated proteins of CRIS Day 45 TAD conducted in IPA.

| Activated Upstream Regulator | Molecule Type |
|---|---|
| 26S PROTEASOME; IGG | complex |
| CPT1B; GSR | enzyme |
| TGFB2; WNT5A | growth factor |
| ERBB3 | kinase |
| RORC | ligand-dependent nuclear receptor |
| RNASEH2B | other |
| INPP5D | phosphatase |
| EZH2; IRF4; LARP1 | transcription regulator |
| FABP1; SPNS2 | transporter |

### 3.8.2 Betamethasone

#### 3.8.2.1 Inhibited proteins

The inhibited regulators proteins for betamethasone treated AD lesions after 45 days consists of molecule types such as complexes, cytokines, enzymes, fusion gene/products, family groups, growth factors, kinases, ligand-dependent nuclear receptors, others, phosphatases transcription regulators and transporters.

**Table 3.9** - Upstream analysis of inhibited proteins of BETA Day 45 TAD conducted in IPA.

| Inhibited Upstream regulator | Molecular type |
|---|---|
| CD3; LH; IMMUNOGLOBULIN | complex |
| PRL; IL-5; IL-4; CD40LG; IL-2; IL-15; IFNA2 | cytokine |
| SMARCAL1 | enzyme |
| ASPSCR1-TFE3 | fusion gene/product |
| G PROTEIN ALPHA I; G PROTEIN ALPHA; INTERFERON ALPHA | group |
| NGF | growth factor |
| ORAI1 | ion channel |
| TRIB1; MKNK1; STK11; EIF2AK2 | kinase |
| MAVS; SCAP; FADD | other |
| TEAD1; E2F1; XBP1; TFEB;IRF7; MITF; PITX2; MLXIPL; MYCN; TCF7L2; MYCL; IRF5; MYC; EIF4E | Transcription regulator |
| SLC15A4; HBB-B1; HBA1/HBA2 | transporter |

### 3.8.2.2 Activated proteins

The activated regulators proteins for betamethasone treated AD lesions after 45 days consists of molecule types such as cytokines, enzymes, family groups, kinases, other, peptidases, transcription regulators and translation receptors.

**Table 3.10** - Upstream analysis of activated proteins of BETA Day 45 TAD conducted in IPA.

| Activated Upstream regulator | Molecular type |
|---|---|
| CLCF1 | cytokine |
| RNF187; EGLN2; KDM5A; TREX1 | enzyme |
| CDK4/6 (family) | group |
| MAP4K4 | kinase |
| BBS4; BANF1; CST5 | other |
| ADAM10 | peptidase |
| CCND1; MXD1; ETV6 | Transcription regulator |
| LARP1 | Translation regulator |

# Chapter 4: Discussion

# 4 Discussion

## 4.1 AD lesions post-treatments compared to the normal skin after 45 days

### 4.1.1 PCA Analysis

The proteomic data underwent PCA to investigate whether the proteins were lesion specific in different groups such as NS, UAD and TAD. The clustering was performed based on the samples, with the samples grouped to their clinical grouped lesions.

PCA is a valuable technique to visualise extensive datasets and identifying patterns or characteristics that may distinguish between different groups. Although the PCA allows in determining whether the protein expression profiles can differentiate between NS, UAD and TAD samples, under the treatments, with either Betamethasone or Crisaborole.

The overlapping of some samples in the clustering could indicate that the treatments (Betamethasone or Crisaborole) were effective in partially normalising the protein profiles of the AD lesions, potentially bringing them closer to the baseline state of the NS group after 45 days of treatment. The results are significant as the PCA showed was able to distinguish between different groups (figure 3.1, 3.2, 3.3 and 3.4).

These findings are significant because they highlight the capacity of PCA to differentiate between the distinct clinical states of AD lesions, even though the treatment may have a partial or complete effect in returning the lesions to a normal-like state. The ability to distinguish between the treatment and control groups further underscores the potential of these proteomic biomarkers to serve as indicators of disease status and therapeutic response.

### 4.1.2 LIMMA analysis

To investigate whether changes in the proteome profile of AD lesions receiving treatment were approaching the normal state compared to lesions that had not received treated. The proteomic data was analysed through LIMMA, and this was done by seeing the amount of proteins that were significantly changed and to see if there was comparisons with PCA. The amount of proteins were found by the different abundant proteins that were significantly different (FC <-2 and >2, adjusted p-value >0.05).

The data in (table 3.2) shows that 7 proteins were found in Betamethasone day 45 normal skin versus Betamethasone day 45 untreated AD in comparison to 18 proteins in in Betamethasone day 45 normal skin versus Betamethasone day 45 treated AD.

Whereas, that 11 proteins were found in Crisaborole day 45 normal skin versus Crisaborole day 45 untreated AD in comparison to 171 proteins in Crisaborole day 45 normal skin versus Crisaborole day 45 treated AD. When comparing the treatments after 45 days, the amount of proteins found in untreated lesion were lower in both treatments. Whereas the amount of proteins found in treated lesions were higher. As a result, a higher number in differential abundant proteins may indicate that the treatment was able to bring back AD lesions back to normal state after 45 days.

## 4.2 Proteomic profile changes

Differential abundance analysis the proteomic identified potentially significant biomarkers that will allow for the differentiation of the proteomic changes between TS samples of AD collected before and after treatment with the PDE4 inhibitor, Crisaborole, and control groups.

### 4.2.1 Crisaborole

Differential abundance analysis was performed using LIMMA analysis software. From the total of 4929 proteins identified, 493 proteins in Crisaborole day 45 treated AD lesions verses Crisaborole day 1 treated AD lesions were found to be differentially abundant. Of those 493 proteins, the top 20 proteins were identified using adjusted p-value <0.05 and fold change -2 < and >2.

In comparison there was no differential abundant found in Crisaborole day 45 NS verses Crisaborole day 1 NS and Crisaborole day 45 untreated AD lesions verses Crisaborole day 1 untreated AD lesions. As a result, this is expected to be positive, when comparing the proteomic profile between normal skin, untreated AD lesions and treated AD lesions of differential abundant proteins, there should be no changes between normal skin and untreated AD lesions as no treatment was applied, whereas there would be changes in treated AD lesions after 45 days.

Antimicrobial Peptide and Skin Barrier Function

As shown in table 3.3, of the top 20 proteins, 2 proteins such as Protein S100-A7A (S100A7) and ribonuclease A family member 7 (RNASE7) were found to be significantly downregulated in AD.

