Novel, non-invasive approaches for the diagnosis of inflammatory skin diseases

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Psoriasis and eczema, among the most common dermatological conditions, are complex inflammatory dermatoses with distinguished underlying pathomechanisms. However, their similar clinical appearance at certain anatomical locations or in case of minimal or chronic inflammation often represents a diagnostic challenge. Establishing the correct diagnosis is essential not only from a therapeutic perspective, but also for correctly assessing, treating and potentially preventing important associated comorbidities. Skin biopsy followed by histopathology is the current diagnostic gold standard. Nevertheless, it is an invasive, costly procedure requiring specialised skills and equipment, which limits its clinical and research use.

This thesis therefore focused on tape stripping and its potential 1) as a non-invasive diagnostic tool to distinguish psoriasis from eczema with the identification of disease specific biomarkers, 2) as a research tool to facilitate the pathomechanistic understanding of these, 3) to detect early molecular changes in subclinical inflammation, 4) in the prediction of therapeutic response and disease follow up.

The main results of the thesis are the identification of IL36γ and elafin as highly specific and sensitive psoriasis biomarkers which allow its differentiation from eczema and the use of tape stripping as a non-invasive diagnostic tool even in clinically challenging cases. Secondly, 644 proteins across lesional, non-lesional and healthy tape stripping samples have been identified through mass spectrometry, with 90 proteins being differentially expressed between psoriasis and healthy samples, with a potential use in molecular subtyping. Furthermore, subtle molecular changes in non-lesional samples were successfully identified. Finally, preliminary data in cell culture experiments showed modulation of IL36γ and/or elafin expression following psoriasis-relevant treatments.

Overall, tape stripping proves to be a viable non-invasive method to identify different patterns of epidermal inflammation, facilitate diagnostic accuracy, treatment follow up and personalized medicine approaches, which could complement and even replace biopsies in the future.

Table of Contents

Table of Figures

List of Tables

Abbreviations

ACD	**allergic contact dermatitis**
AD	**atopic dermatitis**
AK	**adenylate kinase**
AKDR	**AK detection reagent**
ALDOA	**fructose-bisphosphate aldolase A**
AMP	**antimicrobial peptide**
ANOVA	**analysis of variance**
APOA1	**apolipoprotein A-I**
ARG1	**arginase1**
ASPRV1	**retroviral-like aspartic protease 1**
AUC	**area under the curve**
Bak	**pro-apoptotic BCL2-antagonist/killer**
Bax	**BCL2 associated X protein**
BCA	**bicinchoninic acid assay**
Bcl-2	**B-cell lymphoma 2**
Bcl-X	**B-cell lymphoma-X**
BH	**bleomycin hydrolase**
BMP2	**bone morphogenetic protein 2**
BSA	**bovine serum albumin**
BTM	**betamethasone dipropionate**
CALML	**calmodulin-like protein**
CARD14	**caspase recruitment domain-containing protein 14**
CAT	**catalase**
CCL	**CC-chemokine ligand**
CDLE	**cutaneous discoid lupus erythematosus**
CFL1	**cofilin 1**
CRABP2	**cellular retinoic acid-binding protein 2**

CTACK	**cutaneous T cell attracting chemokine**
CVD	**cardiovascular disease**
CXCL	**C-X-C Motif Chemokine Ligand**
CysA	**cyclosporine A**
DAMP	**damage-associated molecular patterns**
DC	**dendritic cell**
DITRA	**deficiency in IL36RA**
DLQI	**Dermatology Life Quality Index**
DMSO	**dimethyl sulfoxide**
EASI	**Eczema Area and Severity Index**
ECL	**enhanced chemiluminescence**
EDC	**epidermal differentiation complex**
EDTA	**ethylenediaminetetraacetic acid**
EGF	**epidermal growth factor**
ELISA	**enzyme-linked immunosorbent assay**
FCS	**foetal calf serum**
FGF	**fibroblast growth factor**
GM-CSF	**granulocyte-macrophage colony-stimulating factor**
GPP	**generalised pustular psoriasis**
hBD	**human β defensins**
HDL	**high-density lipoprotein**
HE	**haematoxylin-eosin stained**
HIV	**Human Immunodeficiency Virus**
HLA	**human leukocyte antigen**
HNP	**human neutrophil peptides**
HRP	**horse radish peroxidase**
HSP	**heat shock protein**
IBD	**inflammatory bowel disease**

ICD	**irritant contact dermatitis**
IFNα	**interferon-α**
Ig E	**immunoglobulin E**
IL	**interleukin**
ILC	**innate lymphoid cell**
iNOS	**inducible nitric oxidase synthase**
IP10	**interferon gamma-induced protein 10**
I-TAC	**interferon-inducible T-cell alpha chemoattractant**
JAK	**janus kinase**
JNK	**c-Jun N-terminal kinase**
K	**keratin**
KBM2	**keratinocyte basal medium-2**
KDR	**kinase insert domain receptor**
KLK	**kallikrein**
LC-MS-MS	**liquid chromatography tandem mass spectrometry**
LDH	**lactate dehydrogenase**
LDL	**low-density lipoprotein**
LIF1	**leukemia inhibitory factor 1**
LIMK1	**LIM kinase 1**
LPS	**lipopolysaccharides**
MAPK	**mitogen-activated protein kinase**
MDC	**macrophage-derived chemokine**
mDCs	**myeloid dendritic cells**
MF	**mycosis fungoides**
MIG	**monokine induced by gamma interferon**
MKP1	**MAPK phosphatase**
mRNA	**messenger RNA**
MTX	**methotrexate**

NA	**nucleic acids**
NFAT	**nuclear factor of activated T-cells**
NF-κB	**nuclear factor-kappa B**
NGF	**nerve growth factor**
NMF	**natural moisturising factor**
NO	**nitric oxide**
nUVB	**narrowband ultraviolet B**
OCT	**optical coherence tomography**
PASI	**Psoriasis Area and Severity Index**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**
pDCs	**plasmacytoid dendritic cells**
PPP	**palmoplantar pustular psoriasis**
PsA	**psoriatic arthritis**
PSAPL1	**proactivator polypeptide-like 1**
PUVA	**psoralen and ultraviolet A**
RCM	**reflectance confocal microscopy**
RePUVA	**retinoid PUVA**
RIN	**RNA Integrity Number**
RIPA	**radioimmunoprecipitation assay buffer**
ROS	**reactive oxygen species**
RT-qPCR	**reverse transcriptase quantitative PCR**
SAg	**superantigen**
SA-PE	**streptavidin-phycoerythrin**
SCLE	**subacute cutaneous lupus erythematosus**
SCORAD	**SCORing Atopic Dermatitis**
SE	**standard error**
SPE	**solid phase extraction**

SPINK5	**serine protease inhibitor Kazal-type 5**
SSCA	**squamous cell carcinoma antigen**
TARC	**thymus and activation-regulated chemokine**
Tc1	**type 1 cytotoxic T cell**
Tc17	**type 17 cytotoxic T cell**
Tc22	**type 22 cytotoxic T cell**
TEWL	**transepidermal water loss**
Th	**T helper cell**
TLR	**toll-like receptor**
TNFα	**tumour necrosis factor**
TSLP	**thymic stromal lymphopoietin**

Chapter 1 Introduction

1.1 Anatomy and physiology of the skin

The skin is a complex organ with numerous functions. It has three main compartments: the ***epidermis***, ***dermis*** and ***hypodermis***. The ***epidermis*** is in continuous contact with the external environment and serves as a protective barrier. The underlying ***dermis*** harbours hair follicles, sebaceous and sweat glands. It is well vascularised and innervated, allowing nutrient delivery and maintenance of a functional epidermis, immune surveillance by circulating leukocytes, and reaction to homeostatic changes such as change in temperature. The deeper ***subcutaneous tissue*** or ***hypodermis*** mainly consists of adipocytes, with energy storing capacity and endocrine function and a role in hair follicle regeneration. Between the epidermis and dermis lies the ***dermal-epidermal junction***, a complex structure in itself.

1.1.1 The epidermis: the journey of a keratinocyte

The epidermis is a constantly renewing squamous epithelium, stratified into 5 different layers:

1) the **basal cell layer** or stratum basale/stratum germinativum;

2) the **squamous cell layer** or *stratum spinosum*;

3) the **granular layer** or *stratum granulosum*;

4) the **clear layer** or *stratum lucidum*;

5) and the **cornified/horny layer** or *stratum corneum*.

Epidermal layers are defined by the differentiation and migration of keratinocytes from the deep germinative layer to the surface of the skin. Other cells are also present in the epidermis but in a relatively smaller number, represented by melanocytes (pigment producing cells), Langerhans cells (the main skin-resident dendritic cell) and T cells (mainly CD8+ cytotoxic lymphocytes in the basal and spinous layers) [1].

The keratinocytes' journey starts with their generation from progenitor cells in the **basal layer**. As they travel upwards their differentiation involves transcription of specific genes which characterise differentiation stages, resulting in the production of a number of proteins with major roles in the physiology of the skin (barrier system, antimicrobial defence) (see Figure 1.1). Within the **spinous layer**, specific lipids and structural proteins of the cornified envelop are produced

and stored in membrane-bound lamellar granules (Odland bodies). These include glycoproteins, glycolipids, phospholipids, free sterols, and hydrolytic enzymes, such as lipases, proteases, phosphatases and glycosidases. At this level, keratinocytes form strong junctions with the help of desmosomal connections, which gives this layer's "spine" like appearance. Keratinocytes in the **stratum granulosum** have a granular appearance due to the abundance of keratohyaline granules in their cytoplasm, containing profilaggrin, loricrin and keratin filaments. Once released, profilaggrin is processed into filaggrin. Terminal differentiation begins in this layer [2]. The **stratum lucidum** only exists at anatomical locations of thick skin of the palms and soles. It is a transient zone between the granular and cornified layers [3]. As keratinocytes are pushed towards the **stratum corneum**, they become flattened and lose their cell nucleus and organelles [2]. Thus, the stratum corneum is composed only of terminally differentiated enucleated corneocytes. Containing keratin filaments and filaggrin, surrounded by a lipid rich matrix [4], this structure is similar to bricks embedded in a mortar framework [5].

Keratins (K) are one of the main components of keratinocytes. These insoluble proteins provide the mechanical strength of the epidermis. Their expression throughout the different layers of the epidermis is determined by the differentiation state of the keratinocyte and the anatomical location. K5 and K14 are predominantly expressed at the germinative level. In the spinous layer, they are replaced by K1 and K10 as the differentiation process starts. K9 is specifically expressed in palmoplantar skin [6, 7].

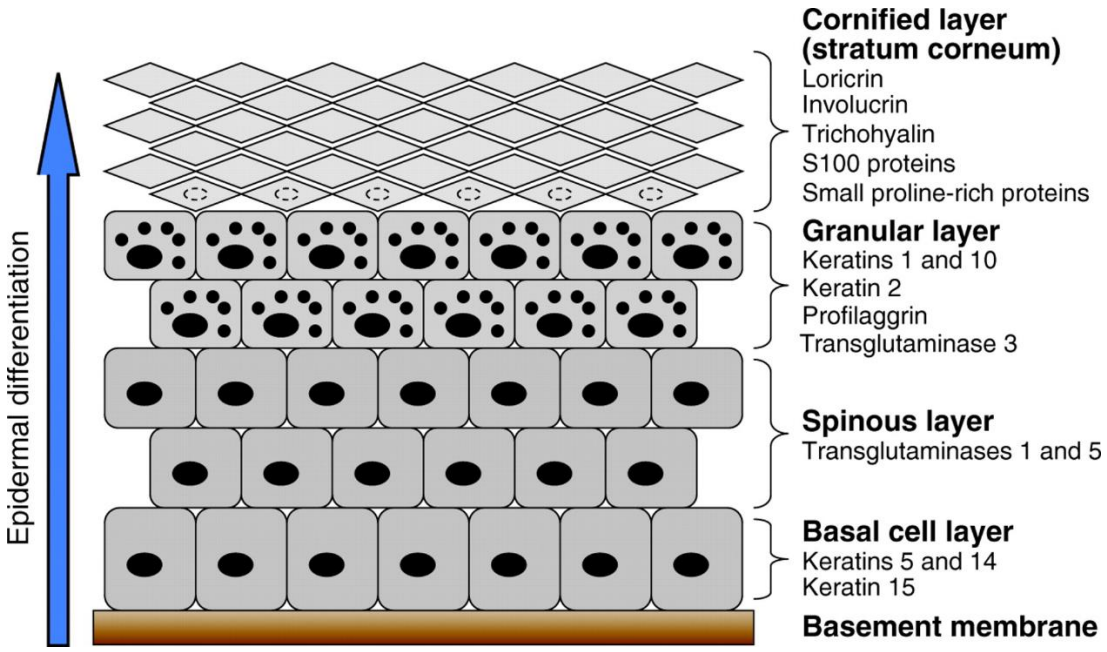

Figure 1.1 The journey of a keratinocyte.