S100A7 is a component of the family of S100 proteins that is known as an antimicrobial peptide that exhibits antimicrobial against bacteria and a signalling molecule that modulates cellular functions. It is notably abundant and actively secreted in skin conditions characterized by excessive cell proliferation such as AD with a disrupted skin barrier function (Son et al. 2016). The primary and frequently observed features of skin conditions such as AD, include an overgrowth of the epidermal layer and irregular differentiation, which are often accompanied by skin dryness due to a dysfunction in the skin's barrier (Son et al. 2016). Given the tendency of S100A7 to be overproduced and highly secreted in these skin disorders, there is a possibility that S100A7 could play a role in the irregular differentiation of the epidermis, thereby contributing to skin dysfunction. Nonetheless, the exact relationship between the secretion of S100A7 and the overgrowth and abnormal differentiation of the epidermis remains ambiguous (Son et al. 2016). Studies have shown that crisaborole modulates key inflammatory markers in AD lesions such as S100A7. Crisaborole has significantly suppressed expression levels of Th17/Th22 IL-17/IL-22–regulated genes S100A7 in AD (Bissonnette et al. 2019)

RNASE7 is a component of the superfamily of ribonuclease A proteins that is a prominent AMP found on the skin (Becknel et al. 2015). RNASE 7 may significantly contribute to host defense through its antimicrobial properties. This enzyme demonstrates a potent broad-spectrum of antimicrobial activity against a variety of microorganisms, thereby aiding in the regulation of bacterial proliferation on the skin's epidermis (Becknel et al. 2015). In the context of AD, a chronic inflammatory skin

disease, there is an impairment in the expression of AMP, including RNase 7. This observation is rooted in the fact that a predominance of Th2 cytokines has been demonstrated to exert a negative regulatory effect on AMP expression (Becknel et al. 2015). Furthermore, it has been noted that RNase 7 possesses the ability to suppress the production of Th2 cytokines in CD4+ T cells. However, this regulatory effect was found to be diminished in CD4+ T cells obtained from AD patients. These findings suggest that RNase 7 plays a crucial role in the negative regulation of Th2 cytokine expression, a function that may be disrupted in AD, consequently fostering a Th2 cytokine-rich environment. Moreover, RNase 7 has been documented to modulate adaptive immunity (Becknel et al. 2015). Studies have indicated that treatment with RNase 7 can selectively diminish the expression of TH2 cytokines (such as IL-13, IL-4, and IL-5) in activated human CD4+ T-cells and TH2 cells. Nevertheless, T cells isolated from individuals with AD exhibit reduced sensitivity to RNase 7 and secrete elevated levels of IL-13, potentially contributing to the pathogenesis of AD (Becknel et al. 2015).

Although the other 18 downregulated proteins may not be associated with Crisaborole and AD, they're involved in T-cell receptor, Inflammation, different pathways, Interleukins and T helper cells. These are relevant to AD, as the infiltration of T-cells into the affected lesional skin, which contributes to chronic inflammation through the activation of T-cells (Agrawal et al. 2011; Carlier et al. 2021). Additionally, interleukins are essential in the regulation for immune responses and abnormal expression of interleukin significantly contribute to the pathogenesis of AD (Makowska et al. 2023; Prados-Carmona et al. 2024).

T-Cell Signaling and Immune Regulation

Several downregulated proteins such as ITSN2, PRSS2;PRSS3P2, TUBA4A, ARAP2, and GOT1 were identified to be involved in T-cell receptor (TCR) signaling and immune cell regulation.

ITSN2 is crucial for the regulation of T-cell receptor (TCR) down-modulation upon its activation. This is further supported by the observation that extended TCR expression leads to persistent TCR signalling, as evidenced by the increased activation of the ERK1/2 signalling pathway (Locard-Paulent et al. 2020).

PRSS2; PRSS3P2 proteins are classified as members of the trypsin family of serine proteases, tasked with encoding anionic trypsinogen. These proteins are situated within a cluster of trypsinogen genes located in the T cell receptor beta locus, playing a significant role in the process of neutrophil degranulation. A study has shown that another treatment of AD has improved PRSSP3P2 in  93% and 99% in PRSS2 of AD after 2 weeks (Rozenbit et al. 2014).

TUBA4A protein is recognized for its role in the formation and stabilization of microtubules in proliferating T cells throughout different phases of the cell cycle (He et al. 2024). This protein plays a crucial part in spindle formation during mitosis, thereby facilitating proper chromosome segregation and ensuring accurate cell division (He et al. 2024).

The findings of research suggest that ARAP2, a newly identified T cell adaptor protein, has a distinct function in T cells, contributing to both the proximal activation signalling

and inside-out signalling pathways that culminate to integrin activation and T cell adhesion (Jung et al. 2016).

The activation, proliferation, functionality, and differentiation of T cells are intricately associated with appropriate metabolic reprogramming and regulation. Our findings highlight the essential role of GOT1 in facilitating the effector differentiation and functionality of CD8+ T cells. GOT1 supports proliferation by preserving intracellular redox balance and facilitating serine-mediated purine nucleotide biosynthesis. Additionally, GOT1 enhances the glycolytic programming and cytotoxic capabilities of cytotoxic T lymphocytes (Xu et al. 2023).

Inflammatory Response Proteins/Modulation

Key proteins involved in inflammatory responses, including CSTB, PTGES, MGST2, and NAMPT, were also found to be downregulated.

CSTB are a small, multifunctional protein that plays a significant role in regulating inflammation and the innate immune response. Beyond these primary roles, cystatin B has been associated with several other functions, including the maintenance of cellular homeostasis, the reduction of oxidative stress, involvement in autophagy, inhibition of apoptosis, and neuroprotective effects (Contini et al. 2023). Research shows that CSTB engages in protein-protein interactions with multiple proteins that are involved in distinct biological functions, including the degranulation of granulocytes, activation of neutrophils, modulation of the cytoskeleton, antimicrobial defence, and glucose metabolism (Contini et al. 2023).

The domain of autoinflammation is evolving from a focus on genetic factors in innate immune-mediated diseases to a more comprehensive systems-based approach. This approach highlights how various converging molecular pathways, including those involving the actin cytoskeleton, play a role to the autoinflammatory processes and in several conditions marked by the simultaneous presence of inflammation, autoimmunity, and compromised immune responses. The intricate regulation of actin remodelling exemplifies the overlap between of autoinflammatory diseases with immunodeficiencies. While these disorders can manifest a wide range of symptoms, certain characteristics may facilitate diagnosis, such as atypical skin manifestations (spanning from severe viral infections to eczema and sterile abscesses), and defects in chemotaxis and lymphocyte proliferation (Papa et al. 2020).

MGST2 is responsible for the production of leukotriene C4, which plays a crucial role in the intracrine signalling pathways associated with endoplasmic reticulum stress, oxidative DNA damage, and cell death. Additionally, it contributes in the production of prostaglandins, which include several significant targets for the development of anti-inflammatory medications and drugs that disrupt the biosynthesis of prostaglandins and leukotrienes (Thulasingam et al. 2021).

Higher serum levels of NAMPT have been correlated with acute and recurrent eczema in patients (Gesing et al. 2017). Research indicates that NAD+ and PAR metabolism plays a significant role in the development of AD (Arroyo et al. 2023). The excessive activation of PARP1 in keratinocytes, presumably stimulated by NAD+ from NAMPT, worsens the inflammatory response and hyperplasia seen in AD (Arroyo et al. 2023). Therefore,

inhibiting NAMPT and PARP1 pharmacologically effectively results in disease-related inflammation and proliferation, aiding in the restoration of skin homeostasis. This observation is consistent with other previous research on other inflammatory skin diseases (Arroyo et al. 2023).

ERK1/2, MAPK, and NF-κB Signaling Pathways

Several proteins, including ITSN2, S100A14, PTGES, and LYPA1, were identified as components of canonical inflammatory signaling pathways.

For the ITSN2 protein, a study utilised the ITSN2 gene, uncovered that the associated coding protein is integral to the modulation of TCR during engagement. Correspondingly, extended expression of TCR was associated with continuous TCR signalling, as indicated by the heightened activation of the ERK1/2 pathway (Locard-Paulet et al. 2020).

S100A14 protein has been associated with the regulation of various cellular functions, including cell proliferation and apoptosis. Research indicates that the extracellular S100A14 can interacts with RAGE, which triggers the activation of the mitogen-activated protein (MAP) kinase pathway, specifically including the extracellular signal-regulated kinase (ERK) and NF-κB signalling pathways (Basnet et al. 2019). When present in low concentrations, extracellular S100A14 interacts with RAGE, which subsequently activates the ERK1/2 and NF-κB pathways, thereby facilitating the proliferation and survival of ESCC cells. Additionally, intracellular S100A14 interacts with the intracellular domain of ERbB2, resulting to the activation of the ERK1/2 pathway (Basnet et al. 2019).

Microsomal prostaglandin E synthase-1 (PTGES) protein functions as terminal enzyme in the PGE2 synthesis pathway and is activated by the ERK1/2 signalling cascade (Kobayashi et al. 2014). Studies have shown that the expression of this gene is promoted by the proinflammatory cytokine interleukin 1 beta. The protein plays an essential role to the inflammatory response mechanisms within the body (Kobayashi et al. 2014). LYPA1 protein is part of the MAPK pathway.

T Helper Cell Modulation

The proteins involved in T helper cells are PERP. PERP protein plays a fundamental role in to the skin's protective barrier against infections. Additionally, it serves as an effector in the TP53-dependent apoptotic pathway, where it positively modulating apoptosis in T-helper 17 (Th17) cell populations through mechanisms involving caspase-dependent signalling pathways (Zhou et al. 2018).

Epidermal Repair and Calcium Regulation

The proteins involved in epidermal barrier are VAT1. VAT1 plays a beneficial role in the regulation of calcium-dependent keratinocyte activation throughout the process of epidermal repair (Ma et al. 2020).

Unassociated Proteins

These proteins were found to be downregulated but have no association with AD and Crisaborole are SSR4, PSMB4 and NCCRP1.

### 4.2.2. Betamethasone

Differential abundance analysis was performed using LIMMA analysis software. From the total of 4929 proteins identified, 359 proteins in BETA DAY 45 vs BETA DAY 1 TAD were found to be differentially abundant. Of those 359 proteins, the top 20 proteins were identified using adjusted p-value <0.05 and fold change -2 < and >2. In comparison there was 3 differential abundant found in BETA Day 45 NS vs BETA Day 1 NS and BETA Day 45 TAD vs BETA Day 1 TAD.

Downregulation of Inflammatory Pathways: NF-κB Signaling

Of the top 20 proteins, 1 protein such as Nuclear factor NF-kappa-B p105 subunit;Isoform 2 of Nuclear factor NF-kappa-B p105 subunit (NFKB1) were found to be significantly downregulated in AD (table 3.4).

The Nuclear factor NF-kappa-B p105 subunit;Isoform 2 of Nuclear factor NF-kappa-B p105 subunit (NFKB1) protein functions as a transcriptional regulator that is activated in response to both numerous inside and outside the cell factors, such as cytokines, oxidative free radicals, ultraviolet radiation, and components from bacterial or viral origins (National Library of Medicine 2024). Upon activation, NFKB migrates to the nucleus, where it initiates the expression of genes that play essential roles in numerous biological processes. Misregulated activation of NFKB has been implicated in several inflammatory conditions, while sustained inhibition of NFKB can lead to abnormal immune cell maturation or inhibited cellular proliferation (National Library of Medicine 2024).

Of the other 19 different abundant proteins found in the LIMMA analysis are not found to be associated with AD. Although these downregulated proteins may not be associated with Betamethasone and AD, they're involved in and immune response, signalling pathways, Interleukins, immunoglobulin, skin barrier and glucocorticoid.

Glucocorticoid Signaling Modulation

The protein that is associated with glucocorticoid is SGTA. SGTA (Small Glutamine-rich Tetratricopeptide Repeat-containing Protein Alpha), identified as a cochaperone, exhibits a distinct regulatory specificity for the glucocorticoid receptors. Studies have shown that SGTA interacts with and precisely modulates the activity of the receptor (Paul et al. 2014).

Immune Response and Innate Immunity Regulation

The proteins involved in an immune response are PAK4, CHCHD6, HOOK1, SRRM2 and RAB4A. PAK is responsible for modulating the expression levels of IRF and TNF-α, both of which play a significant role in the immune response (Naija et al. 2021).