As keratinocytes migrate from the basal cell layer towards the stratum corneum, they express different types of proteins, including keratins, specific to the epidermal layer. By the time they reach the cornified layer, they become anucleated, lose their organelles, and their cell membrane is substituted with the cornified envelop rich in proteins embedded in a keratin framework and cross-linked by transglutaminases. They are no longer interconnected with cell junctions, and are ready for desquamation [8].

Keratinocytes normally take 28 days to migrate from the basal layer to the outermost epidermal layer, where following terminal differentiation they are shed, a process called desquamation. Any disturbance in the differentiation process can lead to visible symptoms at the outer skin. Examples include psoriasis, which is associated with accelerated keratinocyte proliferation and differentiation leading to pathognomonic symptoms of thickened plaques with easy to remove, poorly formed corneal layer [9]. Another example are loss of function mutations in the filaggrin gene present in approximately 25-50% of atopic dermatitis (AD) cases, leading to a compromised skin barrier function [10].

1.1.2 The dermal compartment

The dermis is a connective tissue layer, predominantly consisting of collagen admixed with elastin fibres, glycosaminoglycans, fibroblasts, endothelial and lymphatic cells, nerve related cell types and tissue resident or circulatory leukocyte subtypes. It can be divided into two compartments. The **papillary dermis** lies immediately below the dermal-epidermal junction, nurturing the epidermis via a rich vasculature. The **reticular dermis** forms the major part of the dermis. It consists of thick collagen bundles, and its main function is to support

the overlying epidermis and to protect the underlying structures by providing strength and elasticity to the skin [11].

1.2 Disease burden in eczema and psoriasis

Psoriasis and eczema are amongst the most common dermatology conditions. Both have a significant, often under-recognised disease burden and impairment in quality of life [12, 13] comparable with patients suffering from other major health problems [14], including cancer [15].

Although neither psoriasis nor atopic dermatitis are infectious diseases, due to the visible changes on the skin they can lead to considerable social stigma, resulting in psychological distress including social anxiety, depression and suicidal ideation [13, 16-18]. Patients are not only limited in their everyday activities because of pain and itch (physical discomfort, difficulty in concentrating, sleep disturbance), but due to the aesthetic disfigurement and stigmatisation, also in their life-goals, occupation, leisure and sport activities, social and family relations. Patients often experience difficulties in their personal relationships regarding sexuality, which is even more relevant if they suffer from genital disease [13, 18, 19]. As stress in itself can provoke itching, it can also lead to the exacerbation or worsening of atopic dermatitis [19].

Due to the visibility of lesions, patients with facial, hand and arm involvement experience their disease as more disabling than patients with extensive lesions on the trunk or lower extremities [18, 20]. Similarly, it has been shown that when the disease involves the hands and feet it leads to more significant physical disability due to the loss of function of hands (painful fissures, severe itch, oedema limiting normal movements) than in patients without palmoplantar involvement. Palmoplantar involvement can restrict not only a patient's work ability, but also daily activities of self-care (e.g. brushing, dressing, self-cleaning, difficulty to walk) [21-23]. This is particularly relevant as palmoplantar psoriasis is present in approximately 40% of all psoriasis cases [18], and there is a high prevalence of hand dermatitis (approximately 10% in the general population) [24].

In addition to the health and social aspects of the disease, there is an additional financial burden for patients when it comes to "out-of-pocket costs" of moisturisers, emollients, barrier creams, protective gloves and over the counter medications, such as antihistamines [25, 26].

Early recognition and appropriate management of both eczema and psoriasis is of importance in order to potentially prevent and reduce psychological distress, to offer patients a better chance in life, to prevent the development of systemic comorbidities and to reduce healthcare costs.

1.3 Psoriasis

Psoriasis is an immune mediated, chronic inflammatory disease. It is now recognised that due to the associated systemic inflammation, it is not limited to the skin, but plays a role in development of co-morbidities (rheumatologic, cardiovascular, metabolic, gastrointestinal co-morbidities, malignancies) [27]. Therefore, timely recognition and early initiation of appropriate treatment could have a significant impact on patients' general health and quality of life, but also on reducing healthcare costs.

1.3.1 The epidemiology and etiopathogenesis of psoriasis

Psoriasis is a multifactorial, complex disease, where environmental factors are known to have a triggering effect on the disease in genetically susceptible individuals [28]. At least a 100 million people are affected with psoriasis worldwide, the estimated prevalence ranging from 0.09% (Italy) to 11.43% (Norway) [29].

1.3.1.1 Environmental factors

The etiologic role of streptococcal tonsillitis in the development of guttate psoriasis is well established [30, 31]. The role of trauma in exacerbating psoriasis is also well known, with the tendency of the disease to develop in newly induced cutaneous injuries or existing scars (scratch, surgical scar, around stoma begs, in tattoo, insect bites), recognised as the Koebner phenomenon [32, 33].

Besides microorganisms and trauma, a number of other factors can lead to the exacerbation of psoriasis, such as stress, smoking, excessive alcohol intake [34], drugs (beta-blockers, lithium, anti-malarials, interferon), cold weather, humidity, diet, obesity and Human Immunodeficiency Virus (HIV) infection [35].

1.3.1.2 Genetic predisposition

Genetic predisposition has been recognised in the aetiology of psoriasis. Human leukocyte antigen (HLA)-Cw6 is considered as the major susceptibility allele in guttate psoriasis [36] and in type I psoriasis. Type I psoriasis tends to be familial and more severe, with onset before the age of 40, compared to the type II form with later disease onset (>40 years) [37]. Genome-wide association studies over the last years described a range of susceptibility genes (reviewed by Dand et al) [38]. These include gene variants encoding the interleukin (IL) 23 receptor and IL12B (p40) [39, 40]. The significance of gain in function mutations in the caspase recruitment domain-containing protein 14 (CARD14) gene have been also recognised in association with psoriasis [41, 42], while loss of function mutations in the IL36 receptor antagonist (IL36RN) gene have been linked to pustular

psoriasis forms. Generalised pustular psoriasis (GPP), due to the dysfunction of the IL36 receptor antagonist (IL36RA) [43], is now recognised as “DITRA” or “deficiency in IL36RA” [44].

1.3.1.3 Cutaneous inflammation

Current models suggest that psoriasis is the result of an active interplay between the innate and adaptive immune system in genetically susceptible individuals, which may start in the context of an environmental trigger.

One line of thought focusses on keratinocyte damage and release of nucleic acids (NA) from damaged cells. These NA released from damaged keratinocytes form complexes with LL37 (cathelicidin), an antimicrobial peptide (AMP) highly expressed by keratinocytes in response to cell damage. LL37 can shuttle NA cross the cell membrane into responder cells where it is recognised by toll-like receptor (TLR) 7, 8, 9 or by cytosolic DNA sensors [45]. This recognition may lead to the activation of plasmacytoid dendritic cells (pDCs), which in turn start the enhanced production of interferon-α (IFNα). IFNα activates myeloid dendritic cells (mDCs) [46] in conjunction with pro-inflammatory cytokines produced by keratinocytes (IL1β, IL6, tumour necrosis factor (TNF) α) [47]. Activated dendritic cells (DCs) support the polarisation of T helper (Th) cells, Th1 with IL12 [48] and Th17 and Th22 with IL23 stimulation [49, 50]. The resulting effector T cells migrate to the skin, where they start the production of pro-inflammatory cytokines, namely IL17A, IL17F and IL22 by Th17 and type 17 cytotoxic T cells (Tc17), IL22 by Th22 and type 22 cytotoxic T cells (Tc22) and IFNγ and TNFα by Th1 and type 1 cytotoxic T cells (Tc1) [28, 50-52]. In recent years, the production of the above mentioned cytokines has also been described by tissue resident innate lymphoid cells (ILCs) and this source could be important in early psoriatic events. Feedback loops between IL36 and IL23 have been recognised and further highlight the importance of keratinocyte derived factors [53]. Due to overwhelming results from clinical observation following anti-IL17A and anti-IL23p19 therapy, there is little doubt that the IL23 - IL17 axis is of key importance in psoriatic inflammation [54].

DC and T-cell derived cytokines act on keratinocytes, key cells in psoriatic inflammation. IL22 induces keratinocyte proliferation, and in combination with IL17 alters keratinocyte differentiation and enhances the production of S100 proteins and AMPs [55, 56]. Out of the seven IL17 isoforms, IL17A (referred to as IL17), C, E and F are known as being increased in psoriatic skin. While IL17A is produced by hematopoietic cells (e.g. Th17 lymphocytes), keratinocytes are the main source of IL17B, C and E at skin level, with IL17F being produced by both haematopoietic cells and keratinocytes [57, 58]. Although IL17A is recognised as the key player in psoriasis pathogenesis and it is the main target

of biologic therapies, the role of the other IL17 isoforms produced by keratinocytes is also considerable. IL17F shows most homology with IL17A, and along with IL17C and E have been found to be pro-inflammatory in psoriasis and to play a role in neutrophil recruitment [57]. Psoriatic keratinocytes also express high levels of antimicrobial peptides (LL37, defensins, S100 proteins) and inflammatory cytokines (e.g. IL1 family members, TNFα). Key keratinocyte produced chemokines include C-X-C Motif Chemokine Ligand (CXCL) 1 and CXCL8 (IL8), responsible for neutrophil infiltration; CXCL9, CXCL10, CXCL11 with Th1 and CC-chemokine ligand (CCL) 20 with Th17 chemoattraction [59].

This perpetual cross talk between T cells and activated keratinocytes maintains the psoriatic inflammation (see Figure 1.2).

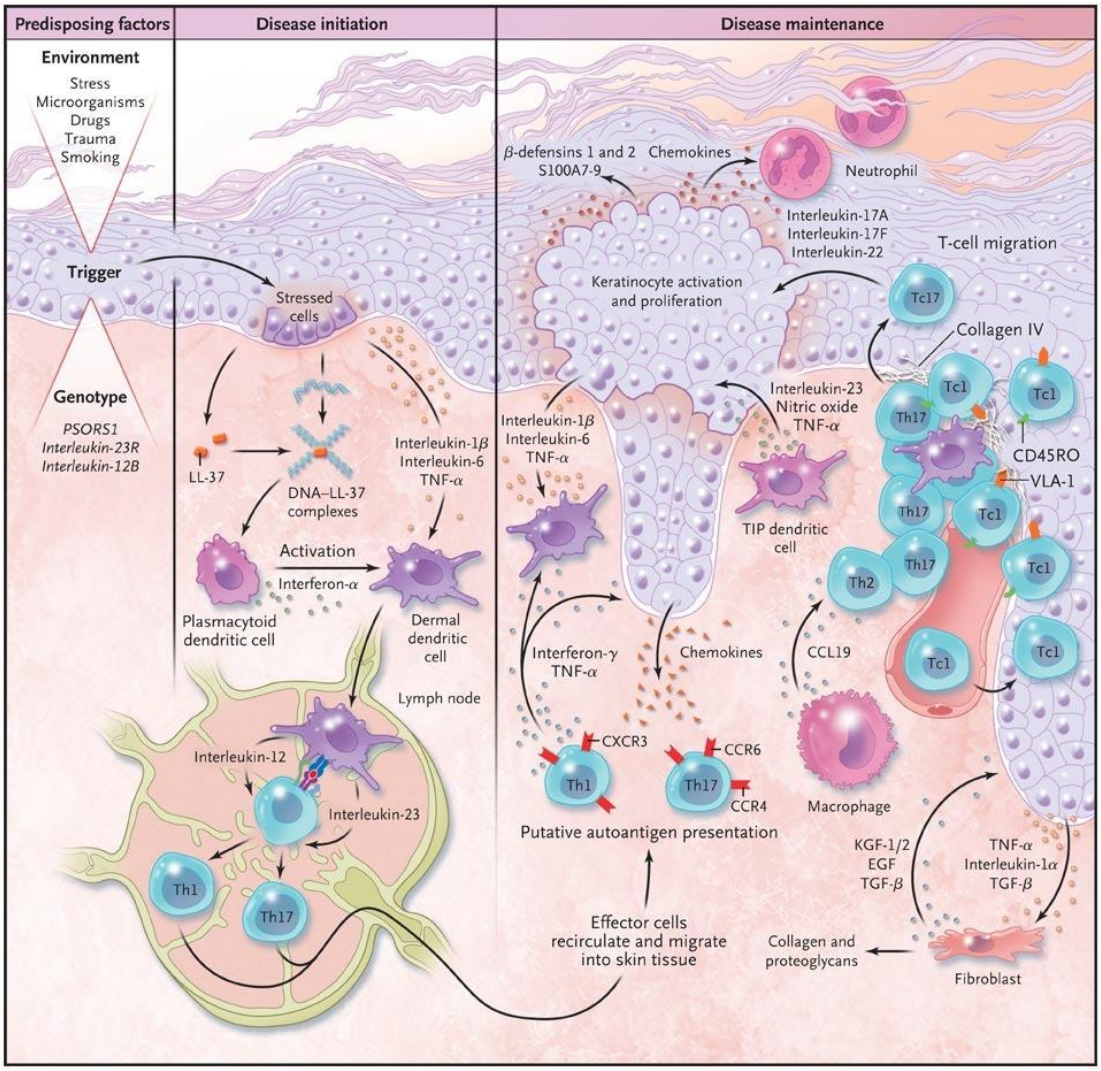

Figure 1.2 Pathogenetic model of psoriasis.