The MICOS complex subunit MIC25 (CHCHD6) is an essential part of the MICOS complex, a significant protein assembly found within the inner membrane of mitochondria (Chen et al. 2023). Beyond their role in metabolism, mitochondria functions as signalling centres that modulate a range of cellular biological processes, particularly those associated with innate immune responses. This multifaceted relationship between mitochondria and innate immunity encompasses several mechanisms that contribute to the preservation of mitochondrial homeostasis (Chen et

al. 2023). Initially, mitochondria functions as scaffolds for signalling molecules, facilitating the activation of innate immune responses through the assembly of signalling complexes. Numerous interactions have been recognised between innate immunity and different characteristics of mitochondria, such as their membranes, dynamics, components, and metabolites (Chen et al. 2023).

HOOK1 proteins facilitate clathrin-independent endocytosis, which is responsible for the uptake of various plasma membrane proteins that are essential for contributing for the maintenance of homeostasis and regulation of immune functions (Maldonado-Báez et al. 2013).

SRRM2 functions as a scaffold that organizes nuclear speckles, thereby playing a significant role in the regulation of alternative splicing associated with innate immunity and the maintenance of cellular homeostasis (Xu et al. 2022).

RAB4A are one of the various Rab GTPases that play a significant role in the regulation in autophagy. This cellular degradation mechanism is essential for modulating various components of both innate and adaptive immunity, as well as inflammation (Prashar et al. 2017). Autophagy allows for the encapsulation of macromolecules, organelles, or invading pathogens within a double-membrane structure known as the autophagosome, which subsequently fuses with lysosomes to facilitate the breakdown of its contents (Prashar et al. 2017).

Interleukin Signaling and Cytokine Regulation

Several proteins such as CARMILI1, SMARCB1, HPGD, PCDH1 and EIF2AK4 are involved in interleukin signaling. CARMILI1 leucine-rich repeat region is associated with Interleukin-1 (IL-1) signalling. The absence of CARMILI1 significantly lowers IL-1 signalling and decrease in collagen breakdown (Wang et al 2013). Cell-permeable peptides that bind to CARMILI1 and prevent its interaction with IL-1 signalling molecules are effective in inhibiting collagen degradation (Wang et al 2013). CARMILI1 knockout reduces IL-1-induced ERK activation. Therefore, CARMILI1 represents a significant target for the advancement of anti-inflammatory therapeutic agents (Wang et al 2013).

SMARCB1 was found to associate with the promoter of IL-6 under steady-state conditions, but it dissociated during an active immune response. This indicates that SMARCB1 functions as a direct repressor of IL-6, a conclusion that was further validated through both loss- and gain-of-function studies. Overall, research reveals that SMARCB1 is an essential regulatory molecule in both the cell cycle and immune response (Choi et al. 2020). Research has unveiled a novel molecular connection between SMARCB1 and IL-6 in the context of immune reactions, emphasizing the significance of SMARCB1 as a pivotal component in the advancement of therapies for inflammation-related diseases (Choi et al. 2020).

HPGD (hydroxyprostaglandin dehydrogenase 15-[NAD]) which is a PGE2 degrading enzyme was found that the expression levels were decreased in the lesional skin of individuals with AD. Furthermore, the expression if HPGD, which is responsible for the degradation of PGE2, exhibited a negative correlation with the expression if IL-22 (Robb et al. 2018).

PCDH1 genes is linked to AD bronchial hyperreactivity, nonatopic childhood asthma, and transient early wheezing, according to genetics associated studies. The dysfunction of PCDH1 that contributes to a compromised epithelial barrier function, which plays a crucial role in the pathophysiology of AD and asthma (Biswas 2018). The mechanism involves the enhancement of epithelial barrier function. The expression of PCDH1 isoform 2 is elevated through glucocorticoid induction. This isoform exerts a beneficial effect on epithelial barrier integrity, resulting in improved epithelial barrier function and increased PCDH1 expression (Biswas 2018). Additionally, the transcription factor NF-E2 related factor 2 (Nrf2), which safeguards cells against oxidative stress, is also instrumental in maintaining the stability of the airway epithelium following glucocorticoid treatment (Biswas 2018).

EIF2AK4 protein has been identified to play a crucial role as a modulator of cytokine production and macrophage activity in various diseases (Yu et al. 2022). In studies involving EIF2AK4 knockout mice that were challenged with lipopolysaccharide, exhibited a significantly reduced in the inflammatory response was observed, with decreased levels of IL-6 and IL-12 (Yu et al. 2022). In the context of inflammatory diseases, EIF2AK4, when activated by IFN-γ, has the capacity to inhibit the production of proinflammatory cytokines and attenuate macrophage activity (Yu et al. 2022).

Immunoglobulin and Humoral Response

ALG1, involved in glycosylation, is essential for proper antibody production. Individuals who are impacted may exhibit unusually low production of proteins referred to as antibodies, with a particular emphasis on IgG (National Library of Medicine 2017).

Skin Barrier Integrity and Pruritus

Proteins associated with skin barrier function and pruritus are VIPAS39 and CCDC47. VIPAS39 protein has exhibited irregular morphology and positioning of lamellar bodies, accompanied by a decrease in the thickness of corneocytes and the accumulation of lipids within the stratum corneum (Lee 2020). By downregulating the VIPAS39 protein, this may increase the thickness of corneocytes and the accumulation of lipids within the stratum corneum of AD (Lee 2020). CCDC47 can be characterised by pruritus (Morimoto et al. 2018).

Additional Signaling Proteins

XPO7, a nuclear export protein involved in NF-κB p65 subunit trafficking, was found to be downregulated(Liang et al. 2013). XPO7 knockdown has been associated with reduced nuclear accumulation of p65 and decreased NF-κB transcriptional activity, reinforcing the suppression of inflammatory signaling observed with NFKB1 (Liang et al. 2013). Additionally, XPO7 was found to interact with p65 in a manner dependent on TNF-α (Liang et al. 2013). While several differentially abundant proteins like AMDHD2, BIN3, NEBL were not directly linked to known pathways in AD or Betamethasone action, their roles in broader cellular processes merit further investigation.

### 4.2.3. Differentially Abundant Proteins found in Betamethasone for NS and UAD

When LIMMA statistical analysis was conducted to find differentially abundant proteins using adjusted p-value 0.05 and fold change -2< and >2, there were 3 proteins found in Beta Day 45 NS vs Beta Day 1 NS and 3 proteins found in Beta Day 45 UAD vs Beta Day 1 NS UAD. Although there should not be any observed changes in NS and UAD after 45 days from baseline, these proteins (table 3.5 ) found may be due to other factors. The following 3 proteins were found in Beta Day 45 NS vs Beta Day 1 NS are SKIC2, PSMD4, SLC17A5 and the following 3 proteins were found in Beta Day 45 UAD vs Beta Day 1 UAD are GRN, PNPT1, STRIP1.

The following 3 proteins were found in Beta Day 45 NS vs Beta Day 1 NS are SKIC2, PSMD4, SLC17A5. The proteins SKIC2 and PSMD4 are not associated with AD or BETA. In addition, SLC17A5 has been found to been associated in the skin as research indicates that free sialic acid has been identified in various tissues, including the skin (Laudau et al. 2004).

Whereas, the following 3 proteins were found in Beta Day 45 UAD vs Beta Day 1 UAD are GRN, PNPT1, STRIP1. The protein STRIP1 is not associated with AD or BETA. The proteins GRN and PNPT1 are associated with immune response and inflammation (Jian et al. 2013; Hsu et al. 2023). GRN and its associated binding partners form intricate networks that are essential for play a role in mediating the pathogenesis of a range of pathophysiological processes, such as immunity, infection, and inflammation. It is likely that additional GRN-binding proteins will be recognised in diverse tissues or under varying physiological or pathological states (Jian et al. 2013). PNPT1 plays a crucial role in modulating the activation of the NLRP3 inflammasome through MAVS and a shift towards glycolysis, thereby underscoring the significance of mitochondrial signalling and glucose metabolism in the regulation of inflammation (Hsu et al. 2023). A deeper exploration of the interactions among PNPT1, mitochondrial stability, and immune responses may provide valuable insights into the mechanisms underlying inflammatory diseases (Hsu et al. 2023).

### 4.2.4 Shared proteins between treatments of Crisaborole and Betamethasone treatments

After LIMMA analysis, when comparing the proteins between the treatments of Crisaborole and Betamethasone, there was 1 shared protein found between Beta Day 45 NS vs Beta Day 1 NS and Beta Day 45 TAD vs Beta Day 1 TAD. Also, there was 3 shared proteins found between Beta Day 45 TAD vs Beta Day 1 TAD and Cris Day 45 TAD vs Cris Day 1 TAD, this shown in table 3.6.

The SKIC2 protein was found to be shared between Beta Day 45 NS vs Beta Day 1 NS and Beta Day 45 TAD vs Beta Day 1 TAD is not associated with AD or BETA. The proteins ARHGEF10L and MINK1 are found to be shared between CRIS Day 45 TAD vs CRIS Day 1 TAD and Beta Day 45 TAD vs Beta Day 1 TAD is not associated with AD. Misshapen Like Kinase 1 (MINK) is a serine/threonine kinase that is part of the germinal centre kinase (GCK) family. It shares structural characteristics with kinases linked to NIK, suggesting that it may constitute a distinct subfamily of NIK-related kinases within the GCK family

MINK has the ability to activate the JNK and MAPK14/p38 pathways, thereby promoting the activation of the stress-activated protein kinase MAPK14/p38 MAPK downstream of the Raf/ERK cascade (Gene card. 2024). Whereas, AP3D1 protein plays a play a crucial role in various physiological functions, such as the skin (Ammann et al. 2016).

The significance of these findings is that these proteins are shared between both treatments of crisaborole and betamethasone. When shared proteins are found in two treatments, it means that the same proteins exhibit significant changes (in expression levels, abundance, or activation status) in response to both treatments. This can indicate common mechanisms of action and shared biological pathways, between the treatments (Geyer et al. 2017; Van den Bossche et al. 2017). For mechanism of action/ mechanist insight, shared proteins can indicate that the treatments influence similar cellular processes or pathways such as immune regulation and skin barrier repair. For biomarker potential, shared proteins could serve as biomarkers for treatment efficacy or disease state, especially if they are consistently modulated across effective therapies (Geyer et al. 2017; Van den Bossche et al. 2017). Although these proteins are not associated with AD and the treatments, they may lead to further research given that these proteins were found to be significant in both treatments.

## 4.3 Ingenuity Pathways Bioinformatic Analysis of Differentially Abundant Proteins

### 4.3.1 Crisaborole

Significant differential abundant proteins (p value <0.05, FC <-2 and >2) were uploaded onto the IPA environment, this was done to assess for potential inhibited or activated upstream regulators. These proteins were found in the upstream analysis and found the following proteins in table 3.7 and 3.8.

#### 4.3.1.1 Inhibited proteins

The inhibited proteins found in the upstream analysis of IPA has shown that there's a link between AD and the PDE4 pathways with the proteome changes in the use of Crisaborole on TAD skin. These proteins are CD cells (CD3), cytokines (IL-1A and IL-1B, IL-2, IL-4, IL-5, IL-13, IL-17A and IL-22), Nuclear Factor Kappa B (NF-KB and NF-kB1), Tumor necrosis factor (TNF) and Interferon (IFN). (table 3.7).

Cytokines and Inflammatory Mediators

Crisaborole significantly suppresses pro-inflammatory cytokines central to AD pathology. Key cytokines inhibited include IL-1A, IL-1B, IL2, IL-4, IL-5, IL-13, IL-17A, and IL-22. Studies demonstrate that Crisaborole markedly reduces IL-1A, IL-1B, and IL-17A levels, and also inhibits IL-2 and IL-5 secretion (Bissonnette et al, 2019; Therapeutic Goods Administration, 2019). Studies have shown that suppression of IL-4 and IL-13 is particularly impactful, as it reverses skin barrier dysfunction and restores expression of differentiation markers like filaggrin, loricrin, and involucrin (Facheris et al, 2023; Turchin et al, 2022). Additionally, there's no disruption to tight cell junctions and no susceptibility to infections (Facheris et al. 2023; Turchin et al. 2022). IL-22 inhibition has also been tied to improvements in epidermal pathology, including a decrease in markers of hyperplasia and proliferation (Bissonnette et al, 2019).

Immune Cell Markers

Crisaborole affects immune cell infiltration, as reflected by reductions in CD3+ T cells, specifically CD31 T-cells and CD111 dendritic cells (Bissonnette et al. 2019). This suggests a broader immunomodulatory role of the drug beyond cytokine suppression. (Bissonnette et al. 2019).