A trigger factor leading to cell damage initiates the psoriatic inflammatory cascade in genetically susceptible patients. Plasmacytoid dendritic cells activate myeloid dendritic cells through IFNα production, leading them to migrate to local lymph nodes to alert naïve T cells. With the release of IL12 and IL23 they stimulate the formation of Th1 and Th17/Th22 lymphocytes, respectively. These lymphocytes home to the skin, where the production of key inflammatory cytokines, such as TNFα, IL17A, IL17F, IL22, prompt keratinocyte hyperproliferation and production of further cytokines (such as IL1α, IL1β, TNFα, IL36), chemokines (such as IL8, CXCL1, CXCL9, CXCL10, CXCL11, CCL20) and antimicrobial peptides (such as S100 family members and human β defensins) perpetuating the inflammatory cycle [28].

1.3.2 Clinical phenotypes

Psoriasis can be classified based on a number of factors, such as: age of onset (type1 and 2), disease severity considering the extent of skin involvement (mild, moderate, severe), distribution pattern (flexural, inverse, seborrheic), phenotype (plaque, guttate, erythrodermic, pustular), anatomical localisation (generalised vs palmoplantar, scalp, flexural, genital, nail), stability of disease (stable vs unstable psoriasis) [29, 60, 61] (see summary Table 1.1).

Age of onset	Type I psoriasis <40 years
	Type II psoriasis >40 years
Phenotype/localisation	
Widespread	Plaque psoriasis, guttate psoriasis, generalised pustular psoriasis, erythrodermic psoriasis (≥90% Body Surface Area)
Localised	Plaque psoriasis (limbs, trunk); scalp psoriasis, seborrheic/facial psoriasis, palmoplantar psoriasis (pustular/non-pustular), genital psoriasis, inverse/flexural/intertriginous psoriasis
	+/- nail psoriasis
Disease stability	Stable plaque psoriasis
	Unstable eruptive psoriasis

Table 1.1 Clinical classification of psoriasis.
Table adapted from [60, 61].

1.3.2.1 Plaque psoriasis

Plaque psoriasis is the most common phenotype of psoriasis, accounting for 80-90% of all psoriatic patients. The typical clinical picture is characterised by well demarcated, erythematous plaques, covered by silvery-white scales. The disease has a symmetrical distribution pattern, and mainly localises to the elbows and knees, extensor surfaces of the limbs, sacral area, scalp, but any part of the body could be affected. Plaques can stay stable and localised for years or become widespread, unstable, ultimately leading to erythrodermia [60, 61]. **Erythrodermic psoriasis** is a potentially fatal condition, consisting of

generalised redness of the skin associated with temperature dysregulation and electrolyte imbalance which can be life threatening [62].

1.3.2.2 Guttate psoriasis

Unlike plaque psoriasis, guttate psoriasis starts with the acute onset of multiple, round shaped, scaly, erythematous, small plaques (<1cm, droplet “guttate” appearance), mainly on the trunk and proximal extremities, typically following a streptococcal pharyngitis [31, 61]. Usually, spontaneous remission occurs, however, the disease can advance to plaque psoriasis [60].

1.3.2.3 Pustular psoriasis

Pustular psoriasis is a less common form of psoriasis, and it can be divided into localised and generalised forms. In GPP (von Zumbusch), the pustules are widespread and can merge to form a lake of pus. The early eruptive phase can be life threatening, as associates with systemic symptoms (fever, dehydration). Approximately 24% of patients have mutations in the IL36RN gene [63]. The localised forms are represented by palmoplantar pustular psoriasis (PPP), where pustules are limited to the palms and soles. This condition can be mistaken for superinfected dyshidrotic eczema [64, 65].

1.3.2.4 Psoriasis phenotypes of distinct anatomical locations

Palmoplantar psoriasis

Beside palmoplantar pustulosis, psoriasis of the hands and feet can manifest as sharply demarcated, thickened, indurated, scaling, erythematous lesions, resulting in painful, bleeding fissures. Due to its resemblance to predominantly hyperkeratotic hand eczema, the two conditions can often be mistaken [66].

Scalp/facial psoriasis

Either as part of the psoriatic disease associated with other skin involvement or as the sole manifestation of the disease, scalp psoriasis is a difficult to treat condition. Its differentiation from seborrheic dermatitis can be challenging, especially if no other skin lesions are present [67, 68].

As a special location and a difficult to diagnose area, psoriasis can also involve the external ear canal. Scaling can lead to the obstruction of the ear canal causing hearing loss and otitis externa [69].

Inverse/flexural/intertriginous psoriasis

Unlike in typical psoriatic lesions, in flexural psoriasis the silvery-white scale is missing due to the particularities of the anatomical location (moist condition). The shiny, erythematous, smooth areas are well delimited and can be localised to the

submammary folds, umbilicus, axillar-, groin-, intergluteal- and anogenital area. Fissuration can occur in these tender regions. Seborrheic dermatitis and candida infection can cause a diagnostic difficulty [61, 68].

Nail psoriasis

Nail lesions are strongly associated with psoriatic arthritis (PsA), occurring in 87% of patients with this comorbidity [70]. Nail pitting is the most characteristic psoriatic nail change, often facilitating clinical diagnosis in challenging cases. Furthermore, the nail plate can become thickened, dystrophic, with subungual hyperkeratosis (accumulation of keratin underneath the nail plate). “Oil spots”, a yellowish-brown discoloration underneath the nail plate would indicate an affected nail bed. Onycholysis, detachment of the distal nail from the nailbed, is a frequent occurrence [61, 68].

1.3.3 Diagnostic workup and assessment tools used in psoriasis

1.3.3.1 Clinical diagnostics

The diagnosis of psoriasis is mainly clinical and based on typical presentation patterns, such as the Koebner and Auspitz signs. A true Koebner sign is when typical psoriatic lesions develop at the site of skin injury (e.g. scratch, surgical scar, tattoo). The Auspitz phenomenon describes the appearance of small, pin-point bleeding following psoriatic scale removal.

If the typical features are missing, a biopsy can be taken for histopathological examination in order to support the diagnosis and to exclude potential differential diagnoses, such as eczema, seborrheic dermatitis, fungal infection, pityriasis rosea, psoriasiform drug eruptions or pityriasis rubra pilaris. However, histopathology is not required if the clinical picture is clear. Other clinical investigations are optional and can be requested if indicated, such as skin swabs for bacteriology (to exclude secondary infection), throat swab for beta haemolytic streptococcus (guttate psoriasis) or skin scrapings and nail clippings to exclude a fungal infection [9].

1.3.3.2 Histopathology

The histopathology of psoriasis reflects the underlying pathogenesis and is characterised by three aspects: epidermal hyperproliferation, the presence of an inflammatory infiltrate and neoangiogenesis. Rapid keratinocyte proliferation, incomplete differentiation with stratum corneum keratinocytes retaining their nucleus (parakeratosis), epidermal thickening (acanthosis), elongated rete ridges and torturous vessels in the dermis are hallmarks of histological diagnosis along with the cellular infiltrate (see Figure 1.3). Neutrophils are seen in all subtypes of psoriasis and their intracorneal accumulation is termed “Munro microabscesses” [71]. The inflammatory infiltrate in plaque psoriasis is dominated by a mixture of $CD4^+$ and $CD8^+$ T cells, with a $CD4^+$ predominance. In contrast $CD8^+$ lymphocytes have higher epidermotropism, representing the majority of epidermal infiltrate [51, 72].

It is worth noting however, that the above described classical histopathologic image of psoriasis is not always present and histopathology in itself does not provide molecular detail. Therefore, although histopathology is the current diagnostic gold standard, it only has real diagnostic value when the constellation of the characteristic morphological changes are present.

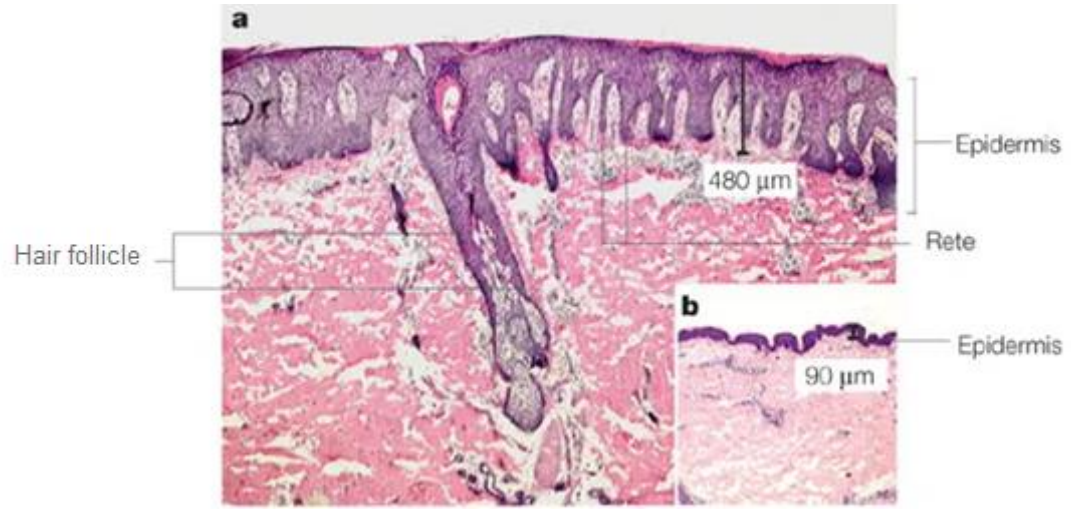

Figure 1.3 Histopathology of psoriasis.

a) Keratinocytes in the psoriatic skin undergo extensive and rapid proliferation, which results in the thickening of the epidermis (acanthosis). The aspect of elongated rete ridges alternates with the thinned suprapapillary epidermis. The stratum corneum is composed of incompletely differentiated keratinocytes, as they no longer lose their nucleus (parakeratosis). The accumulation of neutrophils between the cornified layers (Munro microabscesses) is typical for plaque psoriasis. In the papillary dermis, torturous blood vessels mark the process of neovascularisation and there is a rich lymphocytic infiltrate. b) Histopathology of normal skin for comparison of epidermal thickness and aspect [73].

1.3.3.3 Assessment tools in psoriasis

The most common tool used in a clinical setting to assess and document cutaneous psoriasis severity, progression and response to treatment is the **Psoriasis Area and Severity Index** (**PASI**). The **PASI score** evaluates the degree of erythema, scaling (desquamation) and induration on a score of 0 (no sign) to 4 (very severe) and estimates the extent of these signs at four different body areas (head and neck, trunk, upper and lower extremities) [74]. Biologic treatment can be considered for psoriasis patients with a PASI score of 10 and above according to clinical guidelines. **Dermatology Life Quality Index** (**DLQI**) is generally used in dermatology as a patient reported measure to assess disease impact on patients' daily life [75].

1.4 Eczema

1.4.1 The epidemiology and etiopathogenesis of AD

AD is a complex, multifactorial disease, driven by cell-mediated immune responses and - to a varying degree - immunoglobulin E (IgE) hypersensitivity, where environmental factors contribute to disease development in genetically susceptible individuals. It is estimated to affect up to 20-30% of children and 3% of adults [76, 77]; however some data suggest that there is a higher prevalence, up to 7-10%, in adults [78, 79].

The risk of developing AD is increased 1.5-fold if one of the parents already have an atopic disposition, and it is even higher in children where one or both parents have AD, by 3- and 5-fold respectively [80]. The prevalence of atopic diseases (= allergic asthma bronchiale, rhinitis allergica, AD) significantly increased in industrialised countries and urban areas in recent decades [81], underlining the importance of the environment in disease development.

1.4.1.1 Genetic predisposition

One of the major roles of the skin is to form a barrier against the outside world. Skin integrity is essential for the protection against environmental harm, including pathogens, allergens, pollutants, irritants, toxins and in order to prevent water loss. In AD, a dysfunctional epidermal barrier leads to facilitated entry of these environmental insults and to the increase of transepidermal water loss (TEWL) [82].

The epidermal differentiation complex (EDC) is encoded on chromosome 1q21, including involucrin, loricrin, S100 proteins and most importantly the filaggrin gene on exon 3. On a population scale it is estimated that filaggrin loss of function mutations are responsible for approximately 15% of mild-to-moderate and 50% of moderate-to-severe AD [83]. These patients are more prone to develop AD at an early age, have a more severe and persistent disease phenotype with elevated total IgE levels and allergic predisposition. In the absence of filaggrin and its breakdown products “natural moisturising factor” (NMF), the pH of the skin becomes alkaline, favouring colonisation with pathogen bacterium species, most relevantly with *Staphylococcus aureus.* However, approximately 40% of patients with a filaggrin mutation never develop AD and similarly, a significant number of AD patients have no identifiable filaggrin mutation.

Therefore it is obvious that other genetic and environmental factors also contribute to disease development [82]. One of these mutations is connected to genes encoding IL4 and IL13 (key cytokines in the pathogenesis of AD), and

RAD50 (gene involved in DNA repair), located together on chromosome 5q31.1 [80].

In filaggrin-depleted skin, regardless whether due to filaggrin gene mutation or the downregulatory effect of Th2 cytokines, there is a dysregulation of keratin filaments, disturbed lamellar body formation, reduced intercellular connection at the corneodesmosome and tight junction level, in addition to lipid depletion, increased skin pH and TEWL. Increased TEWL is also noted in the non-lesional skin of patients with AD [82]. All these factors lead to the disruption of skin barrier function and exposure to environmental insults.

1.4.1.2 Cutaneous inflammation

Originally, it was considered that psoriasis is mainly driven by Th1 and Th17 mediators, with critical importance attributed to the role of TNFα, whereas eczema is the result of a dominantly Th2 cytokine response [56, 84, 85]. However, current studies challenged the Th1/ Th2 theory. These highlight that although initial inflammatory response in eczema is predominated by the Th2 cytokine network, the inflammatory set up changes with chronification, featuring Th1, Th17 and Th22 dominance [86-88].

It is now known that the acute phase of eczema is characterized by a Th2, Th22 and Th17 response, although Th2 cytokines dominate the inflammatory landscape while Th17 activity is considerably lower compared to psoriasis. Following a triggering event (barrier disruption, entry of pathogen/irritant), a number of chemokines (such as thymus and activation-regulated chemokine (TARC), also known as CCL17; and macrophage-derived chemokine (MDC), also known as CCL22), and pro-inflammatory cytokines with alarmin properties (such as IL17E, IL33 and thymic stromal lymphopoietin (TSLP)) are released from keratinocytes in order to activate DCs. These activated antigen presenting cells support polarization and clonal expansion of Th2 lymphocytes. Following their migration to the skin, they release IL4, IL5 and IL13 (key cytokines in eczema pathogenesis) as well as IL31 (key inducer of itch). IL4 and IL13 stimulate keratinocytes to produce more TSLP, which in turn further enhance the Th2 response, creating a vicious circle. These cytokines also reduce the production of proteins essential for barrier integrity (e.g. loricrin, filaggrin) and lipids (e.g. free fatty acids, ceramides).

Another role of IL4 and IL13 is the induction of antigen-specific IgE antibodies by B cells [80]. IgE antibodies on the surface of basophils and mast cells have the potential to activate these upon specific antigen binding. This leads to their degranulation and the release of preformed inflammatory mediators. Vasoactive amines (histamine, leukotrienes and prostaglandins) contribute to a type I

(immediate) hypersensitivity reaction, while pro-inflammatory cytokines, IL4 and IL13, promote the Th2 response, and IL5 contributes to eosinophil survival and recruitment. It is a specific finding for AD patients that their Langerhans and dermal DCs express the high affinity receptor for IgE (FcεRI), facilitating uptake of allergens and subsequent presentation to allergen specific T cells, thus increasing allergen specific immunity [89].

1.4.1.3 The microbiome

The skin microbiome is known to be disturbed in AD. A predominant bacterium colonising healthy skin is the commensal *Staphylococcus epidermidis*, which inhibits colonisation with pathogenic species, such as *S. aureus* [90]. Microbial diversity is significantly reduced during an AD flare (dysbiosis), with expansion of *S. aureus* [91] and reduction in commensals such as *S. epidermidis*. However, whether infection with *S. aureus* is an inducer of AD or a secondary phenomenon, is still unclear [92]. The majority of exotoxins produced by *S. aureus* (e.g. staphylococcal enterotoxin B) have superantigen (SAg) properties, potentially increasing the severity of atopic inflammation by oligoclonal T cell activation and this may contribute to increased inflammatory responses not only on the level of skin but also in the upper airways and lungs [93]. A range of other *S. aureus* derived molecules and toxins, such as alpha toxin, have been extensively investigated in the context of AD and may to some extent contribute to heightened inflammatory responses [94].

1.4.1.4 Itch/scratch cycle

Itching and consequent scratching (the “itch-scratch cycle”) has a major role in maintaining AD lesions. Itch is a complex and yet not fully understood sensation. The nerves responsible for the perception of itch in the skin are primary sensory nerves (slow-conducting, non-myelinated C fibres and fast myelinated Aδ fibres).

In histamine-dependent itch, histamine released by mast cells and basophils directly activates histamine receptors found on sensory neurons, causing immediate sensation of pruritus. As H1R blockers fail to be of clear benefit in AD pruritus, other factors contribute to this pathognomic AD symptom. Th2 cytokines themselves may be involved in itch and in particular IL31. IL31 has been shown to directly stimulate sensory nerve endings through their IL31 receptor subunit-α (IL31Rα) [95] and enhances the sensory nerve density of the skin [96].

1.4.2 The etiopathogenesis of contact dermatitis

There are two major types of contact dermatitis, irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Patients with AD have a higher risk of

developing both, due to their already impaired barrier function. Therefore in many instances, patients with AD also present contact dermatitis, making the categorisation of eczema even more difficult. ICD can present in anyone who has contact with an irritant (chemical and/or physical) in a toxic concentration. In acute ICD the offending agent leads to direct and immediate cytotoxicity, causing increased release of damage-associated molecular patterns (DAMP) /pro-inflammatory cytokines and chemokines from keratinocytes and apoptotic cells, including IL1 family members, IL6, IL8 and TNFα. In ACD there is a type IV, cell mediated allergic response. In contrast to ICD, ACD only develops in individuals sensitised to an allergen.

Due to impaired skin barrier function, both patients with active ICD and AD are more prone to acquire additional contact allergies [97].

1.4.3 Clinical phenotypes of atopic dermatitis

The clinical phenotype and predilection sites of AD changes with age. AD tends to start following the second month of life, **infantile AD** (< 2 years), and it has a tendency to affect predominantly the head and neck area. Lesions are usually localised to the cheek, initially with oedematous, red papules, which rapidly develop oozing and consequent crusting. It can extend to the limbs (extensor surface) and trunk, but the diaper area is typically spared. In **childhood AD** (2 - 12 years) the lesions become less serous with the development of more chronic, lichenified lesions (thickened, infiltrated skin), which predilection changes from the extensor to the flexor areas of the limbs, characteristically to the antecubital and popliteal fossa, usually affecting the periorificial areas on the face. The neck, hands and feet, wrists and ankles are often involved. Intense itch and scratching is usually present (suggestive scratch marks) which ultimately lead to the lichenification of lesions. **Adolescent/Adult AD** lesions are also more subacute/chronic in appearance, with the presence of lichenified lesions. The predisposition towards the flexural areas continues. AD in adults often involves the retro-auricular region, neck and chest (see Figure 1.4). In patients where the disease persisted from infancy to childhood and then through adulthood, AD manifests with more severe, difficult to treat, generalised lesions [80].

Atopic hand eczema (intrinsic and extrinsic type), presents in approximately 60% of all adult AD patients, can also involve the feet (**palmoplantar dermatitis**) and can be the only disease manifestation [98]. There is no validated classification for hand eczema. According to phenotype it can present as **predominantly vesicular** (**pompholyx/dyshidrotic eczema**) with deeply seated vesicles on the palms and sides of the fingers, **hyperkeratotic/lichenified** with scaling, induration and frequently with the

presence of deep, bleeding, painful fissures, or **fingertip dermatitis** (**pulpitis sicca**) mainly affecting the area of the last phalanx, usually presenting with deep fissures on an erythematous basis. Based on etiologic factors, **atopic hand eczema** has to be differentiated from **irritant** and **allergic hand dermatitis,** which often represent a challenge as their clinical phenotype is very similar [99].

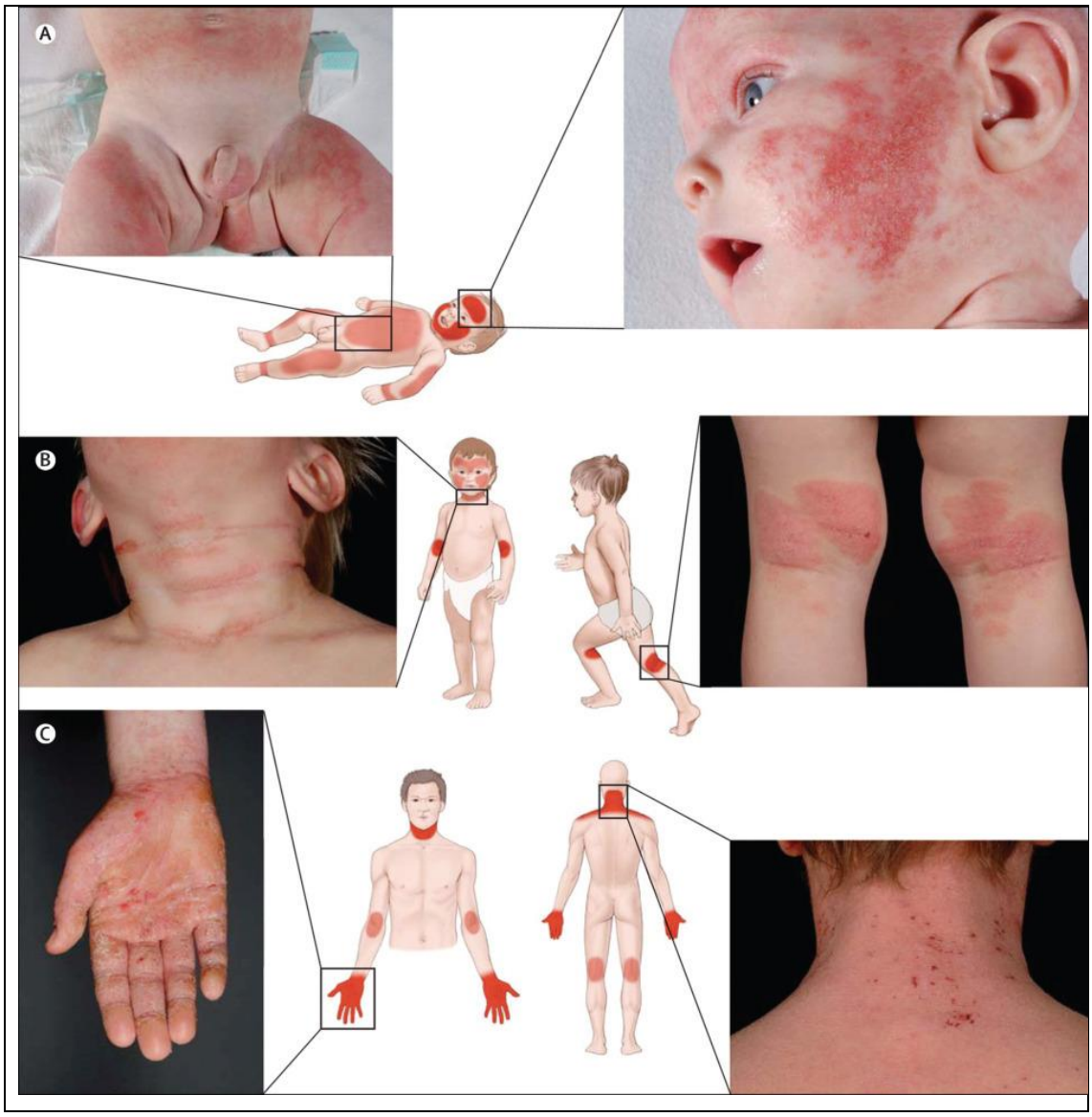

Figure 1.4 Age related distribution pattern of AD.

a) Infantile dermatitis is typically localised to the cheeks and extensor surfaces of the limbs, in a symmetric distribution. The diaper area is characteristically spared. b) The distribution pattern changes in childhood, by a preference to the flexural areas (wrist, ankle, neck, antecubital and popliteal fossa). Lesions tend to have a more chronic appearance opposite to the infantile type, where acute, oozing and crusting lesions dominate the clinical picture. c) Predisposition to the flexural areas continues through adulthood. Anatomical sites, such as the hands, head and neck area, nipple are often involved, and it is not rare to be the sole manifestation of the disease. Lesions are chronic, with lichenification, scaling, fissuration ad excoriations due to uncontrollable itch [92].