NF-κB and TNF Pathways

The inhibition of PDE4 elevates intracellular cAMP, which in turn suppresses NF-κB activity. This suppression downregulates multiple inflammatory mediators/pathways/cytokines, including TNF, further supporting Crisaborole anti-inflammatory efficacy (Zane et al, 2016; Therapeutics Goods Administration 2019.

Interferons

Crisaborole significantly inhibits interferon-gamma (IFN-γ) production, indicating a broad suppression of Th1-mediated inflammatory mediators/responses in AD (Bissonnette et al. 2019).

In contrast, a subset of  inhibited proteins found in the upstream analysis of IPA have shown that there's no direct association between these proteins and the PDE4 pathway with the use of Crisaborole on TAD but are relevant to AD. These proteins that shown the proteomic changes such as Immunoglobulin, IL-6 , CD40LG, VEGF, TGFB1, HBEGF, EGF, IGF1 , EGFR, RAF1, TFEB, BHLHE40, PPARGC1A, STAT4 and STAT5B , EPAS1, STAT3, SIGLEC8, IGF1R, CD28 and LRP1.

Cytokines and Growth Factors

IL-6 shows plays a critical role in the pathogenesis of dermatitis, and it's elevated levels correlate with the clinical severity of the disease pathogenesis of AD (Naji et al. 2022). TGF- β1 shows that there is a dysregulation in the TGF-β1 signalling pathway in AD. Furthermore, protein expression of TGF-β1, along with its correlation with the disease severity, holds considerable clinical significance and emphasizes its potential role in AD pathogenesis (Shafi et al. 2024). VEGF is linked to vascular changes that is associated thickened epidermis (in the chronic phase) or intercellular oedema (in the acute phase) (Lee et al. 2021). The inhibition of CD40L is associated with decreased disease severity (Zheng et al. 2023).

Immunoglobulin and Mast Cell Modulators

For the immunoglobulin complex, IgE plays a role in the pathogenesis  of AD and serum levels of IgE was found to be significantly greater in patients with AD (Juhlin et al. 1969). The inhibition of SIGLEC8 supresses acute and chronic inflammation, mast cells and reduces IgE mediated histamine, prostaglandin D and cytokine release (Youngblood et al. 2020).

Transcription and Signaling Pathways

STAT4 and STAT5B is a Signal Transducer and Activator of Transcription (STAT) pathway that are central components of cytokine-stimulated signalling in inflammatory diseases

(Yan et al. 2022). STAT3 inhibition improves pruritus and inflammation-related cytokines in AD (Takahashi et al. 2023). BHLHE40 was identified as a key regulator of inflammation (Pasanen et al. 2024). PPARGC1A inhibition of PPARy reduces the clinical symptoms of AD (Majewski et al. 2021). For EPAS1, research shows that the genetic or pharmacological inhibition of EPAS1 suppresses IL-31 induction in $CD4^+$ T cells from AD patients (Yamamura et al. 2017). CD28 inhibition is linked to reduced skin lesions of patients with AD (Neuber et al. 2006).

Epithelial and Growth-Related Proteins

While not directly linked to Crisaborole, proteins such as HBEGF, EGF, IGF1, EGFR, IGF1R, RAF1, TFEB, and LRP1 are associated with AD, playing roles in keratinocyte function, epidermal regeneration, and inflammatory signaling.

#### 4.3.1.2 Activated proteins

In IPA analysis, these activated proteins were found in the upstream analysis (table 3.8). These activated proteins found in the upstream analysis of IPA have shown that there's no association between these proteins and the PDE4 pathway with the use of Crisaborole on TAD but may be relevant. These proteins that shown the proteomic changes such as 26S PROTEASOME, IGG ERBB3, RORC, EZH2, FABP1. These activated proteins found in the upstream analysis of IPA have shown that there's no association between these proteins and the PDE4 pathway with the use of Crisaborole on TAD. These proteins that shown the proteome changes such as CPT1B, GSR, TGFB2, WNT5A, RNASEH2B, INPP5D, IRF4, LARP1, SPNS2.

Proteins Involved in Cellular Regulation and Gene Expression

Among the activated proteins, ERBB3, RORC, and EZH2 are associated with transcriptional regulation and signal transduction. ERBB3 is a member of the epidermal growth factor receptor family and plays a role in cell proliferation and survival (Palaniswamy et al, 2021). Though not linked to Crisaborole PDE4 inhibition, its activation may reflect regenerative or compensatory signaling in keratinocytes. RORC (RAR-related orphan receptor gamma), a key regulator of Th17 cell differentiation, may signal ongoing immune activation or a shift in T cell populations (Ivanov et al. 2006). EZH2, a histone methyltransferase, contributes to epigenetic regulation and has been implicated in inflammatory responses and skin barrier function (Yang et al. 2022, Gan et al. 2018).

Metabolic and Proteolytic Pathway Regulators

Proteins such as FABP1 (fatty acid binding protein 1), 26S proteasome, and CPT1B (carnitine palmitoyltransferase 1B) suggest metabolic reprogramming and protein turnover in the skin. FABP1 is involved in lipid metabolism, possibly reflecting changes in the lipid barrier following treatment (Smathers and Petersen. 2011; Shamaprasad et al. 2024). Activation of the 26S proteasome may indicate increased protein degradation and cellular turnover (Kloetzel. 2004). Similarly, CPT1B is a mitochondrial enzyme essential for fatty acid oxidation, potentially responding to altered energy demands in inflamed or healing skin (GeneCard 2014; Wang et al. 2024).

Immune Modulators and Inflammatory Regulators

Several proteins activated in the IPA analysis such as TGFB2, WNT5A, IRF4, and INPP5D are known immune modulators. TGFB2, a member of the transforming growth factor-beta family, plays a dual role in inflammation and tissue repair. Its activation might suggest compensatory tissue remodeling (Letterio and Roberts. 1998). WNT5A, part of the non-canonical Wnt signaling pathway, is associated with inflammation and skin morphogenesis (Kim et al. 2010; Zhao et al. 2014). IRF4 is critical in the regulation of immune cell differentiation and may reflect ongoing immune responses (Nam and Lim. 2016; Lu et al. 2023). INPP5D, also known as SHIP1, negatively regulates PI3K signaling, suggesting modulation of inflammatory pathways (Gold et al. 2015; Olufunmilayo and Holsinger. 2023).

Nucleic Acid and Membrane Transport Proteins

RNASEH2B, LARP1, and SPNS2 represent proteins involved in nucleic acid metabolism and cellular transport. RNASEH2B plays a role in genome stability and may be upregulated as a response to inflammation-induced stress (Crow et al, 2006). LARP1 is involved in mRNA stability and translation, possibly reflecting altered protein synthesis in the skin (Ogami et al. 2022; Lui et al. 2025). SPNS2, a transporter of sphingosine-1-phosphate, regulates lymphocyte trafficking and could influence inflammatory cell migration (Baeyens et al. 2015; Okuniewska et al. 2021).

## 4.3.2 Betamethasone

### 4.3.2.1 Inhibited proteins

Significant differential abundant proteins (p value <0.05, FC <-2 and >2) were uploaded onto the IPA environment, this was done to assess for potential inhibited or activated upstream regulators. These proteins were found in the upstream analysis and found the following proteins in table 3.9 and 3.10.

The inhibited proteins found in the upstream analysis of IPA has shown that there's a link between  with the proteomic changes in the use of Betamethasone on TAD skin. These proteins that shown the proteomic changes  such as Immunoglobulin, CD cells (CD3), cytokines ( IL2, IL-4, IL-5, IL-15) and CD40LG (table 3.9).

Cytokines: IL2, IL-4, IL-5, and IL-15

Betamethasone significantly suppresses pro-inflammatory cytokines, including IL2 and IL-5, reducing inflammatory cell infiltration and skin inflammation (Strehl et al. 2019). IL-4 inhibition has been shown to reverse skin barrier dysfunction by promoting the expression of key epidermal differentiation proteins—filaggrin, loricrin, and involucrin—while preserving tight junction integrity and reducing infection susceptibility (Facheris et al. 2023; Turchin et al. 2022). Inhibition of IL-15 contributes to a decrease in epidermal hyperplasia, commonly observed in AD (Karlen et al. 2020).

T-Cell Markers and Co-stimulatory Molecules: CD3 and CD40LG

Betamethasone also leads to reductions in CD3+ T-cell infiltration and CD111+ dendritic cells, aligning with suppressed cytokine activity (Bissonnette et al. 2019). The inhibition of CD40LG, a critical co-stimulatory molecule, correlates with reduced disease severity in AD (Zheng et al. 2023).

Immunoglobulin Complex

Although there is no direct evidence linking Betamethasone to immunoglobulin inhibition, IgE remains a key player in AD pathogenesis, with elevated serum levels noted in affected individuals (Wollenberg et al. 2021). The downstream suppression of inflammation may thus indirectly influence IgE-related pathways.

Some proteins, though not clearly associated with Betamethasone or PDE4 inhibition, were inhibited and may contribute to the broader TAD phenotype. These include INFA2, SCAP, TFEB, MYC, and EIF4E. These regulators are associated with transcriptional control, immune signaling, and stress response pathways.

Several proteins were found to be inhibited in the upstream analysis of IPA have shown that there's no association between these proteins and with the use of Betamethasone on TAD. These include hormonal and signaling molecules such as PRL, LH, and G-protein alpha subunits (Gαi, Gα); transcription factors and regulators like MYCN, MITF, E2F1, XBP1, TEAD1, MLXIPL, TCF7L2, PITX2, and TRIB1; immune and signaling mediators including SMARCAL1, ASPSCR1-TFE3, STK11, EIF2AK2, MAVS, and FADD; as well as transport and metabolic proteins such as SLC15A4, Hbb-b1, and HBA1/HBA.

#### 4.3.2.2 Activated proteins.

In IPA analysis, these activated proteins were found in the upstream analysis (table 3.10) These activated proteins found in the upstream analysis of IPA have shown that there's may have an association between these proteins and TAD but not with Betamethasone. These proteins that shown the proteomic changes such as CLCF1 which is a cytokine implicated in immune cell communication; CDK4/6 are cell cycle regulators possibly linked to epidermal proliferation and ADAM10 which is a metalloprotease involved in shedding of membrane proteins and skin barrier integrity.

These activated proteins found in the upstream analysis of IPA have shown that there's no association between these proteins and with the use of Betamethasone on TAD. These proteins include epigenetic and transcriptional regulators such KDM5A, ETV6, MXD1, BANF1; stress and DNA repair-related proteins such as TREX1, RNF187, EGLN2, MAP4K4 and transport/metabolic proteins: CST5, BBS4, LARP1.

## 4.4 Canonical Pathways

The IPA bioinformatic analysis of canonical pathways provide a better understanding of the mechanisms involved in Crisaborole TAD and Betamethasone TAD after 45 days (Figure 3.5 and 3.6).

### 4.4.1 Crisaborole

211 canonical pathways were predicted to be significantly changed in CRIS Day 45 TAD lesions (p-value <0.05 and absolute z-score ≥2) samples. Of these, 5 pathways,

including RHOGDI signalling, Mitochondrial Dysfunction, Antioxidant Action of Vitamin C, SPINK1 Pancreatic Cancer Pathway, Sleep NREM Signalling pathway were upregulated. The remaining 206 included neutrophil degranulation, SRP-dependent cotranslational protein targeting to membrane, EIF2 were downregulated. The top 10 most active canonical biological pathways are illustrated in Figure 3.5.

### 4.4.2 Betamethasone

64 canonical pathways were predicted to be significantly changed in CRIS DAY 45 TAD AD lesions (p-value <0.05 and absolute z-score ≥2) samples. Of these 1 pathway, including granzyme A signalling were upregulated. The remaining 63 included were Processing of Capped Intron-Containing Pre-mRNA, ISG15 antiviral mechanism, SUMOylation of DNA replication proteins were downregulated. The top 10 most active canonical biological pathways are illustrated in Figure 3.6.

When comparing the canonical biological pathways between Crisaborole and Betamethasone Day 45 TAD. The upregulated the canonical biological pathways between Crisaborole and Betamethasone Day 45 TAD were different. In Crisaborole, the 5 pathways, including RHOGDI signalling, Mitochondrial Dysfunction, Antioxidant Action of Vitamin C, SPINK1 Pancreatic Cancer Pathway, Sleep NREM Signalling pathway were upregulated. Whereas in Betamethasone, 1 pathway, including granzyme A signalling were upregulated. Although these upregulated canonical pathways may not be associated with AD, this might provide an understanding for future research.