1.4.4 Atopic march - comorbidities in atopic dermatitis

The tendency to respond to common environmental triggers (allergens) with the hypersensitisation and increased production of IgE antibodies is termed atopy. Atopic individuals are prone to develop **allergic asthma**, **rhinoconjunctivitis** and **AD** [100]. **The atopic march** describes the sequence when an individual having childhood AD, progressively develops allergic rhinitis and asthma (atopic triad) [93]. The early age of onset of AD (by the age of 2-4 years) combined with IgE sensitisation and disease severity are associated with a significantly higher risk for progressing towards the atopic march [101]. Asthma develops in approximately 8% of the general population, in 20-30% of patients with mild, and in 70% of cases with a history of severe childhood AD [93, 102]. Early age of onset with severe AD is characteristic in patients with filaggrin null mutations, and it is known that these cases have a higher risk of developing multiple allergies and asthma [82].

1.4.5 Diagnostic workup and assessment tools used in atopic dermatitis

Currently, there is no validated serum or tissue diagnostic biomarker for AD. Hence, the diagnosis of AD is based on the clinical observation (history, presentation, associated co-morbidities). Available guidelines mainly recommend laboratory testing (including genetic testing) and histopathology in order to exclude other potential diagnostics, but not to establish the diagnosis of AD [103, 104]. A positive skin prick test or detection of specific IgE antibodies in serum can confirm the atopic background [100].

1.4.5.1 Clinical diagnostic criteria

A number of clinical diagnostic criteria have been suggested for the diagnosis of AD and these include: age-specific distribution pattern; pruritus; chronic, relapsing course; personal and family history of atopic disposition [105].

1.4.5.2 Testing the atopic background

Total and specific serum IgE levels

IgE levels are the most frequently used serum markers to identify atopic individuals. High serum levels of total IgE seems to correlate with the severity of the disease; however, high total IgE on its own is not sufficient to determine atopy as this can be elevated for other reasons including infection with parasites. However, an elevated total IgE level, or even normal levels of total IgE in association with the presence of specific IgE to common allergens, such as house dust mite, pollen, pet dander, are highly suggestive of atopy [106]. Yet, this

feature is not present in approximately 20% of affected patients, which lead to the still controversial classification of **extrinsic (allergic)** and **intrinsic (non-allergic)** type of AD [103, 107]. Extrinsic individuals have high total IgE and/or specific IgE levels, with a disturbed barrier function (potential consequence of filaggrin loss-of-function mutation), while intrinsic AD individuals show no elevated total or specific IgE levels [107]. Nevertheless, this concept is not commonly endorsed and other authors suggest that the extrinsic and intrinsic forms are not two different categories but only variations, especially as some patients can later present elevated total/specific IgE levels [103, 108]. In addition, it has been shown that a subset of patients can develop specific IgE antibodies against self-proteins, suggesting an autoimmune background [109]. Testing for these specific IgE antibodies against self-proteins is not available yet in routine settings, however could be promising [108].

Skin prick testing

While blood IgE determines specific sensitisation, prick test results are believed to better inform on clinical relevance as the *in-vivo* response of a patient is shown. The method consist of introducing allergens into the skin, carried out with a small needle prick. If specific IgE is present to the allergen in question, it will cause local mast cell degranulation and the release of histamine, leading to an immediate skin reaction, which clinically presents in form of a wheel > 3mm (positivity). It is a reliable test with rapid outcome, involving the use of a negative (physiologic serum) and positive control (histamine) [110].

Patch testing

Patch testing is performed following national and international guidelines regarding testing procedures and applied allergens in order to identify contact allergens which may cause or aggravate an existing dermatitis. Patients with AD may also develop contact dermatitis, in which case standard patch testing is useful. The most common allergens (standard battery), such as natural rubber latex, preservatives, metals, perfumes, cosmetics, leather chemicals, lanolin and plants as well as substances relevant to the individual patient, are placed in small discs on the skin of the back. These are left on under occlusion for 48 h and thereafter until 96 h, when the test sites are assessed for positive reactions by a specially trained clinician [9].

1.4.5.3 Histopathology

Histopathologic examination is not necessary for the diagnosis of AD, and it is more used to exclude other pathologies [103]. The histopathologic differentiation of different eczema types is non-reliable.

In accordance with the disease phase, **acute, exudative lesions** are characterised by spongiosis (intraepidermal intercellular oedema), various degrees of acanthosis and dermal oedema. The dermal infiltrate is composed by lymphocytes, monocytes, mast cells, with or without the presence of eosinophils. With the development of **chronic, lichenified lesions** the acanthosis becomes more dominant with hyperkeratosis and parakeratosis (psoriasiform pattern), with the elongation of rete ridges and marked papillary dermis [111, 112]. However, these findings are non-characteristics and therefore non-diagnostics [80].

1.4.5.4 Assessment tools in atopic dermatitis

There are a number of eczema specific assessment tools, however, the most widely used in clinical practise are the **Eczema Area and Severity Index** (**EASI**) and the **SCORing Atopic Dermatitis** (**SCORAD**). Similarly to the PASI score in psoriasis, they both asses intensity of disease signs on a scale from 0 (no sign) to 3 (severe) and extent [113].

1.5 Differentiating eczema and psoriasis in clinically challenging cases

Although eczema and psoriasis usually present with distinguished clinical characteristics, their differential diagnosis can represent a real diagnostic challenge. This is the case in certain anatomical phenotypes, such as in isolated palmoplantar, flexural or scalp involvement, or when the disease presents with minimal activity or with an atypical clinical presentation.

1.5.1 The importance of accurate diagnosis

Early and accurate diagnosis of psoriasis and eczema is important to start appropriate, disease specific treatment pathways and to allow for screening and possibly prevention of relevant associated co-morbidities, such as PsA or cardiovascular diseases for the psoriatic disease continuum.

The diagnosis of PsA in the absence of skin lesions (approx. 15%) is extremely difficult for rheumatologists, as there is yet no available serum marker to facilitate the diagnosis [114, 115]. In cases of a positive family history of psoriasis and arthritis changes suggestive of PsA, the diagnosis of “psoriatic arthritis sine psoriasis” can be made [116], however, taking a family history is not always simple nor reliable, as patients are often confused if their family members did indeed had psoriasis or eczema. Therefore when even minimal skin lesions are present, the certainty of the dermatology diagnosis can assure a better rheumatology outcome.

Accurate diagnosis is also relevant because of the available therapeutic options. Although “conventional”, non-biologic, immunosuppressive treatments are generally shared between eczema and psoriasis, many patients move onto biologics due to limited therapeutic effect and/or significant side effects.

Early diagnosis with appropriate and efficient management means more rapid recovery for the patient, decrease in loss of work hours, return to normal physical and social activities, reducing significantly the disease burden but also healthcare costs [117, 118].

1.5.2 The diagnostic challenge

Among the most frequent sites causing a diagnostic challenge are hands and feet. Both psoriasis and eczema can localise exclusively to these areas and both their clinical and histological phenotypes can overlap. Pustular psoriatic lesions can be mistaken for superinfected eczematous vesicles, and vice versa, while hyperkeratotic, scaling, lichenified lesions, exhibiting fissures and erosions, are common manifestations in both diseases (see clinical phenotypes). Furthermore,

histopathologic differentiation of palmoplantar psoriasis from hand eczema is particularly difficult due to the unique anatomical characteristics of the palmoplantar skin and overlapping histopathologic features of these conditions at this skin area. Typical histopathological traits such as spongiosis in eczema and loss of granular layer in psoriasis are non-pathognomic signs in the volar and plantar skin as it can be present with various degrees in both pathologies [119, 120].

Mild scalp and facial psoriasis can be mistaken for seborrheic dermatitis [68] and mistreated, often delaying the diagnosis of PsA [67]. Similarly, inverse psoriasis and seborrheic dermatitis can be both mistaken for a candida infection in the skin fold areas.

1.5.3 Serum, urine and tissue markers in eczema and psoriasis

There are a number of markers upregulated in the blood and/or urine samples of patients with AD and psoriasis. Although they have been shown to correlate with disease flare and severity, almost none of these markers have been compared between different inflammatory diseases but have been compared to healthy controls. In addition, their detection levels may be unreliable and can be influenced by other variables. Therefore, although they could facilitate the diagnosis and disease follow up, none of these markers has yet entered routine diagnostics in any healthcare setting.

1.5.3.1 Serum and urine makers in atopic dermatitis and psoriasis

Correlations with elevated C-reactive protein (CRP) levels, a well-known general inflammatory marker, and disease activity have been found for both AD and psoriasis [121, 122]. In AD high levels of serum eosinophils and eosinophil degradation products [123], in psoriasis neutrophil derived proteases (e.g. elastase) and their inhibitors (α_1-antitrypsin and α_2-macroglobulin) [121] and P-selectin (marker of platelet activation, involved in leucocyte extravasation) [124] have been described to associate with disease severity. However, none of the above are disease specific and as such cannot be used as diagnostic markers.

So far CCL17 (TARC), a chemokine binding to CCR4 which is preferentially expressed on Th2 cells, seems to be the most specific serum marker to correlate with AD severity and to reflect disease activity [125] also showing elevated levels even when compared to other inflammatory skin diseases [126, 127].

A myriad of other serum markers have been described to correlate with AD activity, including CCL27, the cutaneous T cell attracting chemokine (CTACK, responsible for memory T lymphocyte homing to the skin) [128], MDC (CCL22, predominantly Th2 lymphocyte chemoattractant through CCR4) [129], IL18 (IL1

family member, produced mainly by macrophages and keratinocytes, with potent immunomodulatory effects) [130], CD30 (indicator of Th0/Th2 lymphocyte activity, a member of the tumour necrosis factor/nerve growth factor (TNF-NGF) receptor superfamily) [131-133] and IL31 (pruritogen cytokine) [134, 135]. Recent findings highlight periostin (produced by dermal fibroblasts and upregulated by Th2 cytokines IL4 and IL13, playing a role in keratinocyte activation, amplification and maintenance of the Th2 inflammatory response, with a role in tissue remodelling and fibrosis) [136, 137] and serpin B3 and B4, also known as squamous cell carcinoma antigen 1 (SCCA1) and 2 (SCCA2) (serine protease inhibitors physiologically found in the spinous and granular layers of normal squamous epithelium, known to modulate the host immune response enhancing tumour growth) [138]. Many of these proteins have been also found to be upregulated in psoriatic patients when compared to healthy controls, such as MDC [139], IL18 [140], periostin [141] and serpin B3 and B4 [138].

Other proteins significantly higher in psoriatic compared to healthy serum include TNFα, IFNγ, IL6, IL8, IL12 [140], S100A12, IL1 receptor antagonist (IL1RA), vascular endothelial growth factor (VEGF), CXCL5 (also known as epithelial-derived neutrophil-activating peptide 78 (ENA78)) [139]. Serum levels of IL22 [142, 143], as well as the relatively recently discovered IL36γ [144] have been also correlated with disease activity, and their reduction has been associated with therapeutic response.

IL17A levels are reported to be inconsistently elevated in psoriatic serum [139, 140] and it seems to show stronger association with eruptive, inflammatory forms rather than with stable plaque psoriasis, together with IL1RA [145]. Not surprisingly, there is an increase in the serum levels of Th1, Th17 and Th22 lymphocytes in psoriatic patients, with the decrease of Th1 and Th17 cells following anti-TNFα therapy [146]. Serum proteomics also demonstrated, between other molecules, the upregulation of kallikrein 8 (KLK8) (serine protease), K17 (reflecting non-matured, pro-inflammatory keratinocytes), desmoplakin, complement C3, polymeric immunoglobulin receptor and downregulation of Zn-α2-glycoprotein (ZAG) in psoriasis compared to healthy [147].

Adipokines (cytokines produced by adipocytes) also have an altered secretion in the serum of psoriatic patients. Leptin, resistin and lipocalin (increased in insulin resistance) have been found to be elevated in psoriatic serum [148], while adiponectin levels (involved in glucose regulation) are decreased, with normalisation upon psoriasis treatment [149], potentially connecting psoriasis with insulin resistance, the development of atherosclerotic plaques and cardiovascular diseases. The levels of adipokines seem to correlate with the level

of Th17 related cytokines (TNFα, IL6, IL22) [150]. Another metabolic link is reflected by the increase of serum lipid levels (total cholesterol, triglycerides, very low-density lipoprotein and low density lipoprotein cholesterol) and increased oxidative stress (reduced anti-oxidant enzyme activity) in psoriatic patients [151].

Nevertheless, serum markers do not necessary correlate with tissue inflammation and are not reliable to be used for diagnostic purposes [103].

1.5.3.2 Tissue markers in atopic dermatitis and psoriasis

A number of tissue proteins were found to be elevated in both AD and psoriatic lesional skin, reflecting the perturbed proliferation and differentiation status of keratinocytes as well as the presence of inflammation.

There is an altered keratin expression in both pathologies, with significant upregulation of K16 and Ki67, markers of proliferation [152-154], paralleled with the downregulation of K1 and K10, markers of terminal differentiation [152, 155]. In psoriasis there is also an overexpression of K14, normally found in the germinative layer and K17, a keratin found in epidermal appendages but normally not present in healthy skin [152, 156]. Furthermore, marked increase in the gene expression of S100 family members have been found in both pathologies, however significantly higher in psoriasis compared to AD, with increased S100A7 (psoriasin), S100A8, S100A9 and S100A12 protein expression in the upper epidermal layers [152, 154, 157-159]. The S100 family is involved in a variety of intra- and extracellular functions, including cell growth, differentiation, inflammatory response and antimicrobial properties [159]. Other molecules with antimicrobial properties have been also found to be increased, especially in psoriasis, explaining for the reduced, almost non-existent skin infections in psoriatic lesions compared to eczema. Between these antimicrobial peptides, beside S100 family members, are human β defensins (hBD), LL37/cathelicidin and elafin (also known as peptidase inhibitor 3 (PI3) or skin-derived antileukoproteinase (SKALP)) [160]. Increased hBD genomic copy numbers have been found to indicate predisposition for psoriasis [161]. Although increase in hBD2 levels have been shown to be present in acute lesional AD compared to non-lesional and healthy skin, this was still lower compared to levels detected in lesional psoriasis, while no significant difference compared to healthy could be detected in case of chronic lesions [162].

Gene expression profiles have shown that both Serpin B3/B4 are upregulated in eczematous and psoriatic lesional skin compared to healthy, with higher expression in psoriasis [163]. Furthermore, increased activation of triggering receptor expressed on myeloid cells 1 (TREM1) signalling has been found in lesional compared to non-lesional AD skin [164], as well as in psoriasis compared

to healthy, with TREM1 blockade inducing the reduction of Th17 activation and IL17 production in *in vitro* models [165]. TREM1 is a cell-surface receptor involved in innate and adaptive immunity by enhancing cytokine production, including IL8, MCP/CCL2, and TNFα, and connected with infection-related inflammation [164].

Other markers significantly increased in both pathologies, especially in the stratum corneum, include enolase 1 (ENO1) also known as alpha-enolase (a glycolytic enzyme expressed in most tissues) [166-168] and galectin 7 (specifically expressed in keratinocytes, implicated in cell-cell and cell-matrix interactions, keratinocyte proliferation, differentiation and apoptosis, assuring normal growth) [152, 169].

CCL17 (TARC) and CCL27 (CTACK) are considered more disease specific markers for atopic inflammation. Parallel to their enhanced serum levels [128], increased CCL17 [170, 171] and CCL27 [172] levels have been also detected in lesional AD skin compared to healthy and psoriasis, which presents with low CCL27 levels [173]. Decreased CCL27 in association with increased NOS2 (encoding for inducible nitric oxidase synthase/iNOS) gene expression, correlating with histopathologic immunofluorescence, have been also highlighted as potential molecular classifiers to distinguish eczema from psoriasis [174]. iNOS has been previously described as significantly upregulated in psoriasis compared to healthy and eczema [175].

Although there is an increase in the expression of S100 family members in AD skin, other EDC genes, such as filaggrin, loricrin, involucrin, corneodesmosin and late cornified envelope 2B (LCE2B), were found to be downregulated already in the non-lesional AD skin in contrast to healthy controls, both on messenger RNA (mRNA) and on protein levels [154, 176]. Similarly, the presence of Th2 (IL4, IL13, IL31) and Th22 (IL22) cytokines is already detectable in non-lesional AD skin [176] and their gene expression levels are significantly increased in lesional AD skin, with only a small increase in Th17 cytokines (IL17, IL23). However, there is a noticeable increase in molecules which have previously been described to be IL17 regulated (CCL20, elafin and lipocalin 2 (LCN2) [154, 164]. It has been also shown that epidermal keratinocytes of lesional AD skin have enhanced IL31 receptor A (IL31RA) expression compared to healthy keratinocytes, suggesting a lower threshold for itch sensation among atopic patients [177].

In psoriatic skin immunohistochemical staining revealed a significant increase in anti-apoptotic B-cell lymphoma-X (Bcl-X) and pro-apoptotic Bcl-2 antagonist/killer (Bak) and Bcl-2 associated X protein (Bax), together with a decrease in anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein expression compared to healthy skin, reflecting the presence of perturbed apoptosis and hyperproliferative status. Bcl-

X expression is present in uninvolved psoriatic skin [178, 179]. In one study depleted Bcl-2 levels have been also found to differentiate psoriasis from eczema in immunohistochemistry [180].

Increased levels of inflammatory mediators in psoriatic lesional skin confirm their essential role in disease pathogenesis. There is an enhanced expression of leukemia inhibitory factor 1 (LIF1, an IL6 class cytokine, which promotes proliferation by inhibiting differentiation, and can potentially enhance IL8 production) [181], TNFα, IFNα, IL2, IL6, IL8, IL12 [182], IL23, IL23R, and Th17 cytokines (IL17A, IL17F, IL22, IL26 and CCL20) [183] and reduced levels of IL1, IL4, IL5, IL10 (anti-inflammatory cytokine) [182] and IL15 (structurally similar cytokine to IL2, with anti-apoptotic function and effects on T cell activation and proliferation) [184].

Ever since their discovery, IL36α, IL36β, IL36γ and their counter regulator IL36RA (new IL1 family members) have been placed into the spotlight of psoriasis research [185-188]. Evidence indicates an upregulation of IL17/TNFα-associated genes, with specifically high expression of IL36γ in psoriasis compared to other chronic inflammatory skin diseases, including AD, contact eczema and lichen planus. Furthermore, IL36γ immunohistochemistry allowed differentiation of clinically unclear cases of psoriasis from eczema presenting enhanced expression of IL36γ in the upper layers of the epidermis in psoriatic lesional skin [144].

Renin was also shown to be upregulated in psoriatic lesional skin, potentially connecting psoriasis with the already recognised increased cardiovascular risk [139].

1.6 Non-invasive diagnostic tools in dermatology

The accessibility of the skin as an organ combined with the availability of highly sensitive and specific tools and analysis systems (mass spectrometry, high sensitivity enzyme-linked immunosorbent assay (ELISA), bead based multiplex systems, polymerase chain reaction (PCR) techniques) opened new avenues into dermatology research. As traditional dermatology diagnostics mainly relies on clinical judgement, clinicians rely on invasive biopsies and on histopathologic examination in clinically challenging cases. There is an unmet need in dermatology for the development of easy to perform, accessible, non-invasive diagnostic tools, which could allow for fast and accurate diagnosis, disease follow up and evaluating therapeutic response. A number of diagnostic tools are currently being researched, developed and optimised in order to meet these needs.

1.6.1 Imaging

Dermatology imaging has significantly evolved in recent years, from the use of simple magnifying lenses, through dermatoscopy, to high resolution, non-invasive imaging techniques which allow for *in vivo* scanning of the skin in depth.

Reflectance confocal microscopy (RCM) is a new imaging technique in clinical dermatology. It is particularly suitable for the assessment of malignant changes of the epidermis, as it provides high cellular and even sub-cellular resolution. It uses a laser beam to scan the epidermis, and the imaging is based on light reflection. However, it only provides clear images of the epidermis as due to light scattering it loses resolution with depth.

Optical coherence tomography (**OCT**) is based on the reflection of low-coherent light. It does not provide high cellular resolution, but by compensating for light scattering it can penetrate deeper in the skin and allows for a wide field of view and detection of primarily morphological changes in the skin. Visualisation of the skin microvasculature and blood flow is also possible by **dynamic OCT**.

Other, similar techniques are also available and under development. **Multiphoton microscopy** is similar to RCM and uses light absorption for the detection of epidermal changes in the cellular compartment. **20-MHz high-frequency ultrasound** is based on reflection of ultrasound waves and has the deepest skin penetrance. **Raster-scan optoacoustic mesoscopy** is an emerging method using light absorption, which allows for the detection of morphological, molecular and functional modifications [189, 190].

Unfortunately, these imaging techniques are still expensive and do require specialised training for the recognition of distinct imaging features. So far the main focus was on their diagnostic value in recognising skin malignancies and their use in inflammatory skin diseases has not been widely investigated. However, as they are highly promising future technologies allowing for "*in vivo* histology" of the skin, they could prove useful in differentiating eczema from psoriasis in clinically challenging cases and anatomical locations, such as the hands and feet.

1.6.2 Functional skin tests

The non-invasive measurement of **TEWL** with a Tewameter® and **skin elasticity** with a Cutometer® probe can provide valuable information on the function of the skin, but they do not provide molecular data. **TEWL** has been used for the assessment of barrier function. Water evaporates from the skin as part of the

normal homeostasis. However, increased water loss has been recorded in case of disrupted barrier function not only in AD but also in psoriasis. In addition, TEWL is often used to study the beneficial effect of different topical treatments (emollients and corticosteroids), reflected by the restoration of normal TEWL. **Skin elasticity** is altered in a number of skin diseases and does not allow for specific diagnostic correlation regarding inflammatory skin diseases [190].

1.6.3 Suction blister

In this technique a suction blister is formed with the help of a negative pressure instrument within 2-3 h (depending on site), by separating the epidermis from the dermis. The blister roof is collected (epidermis) and can be used for gene, protein and lipid studies [191].

1.6.4 Microneedle technique

The microneedle technique utilises micron-scale needles to create micropores into the skin. It is an emerging new method, which has been used for transdermal drug delivery (e.g. insulin, heparin), monitoring pharmacokinetics, but also for the study of immunologic markers in the interstitial fluid [192].

1.6.5 Skin scraping and washing fluid

Similarly to tape stripping, the skin scraping technique collects scales scraped away from the skin surface. It is simple and non-invasive, however it is difficult to standardize and it is only suitable to be used in pathologically involved skin [190]. Washing of standardised areas of lesional as well as non-lesional and healthy skin, with the collection of the washing liquid, has been successfully used for the measurement of antimicrobial peptides [193, 194].

1.6.6 Tape stripping

Tape stripping is the collection of the superficial layers of the skin by adhesive tapes. Various tapes are available, however the most frequently used are the acrylic-based (D-Squame discs−1.4 cm diameter, CuDerm, Dallas, TX, USA or 2.2 cm diameter, Monaderm, Monaco), and synthetic rubber-based tapes (Adhesive Research, Glen Rock, PA), but barrier and cellophane tapes are also available. Tapes are applied to the skin for 2-10s by gentle circular motions, followed by brisk removal. Tape stripping does not allow to study full thickness skin. The quantity of biological material obtained by tape stripping is reduced towards the living compartments of the epidermis, hence with the number of tapes applied. Approximately a minimum of 30 consecutive tapes are necessary to completely remove the stratum corneum in healthy skin.