By highlighting the similarities and differences of Crisaborole and Betamethasone, this will show how the 2 medications work. Crisaborole is a nonsteroidal phosphodiesterase inhibitor, whereas Betamethasone is a topical corticosteroid. Due to its nonsteroidal nature, the local side effects associated with crisaborole are not expected to be as severe as those associated with topical corticosteroids (Danby et al. 2023; Eichenfield et al. 2017). However, the extensive alterations in dermal vascularity and collagen matrix that were observed in patients treated with betamethasone valerate compared with crisaborole may be indicative of the development of clinical adverse reactions, including striae (strain of the blood vessels in the extremities), telangiectomy, and the development of skin atrophy (skin lesions) (Danby et al. 2023; Eichenfield et al. 2017). As a result, it is likely that crisaborole should be used as part of a proactive, long-term treatment plan when TCS-related adverse reactions are more likely to occur due to a positive profile (Danby et al. 2023; Eichenfield et al. 2017). Crisaborole and Betamethasone treatments have the same inhibited regulators that were found in the IPA such as immunoglobulin, CD3, IL-2,IL-4, IL-5. NF-KB was found to be inhibited in Betamethasone via LIMMA analysis and in IPA for Crisaborole . Also, these shared proteins, were found in both treatments such as AP3D1, ARHGEF10L and MINK1.

The overlap of findings from IPA and LIMMA analyses highlights different yet complementary insights into how Crisaborole and Betamethasone modulate inflammatory pathways in AD. Some regulators, like immunoglobulins and T-cell–related cytokines (IL-2, IL-4, IL-5), were revealed by IPA as inhibited in both treatments, suggesting that each drug suppresses common immune activation signals, likely contributing to reduced inflammation. The identification of NF-κB as inhibited by

Betamethasone through LIMMA and by Crisaborole via IPA emphasizes how a single pathway can be detected through different analytical lenses.

## 4.5 Limitations

The limitation of this study involves the sample collection from only one study location (Westmead hospital), this limited the number of patients being recruited as the clinic has the same continuous patients that attended, and majority of patients were managing their AD that didn't require the study, which resulted in a small sample size. Other factors that can occur during collection and preparation of samples include a number of people being involved, this results in data analysis and protein identification being affected. The factors involved could be due to how various people applying pressure on the tape strip when collection samples from patients, misplaced samples, laboratory issues when preparing the samples and technical issues with data acquisition from sample runs on MS. Additionally, there were 2 people involved at the beginning of the study with the sample collection before I was given the project to continue the study which affects the pressure applied on tape strip when collecting skin samples, the proteomics sample preparation was done myself and MS data was acquired after the sample preparation process.

Limitations of tape stripping involves inconsistent attachment of the skin to adhesive tape could result in a poor association between the evaluation of proteins levels and the thickness of the epidermal layer, and this technique exhibits a constrain in its depth for action and predominantly focuses in the SC. It may be challenging to attain deeper layers of the epidermis. Low abundance, along with the presence of other functional and structural proteins, may have interfered with the detection of PDE4 proteins, making it difficult to measure them or observe any changes. To minimise human error during sample collection, a D-Squame pressure applicator can be used to ensure consistent pressure when applying adhesive discs. This spring-loaded device minimises pressure fluctuations that might otherwise affect the number of proteins adhering to the disc.

A key limitation of this study is the small sample size, which reduces the statistical power and may limit the reliability of the results. With fewer samples, there is a higher chance that observed differences are due to random variation rather than true biological effects. It also makes it harder to capture the natural variability seen in atopic dermatitis across individuals. As such, the findings should be considered preliminary and need validation in larger, independent cohorts to confirm their biological and clinical relevance.

Additionally, it was observed that the BSA of several patients may have worsened whilst on treatment. This may be due to factors such as natural disease fluctuation, a delayed or insufficient response and non-adherent to treatment. Furthermore, there were no proteins quantified by 1 peptide and no  proteins were found to be significantly altered that were the ones identified by 1 or 2 peptides. Proteins identified by a low number of peptides can still be significant on LIMMA analysis, but their abundance  can be overestimated or underestimated due to low detection.

## 4.6 Future Direction

The use of a non-invasive, scarless technique offers a cost effective and efficient method for sampling skin lesions. This approach holds significant potential to advance research in AD and facilitate the development of future interventions. Australia is home to many rural and remote communities that have limited access to specialised healthcare resources. The technique like adhesive discs, which could be employed by general practitioners or the community for sample collection and subsequent analysis, would offer substantial benefits. The results of this study are very exciting but require further validation and wider cohort. Further analysis of the data may help to understand better the outcomes of the scarless samples.

Other techniques to obtain skin samples might be used is suction blistering, punch, incisional, or excisional. Suction blistering is a type of sampling that facilitates access to the deeper layers of the epidermis by employing a vacuum pump to separate the epidermis from the dermis. Although this technique requires a considerable amount of time, it is associated with minimal discomfort and is less invasive compared to punch biopsies. Other techniques such as 2D Gel Electrophoresis (2D-GE), protein microarrays, western blotting/immunoassays could be used to discover changes in proteins. Additionally, future research would benefit from an increased sample size and the collection of samples from diverse centres and international cohorts, as this would enhance statistical power and population representation, potentially leading to the discovery of new research directions.

## 4.7 Conclusion

The use of tape stripping sample collection coupled with proteomics and extensive bioinformatics analysis allows an understanding of the proteomic differences between TS samples of AD collected before and after treatment with the PDE4 inhibitor, Crisaborole, and control groups. As a result of this study, this allows the discovery in the effect of PDE4 inhibitors and how Crisaborole effects adaptive immunity, inflammation, skin barrier and repair, and its potential mechanism of action and potentially investigate and identify alternative or complementary mechanism of action of Crisaborole.

This study showed that tape stripping was able to find the proteomic changes of between NS, UAD and TAD after the use if Crisaborole after 45 days. The proteins found has shown that there's a link between AD and the PDE4 pathways with the proteome changes in the use of Crisaborole on AD skin. The effect of Crisaborole on AD showed that inflammatory pathways, mediators and cytokines were inhibited. Furthermore, there's improvement of the skin barrier that activates the expression of keratinocytes differentiation molecules such as filaggrin, loricrin, and involucrin. Additionally, there's a decrease in disease severity, supresses itch, clinical symptoms of AD and skin lesions.

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