This work chose to use and further advance tape stripping over the other methods described in this chapter, due to its true non-invasive nature compared to other techniques (e.g. suction blister), as well as its ease of use. Tape stripping does not require special equipment (besides tapes), specialist knowledge or extensive training. It is a technique which can be easily learnt and performed by any personnel, and potentially even by patients in the future. It does not result in scarring, cause pain or discomfort, and it can be applied to virtually any anatomical location, also allowing for multiple or repeated sampling. Due to these properties it is highly suitable to be used also in the paediatric population. Although it does not allow for the study of full thickness skin, it is particularly suitable for the study of skin diseases with epidermal involvement, such as psoriasis and eczema.

Tape stripping has been previously used for transcriptomics, proteomics and metabolomics studies of the epidermis [190]. It has been also shown that tape stripping can successfully detect molecular changes in the stratum corneum of AD (including elevated TARC [171, 195], TSLP [196], hBD2 [197, 198] as well as a number of other markers with large proteomic profiling [199, 200]), and psoriasis patients (including high TNFα, IFNγ, K16, CD2, IL23A, IL12B, VGEF) [201]. However, none of the previous studies directly compared these two disease entities, but rather used healthy controls as comparators. As such, although ‘potential biomarkers’ have been highlighted previously by using tape stripping, these were mainly markers for lesional skin rather than disease specific.

1.6.7 Hair plucking

Hair plucking is an emerging technique to study skin pathologies involving the hair follicle (such as lupus erythematosus). The hair follicle derived epithelium, obtained by hair plucking, has been shown to provide valuable diagnostic information [202, 203].

1.7 Project Aims

As presented, the diagnosis of both psoriasis and eczema are strongly reliant on the clinical judgement of the examining physician. When the typical disease phenotype is missing, in case of minimal or very chronic inflammation, or in certain anatomical locations where clinical signs overlap (such as the palms and soles, flexural areas, scalp, face), the clinical diagnosis is challenging even for experienced dermatologists.

Histopathological examination of the pathologically involved skin is the current diagnostic gold standard and can potentially aid the diagnosis, however, in these cases histopathology reports are often inconclusive (overlap of histopathologic features just as clinical signs in distinct anatomical locations and in chronic inflammation, or minimal, undifferentiating changes in mild inflammation) and require repeated biopsies. Skin biopsies however are invasive procedures, which often induce anxiety in the patient (needle phobia, pain, increased worry of the disease), with potential complications (anaphylactic shock from the anaesthetic, bleeding, infection, scar formation – especially relevant on the face and genital area). Because of its invasiveness, it is often avoided in children. In addition, biopsies are costly. When taking sterile material/theatre room, staff costs for nurses, medics, histopathologists, laboratory costs for sample preparation, cutting and staining and storage into account, a biopsy will cost around £400. With the need for special equipment and aseptic conditions, a surgically trained staff, and trained dermatohistopathologists, as well as prioritisation of samples with potential malignancy, delays in reporting the diagnosis are common and can take up to 8 weeks from the time of the biopsy in UK clinical reality. A number of diagnostic markers have been described with microarrays and qPCR, but these approaches are also based on biopsy material, and are costly.

Although many serological markers have been proposed for disease severity for both eczema and psoriasis, so far none of these have been found to provide high enough sensitivity and/or specificity to be used as diagnostic markers. In general very severe disease presentations show reliable blood “inflammation” profiles. However, diagnostically challenging scenarios often involve mild or unusual disease presentations.

There is a clear need for a non-invasive diagnostic tool which would facilitate diagnosis in routine practise situations and allow for personalised, evidence based treatment decisions.

The main aim of this project was:

- The further development of tape stripping as a non-invasive diagnostic tool to allow for the identification of a reliable epidermal biomarker which differentiates psoriatic from eczematous inflammation.

Additional aims were as follows:

- Investigate if tape stripping could be used to gain further pathomechanistic insights into disease development.
- Examine if early molecular changes can be detected by tape stripping in the non-involved skin of psoriasis and eczema patients, with potential future use for prevention and diagnostic in subclinical inflammation.
- Assess if tape stripping markers could be used as indicators of treatment response, in order to facilitate personalised, evidence based treatment options.

Chapter 2 Materials and Methods

2.1 Participant recruitment

2.1.1 Ethical approval

Ethical approval for the recruitment of volunteers was obtained from the ethical committee of the University of Leeds (BIOSCI 14-001). Ethical approval for the recruitment of patients was approved by NRES Committee North East-York (ILTIPP study: REC 14/NE/1199, PDAR study REC 16/YH/0086, ALPHA study: REC 14/YH/1259, CONVAS study: REC 10/H1306/88). Full informed written consent was obtained from all participants. The study was conducted in accordance with the declaration of Helsinki.

2.1.2 Participant recruitment

Patients were recruited from specialist dermatology clinics from St Luke's Hospital, Bradford Teaching Hospitals NHS Foundation Trust and from Chapel Allerton Hospital, Leeds Teaching Hospitals NHS Trust. Only patients over the age of 18 were eligible. As the focus of the study was on psoriasis and eczema, mainly patients with a clear clinical diagnosis of psoriasis (including plaque, guttate, scalp, inverse, palmoplantar subtypes) or eczema (atopic and non-atopic dermatitis) were recruited. However, patients suffering from other inflammatory skin diseases including cutaneous discoid lupus erythematosus (CDLE), subacute cutaneous lupus erythematosus (SCLE), pityriasis rubra pilaris and fungal infection were also included. The diagnosis was made by specialist dermatologists, based on the characteristic morphological and clinical features and, where available, routine dermatohistopathology. Systemic treatment naïve as well as patients on active systemic treatment were recruited, with the exception of patients under systemic steroid or active biologic treatments. However, an exception was made if the patient was non-responsive to therapy, showing signs of disease reactivation or had an unusual phenotype.

Healthy volunteers over the age of 18 years, with no personal history of any skin diseases were also recruited.

2.1.3 Clinical data collection

A case report form (CRF, appendix) as developed for the ILTIPP trial was completed for each patient and information on clinical diagnostic, disease phenotype and lesion presentation from this CRF were used. All samples analysed in this project also had information on the intensity of inflammation in the sampled area (local lesions score).

2.2 Materials

Reagents were obtained from Sigma-Aldrich, UK, unless stated otherwise.

2.2.1 Buffers used

Tape stripping lysis buffer for samples analysed by ELISA based techniques: 20 mM Tris pH 7.4, 150 mM NaCl, 1% Trition X100, 5 mM EDTA, 1 X protease inhibitor cocktail (Roche, Welwyn Garden City, UK);

Tape stripping lysis buffer for samples analysed by mass spectrometry: 6M Guanidine HCl, 150mM NH_4 HCO_3;

RIPA buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA ,1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 1 µg/ml leupeptin;

RLT buffer: high concentration of guanidine isothiocycanate (exact composition confidential- Qiagen);

PBS Tween (for washing Western blot membranes): PBS, 0.1% Tween-20.

2.2.2 Cell lines used

HaCaT cells are an adherent spontaneously transformed cell line derived from adult human keratinocytes [204].

Human primary keratinocytes were obtained from healthy volunteers and psoriatic patients in accordance with the Declaration of Helsinki as stated previously.

2.3 Non-invasive sample collection and processing

2.3.1 Tape stripping

D-squame adhesive discs of 3.8 cm^2 (Cuderm corporation, Dallas, Texas, USA) were used to non-invasively sample the skin. The sampled area was marked following the placement of the first tape by applying four marks with a pen around the outside of the tape (at 12, 3, 6 and 9 o'clock). In order to remove any impurities from the surface area the first tape applied to the skin was discarded. 10 subsequent tapes were collected from the same location. Tapes were applied with gentle pressure for approx. 5 seconds, removed with a sudden movement and placed in a collection tube. Samples for protein extraction were immediately placed on dry ice following sampling for transport and stored at -80 until processing. For samples intended for RNA isolation, RNAlater (Sigma-Aldrich, Gillingham, Dorset, UK) was used in order to avoid RNA degradation.

2.3.2 Hair plucking

Anagen hair – as verified by macroscopic inspection of the hair follicle – were plucked from lesional areas or areas just adjacent to lesions. Tweezers were used to pluck out individual hairs. An average of 5 lesional and 5 non-lesional hair follicles (by preference collected from the occipital area) were collected from each patient. After cutting off the hair follicle, Optimal Cutting Temperature (OCT) compound (Tissue-Tek; Sakura Finetek Europe, Alphen an den Rijn, The Netherlands) was used to preserve the quality of RNA. If not processed immediately, samples were stored at - 80°C.

2.3.3 Selection of sampled area

The location of tape sampling depended on the pathology and lesion localisation. Therefore, lesions from the elbow or knee were sampled in typical plaque psoriasis, the retro auricular area for scalp psoriasis, the popliteal fossa in typical AD or palmar/dorsal hand in hand eczema. However, if no active skin lesion was present at any of the disease specific sites, samples of the most representative lesion were taken independently of body location. In addition, samples were preferentially taken from nontreated sites, or at sites with no topical treatment for a minimum of 2 days. However, if this was not feasible a sample was taken if an active lesion was present. Lesions with oozing or broken skin (erosions, vesicles, bleeding/oozing fissures) were avoided. Sampling was stopped if any bleeding or oozing occurred during the procedure.

Samples from healthy volunteers were taken from matching areas: elbow, forearm and hands. Non-lesional samples were collected from the forearm of patients. Para-lesional samples of typical plaque psoriasis cases were collected 1 cm from the actively involved skin.

2.3.4 Local lesional severity score

In order to assess the severity of local inflammation of the sampled area a local severity score was developed. This was based on clinical signs in line with severity assessment tools such as PASI or EASI (Eczema Area and Severity Index). For each tape stripped area, signs including erythema, infiltration/lichenification, scaling and vesiculation (in case of eczema) were scored on a scale from 4 to 0, where 4 = very severe; 3 = severe; 2 = moderate; 1 = mild; 0 = absent. In addition, the severity of itch, a symptom often described in both psoriasis and eczema, was also recorded in the same manner.

2.3.5 Sample processing and protein extraction

Tape stripping samples for protein quantification by ELISA based techniques were transferred tape by tape into an Eppendorf tube containing 1.5 ml lysis buffer. Following transfer, samples were labelled and incubated on ice for 30 min. Subsequently, samples were sonicated 3 times for 20sec, with 20sec intervals of cooling on ice between each sonication step in order to avoid overheating of sample and protein denaturation. Finally, samples were centrifuged at 15000g (max rpm) for 10 min and the supernatant was carefully collected. Samples obtained were stored at -80°C.

The same methodology applied for samples collected for mass spectrometry, however the composition and amount of lysis buffer used (0.75 ml) differed.

2.3.6 Total protein content determination

The total soluble protein content of each tape stripping sample was determined using bicinchoninic acid assay (BCA - microplate procedure, Life Technologies Ltd, Paisley, UK).

Tape stripping samples processed for mass spectrometry were diluted with distilled water 1:6 to reduce the sample's content of 6M GuHCl 150mM NH_4HCO_3 to 1M GuHCl 25mM NH_4HCO_3, in order to avoid interference with the BCA assay reagent. In this case a solution of 1M GuHCl 25mM NH_4HCO_3 was also used for the preparation of standards.

2.3.7 RNA extraction

2.3.7.1 Tape stripping samples

Similarly to the previously described method for protein extraction, tape samples for RNA extraction were transferred into a 2 ml Eppendorf tube containing 0.5 ml RLT buffer (Qiagen). Following a 1 min sonication step, samples were centrifuged at 15.000 g for 1 min. Lysates were carefully collected. Following the manufacturer's recommendations, total RNA was extracted with RNeasy Mini extraction Kit (QIAGEN, Manchester, UK).

The acquired RNA quality was assessed by Agilent 2200 bioanalyzer, according to the obtained RNA Integrity Number (RIN) (Agilent Technologies, Stockport, UK).

2.3.7.2 Plucked hair samples

Plucked hair follicles were first cut to frozen sections of 3-5 µm thickness with a Leica CM3050S cryostat (Leica Microsystems, Buckinghamshire, UK) prior to RNA extraction. Sections were then transferred into RNase free universal tubes containing lysis buffer. Total RNA was extracted with ReliaPrep RNA Cell Miniprep System (Promega, Wisconsin, USA). RNA quality was assessed using an Agilent bioanalyzer.

2.3.8 Reverse transcriptase quantitative PCR (RT-qPCR)

Reverse transcriptase quantitative PCR (RT-qPCR) was used to quantify mRNA expression of genes of interest in hair follicle samples. cDNA was generated from 50-100 ng RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Loughborough, UK). Primers for genes of interest were purchased from Qiagen (Hilden, Germany), except for U6 snRNA32 (Sigma-Aldrich, Poole, UK). RT-qPCR was carried out on a QuantStudio5 Real-Time PCR system (Applied Biosystems, California, USA) using Qiagen QuantiTect SYBR Green Master Mix (Qiagen, Manchester, UK), according to the manufacturer's protocol, with the following parameters: initial heat activation, 95°C for 5 min; denaturation, 95°C for 10 s; combined annealing and elongation, 60°C for 30 s for a 40 cycle run. The comparative or ΔΔCt method was used to analyse the acquired RT-qPCR data. The mRNA expression of target genes were normalised to the housekeeping gene U6snRNA.

2.4 Mass Spectrometry

2.4.1 In-solution digest protocol

For mass spectrometry analysis 70µg protein was used per sample, with a minimum of 50µg protein where total protein concentration was lower. Proteins were denatured, followed by reduction and alkylation steps, and finally by trypsinisation in order to generate peptides within the mass range detectable via mass spectrometry.

Samples were diluted from 6M GuHCl 150mM NH4HCO3 to 1M GuHCl 25mM NH4HCO3 with distilled water. GuHCl initiated protein denaturation. Dithiothreitol was used at a final concentration of 10mM for the reduction of disulphide bounds (reduction step). Samples were incubated at 56 °C for 1 hr with shaking in order to enhance protein denaturation and reduction. This was followed by alkylation with iodoacetamide at a final 55mM concentration, in order to prevent re-formation of disulphide bonds. Samples were covered with a foil in order to avoid photoexposure and incubated at 20 °C for 1 hr with shaking (alkylation step). The reduction and alkylation of disulfide bonds was necessary for protein denaturation, allowing proteolytic enzymes (trypsin) to access proteolytic sites. Trypsin with high cleavage activity (Pierce Trypsin Protease, MS Grade, Thermo Fisher Scientific), was used for enzymatic degradation of proteins to peptides. 1µg trypsin was used for each µg of protein (e.g. 50µg of trypsin was added to 50 µg protein). Samples were incubated at 37 °C for 18 hours with shaking. The reaction was quenched by adding 5 µl of 1.0% trifluoroacetic acid (TFA).

2.4.2 Solid Phase Extraction (SPE)

WAT054960 Sep-Pak tC18 Sep-Pak C18 Vac cartridges (Water LTD), with hydrophobic properties, were used to adsorb analytes (peptides) from samples. Each cartridge was fixed and an empty Eppendorf tube was placed beneath. First the cartridge was wet by forcing 1 ml acetonitrile through at less than 1 drop a second (**column conditioning**). In a similar way, the cartridge was wet with 1 mL 0.1% TFA (**column equilibration**). The sample's pH was adjusted by adding 500µL 0.1% TFA to each sample before passing it through the cartridge (**sample loading**). Following sample application, the cartridge was washed with 1 ml 0.1% TFA to remove undesired contaminants (**column washing**). Finally, recovery of the analytes from the sorbent column was obtained by elution with 500µL: 50/50 0.1% TFA (**analyte elution**). The eluent was subsequently dried down in a speed

vacuum (~3 hours), reconstituted in 0.1% TFA (20µl) and stored at -80°C until mass spectrometry analysis.

2.4.3 Mass spectrometry

Liquid chromatography tandem mass spectrometry (LC-MS-MS) using an **Orbitrap system** was performed in-house by the Mass Spectrometry Research Facility, University of Leeds, by Rachel George and James Ault.

2.4.4 Mass spectrometry data acquisition and analysis

MaxQuant proteomics software package and Perseus software version 1.6.1.3 were used to analyse the obtained large mass spectrometry data. The data was cleaned by filtering and removing false hits, including proteins only identified by site, reverse and potential contaminants. This was followed by data normalisation using log2(x) transformation. Missing values were replaced randomly from normal distribution.

2.5 Protein quantification

2.5.1 IL36γ ELISA

A custom made sandwich ELISA was used for the quantification of IL36γ in samples following general ELISA protocols. 96 well immunosorbent ELISA plates (Nunc. Life Technologies, Paisley, UK) were coated with 100µl/well B5A2 monoclonal capture antibody in phosphate buffered saline (PBS) at a concentration of 2 µg/ml. The IL36γ capture antibody B5A2 was generated and provided by Dr Tom Macleod from the University of Leeds. The plate was incubated at 4°C overnight. Plates were then washed 4 times with 0.1% Tween-20/PBS and blocked for 1 hour with 2% bovine serum albumin (BSA) in 0.1% Tween-20/PBS. Following repeated washing, samples were added to the plate and subsequently incubated for 2 hours at room temperature with agitation. At the end of the incubation period samples were discarded, followed by repeated washing. Biotinylated monoclonal IL36γ detection antibody HCL17 was added to each well at a concentration of 250 ng/ml and incubated for 1 hours. After subsequent washing steps, streptavidin-HRP was added to the wells and the plate was incubated for 20 min. Plates were then washed and TMB was used as chromogenic substrate for HRP detection. Plates were incubated in the dark until colour development before addition of equal amount of 2N H_2SO_4. The absorbance of each well was read at 450 nm and 550 nm, followed by subtraction

of 550 nm values from 450 nm values to correct for optical imperfections in the microplate.

2.5.2 Commercially available ELISAs

Elafin, cathepsin S (R&D systems, Abingdon), S100A8/A9 (Biolegend, London), CCL27 (Bio-Techne Ltd, Abingdon), hBD2, hBD3 (Peprotech, London) and RNAse 7 (Abcam, Cambridge) proteins from the tape stripping samples were also quantified by ELISA according to the manufacturers' protocols.

2.5.3 Multiplex bead-based quantification assays

Multiplex bead-based quantification immunoassays (LEGENDplex, Biolegend, London, UK) were used to detect and measure cytokines and chemokines in tape stripping samples, following the manufacturer's recommendations. These assays are based on the principles of sandwich immunoassays, capturing a soluble analyte between two antibodies. Beads are differentiated by size and fluorescence intensities. Each bead set is combined with a specific capture antibody (capture bead), allowing capture of the target analyte from the sample. This way multiple proteins of interest can be identified and quantified by a selected panel of capture beads. To the capture bead-analyte complex, biotinylated detection antibodies are added. Finally, by adding streptavidin-phycoerythrin (SA-PE) a fluorescent signal is generated with intensities correlated to the proportion of the amount of bound analyte. The fluorescence signal intensity was acquired by flow cytometry (LSRII, Oxford, UK). The results are analysed using LEGENDplex™ data analysis software (Biolegend), determining the concentration of an analyte based on a given standard curve. A range of cytokines and chemokines were measured in this manner by using the following ready-made kits: Human Cytokine Panel 2 (13-plex), Human Th Cytokine Panel (13-pex), Human Growth Factor Panel (4-plex), Human Proinflammatory Chemokine Panel (13-plex). Customised kit, containing 10 cytokines of interest (IL1α, IL1β, IL8, IL18, CCL17 (TARC), CXCL1 (GROα), CXCL10 (IP10), CCL20 (MIP-3α), IFN-λ1, IFN-λ2/3) was also purchased from Biolegend.

2.6 Cell Culture

2.6.1 Cell culture

HaCaT cell lines were grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Slough, UK) supplemented with 10% foetal calf serum (FCS) (Gibco, Paisley, UK), 0.5% L-Glutamine (Sigma-Aldrich, UK), 0.1 mg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, UK).

Primary keratinocytes were cultured at 37°C in keratinocyte basal medium-2 (KBM-2) (PromoCell, Heidelberg, Germany) supplemented with Bovine Pituitary Extract (0.004 ml/ml), Epidermal Growth Factor (0.125 ng/ml), Insulin (5 µg/ml), Hydrocortisone (0.33 µg/ml), Epinephrine (0.39 µg/ml), Transferrin (10 µg/ml) and $CaCl_2$ (0.06 mM). 0.1 mg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, UK) were added to the culture medium.

2.6.2 Cell passage

Primary keratinocytes were grown to 70-80% confluence, whilst cell lines to 80-90% confluence before passaging. All reagents used were previously warmed up in a 37°C water bath for a minimum of 15 minutes.

Used culture medium was removed from the growth flask with a serological pipette (stripette). Cells were washed with 5-7 ml (for T75 culture flask) pre-warmed PBS (Gibco, Paisley, UK) by shaking the flask gently for about 4-5 times, then PBS was removed.

For primary keratinocytes 5 ml pre-warmed 0.02% EDTA/Versene (Gibco, Paisley, UK) was added to the growth flask in order to disrupt cell-cell interaction, and incubated at 37ºC, 5% CO2 for about 5 minutes. When signs of complete detachment by rounding up of the cells was complete, the 0.02% EDTA/Versene was removed and 3 ml pre-warmed trypsin/EDTA was added. Following another incubation step of 3-5 mins, allowing the majority of the cells to detach from the flask, 3 ml pre-warmed trypsin neutralising solution (TNS, Lonza) was added. The liquid containing the cells was pipetted up and down 3 times in order to wash the flask thoroughly and then transferred into a 15 ml Falcon tube. After centrifugation at 1500xg for 3min, the supernatant was removed gently and the pellet resuspended in 10 ml pre-warmed keratinocytes growth medium.

In a similar manner, for cell lines either 3 ml (for T75 culture flask) or 5 ml (for T150 culture flask) of pre-warmed 0.05% trypsin-EDTA (Gibco, Paisley, UK) was

added to the growth flask, following the initial washing with PBS. The flask was incubated for about 5 minutes at 37ºC with 5% CO2, until the majority of cells appeared to be detached from each other and from the flask. An equal volume of DMEM containing 10% FBS was used to stop the trypsinisation. The suspension was pipetted up and down 3 times in order to wash the flask thoroughly and then transferred into a 15 ml Falcon tube. After centrifugation at 1500xg for 3min, the supernatant was removed gently and the pellet resuspended in 10 ml pre-warmed DMEM containing 10% FBS and 1% streptomycin/penicillin.

2.6.3 Psoriatic phenotype induction and drug efficacy assays

HaCaTs were plated at 15.000 cells/well in 0.5 ml DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin (24 well plates) and cultured in a humidified incubator containing 5% CO_2 at 37°C to 70-80% confluence. Prior to stimulation with “psoriatic” cytokines (IL17A, TNFα), media was changed to DMEM supplemented with 1% FBS, and 1% penicillin/streptomycin. HaCaTs were stimulated with IL17A (20 ng/ml) and with TNFα (50 ng/ml) and incubated for either 2h or 24 h depending on experimental setup. Following the incubation period, cells were treated with drugs used for psoriasis treatment as follows: hydrocortisone (0.1 μg/ml, 0.5 μg/ml, 5 μg/ml – in repeated experiments this was increased to 0.5 μg/ml, 5 μg/ml, 7.5 μg/ml); betamethasone (10^{-4} M, 10^{-6} M, 10^{-8} M); calcipotriol (10^{-6} M, 10^{-7} M, 10^{-8} M); betamethasone (10^{-4} M, 10^{-6} M, 10^{-8} M) combined with calcipotriol (10^{-6} M, 10^{-7} M, 10^{-8} M); cyclosporine A (1 μg/ml, 10 μg/ml, 50 μg/ml); methotrexate (MTX) (0.1 μM, 1 μM, 10 μM); cyclosporine A (1 μg/ml, 10 μg/ml, 50 μg/ml) combined with MTX (0.1 μM, 1 μM, 10 μM); acitretin (1 μM, 5 μM, 10 μM); dimethyl fumarate (1 μM, 10 μM, 20 μM); apremilast (0.5 μM, 1 μM, 1.5 μM); tofacitinib (0.5 μM, 1 μM, 2 μM); narrowband ultraviolet B (nUVB) (20mJ/cm^2).

Following treatment, cells were incubated with the selected drugs at the required concentrations for 24h and also for 48h in the case of MTX. The culture media was temporarily removed for the treatment with nUVB (310-311 nm) 20mJ/cm^2, then the same media was replaced immediately following treatment. After incubation, the cell supernatants were collected in a 1.5 ml Eppendorf tube and centrifuged at 13.000 x g for 5 mins at 4°C. The cell free supernatants were transferred in a new 1.5 ml Eppendorf tube and store at -80°C.

After removing the supernatants from the plate, cells were washed with cold PBS (1 ml/well), followed by adding cold RIPA lysis buffer (500 μl/well). Plates were placed on ice for 10 mins followed by incubation on a shaker for an additional 20

mins. Cells were scraped with a silicon spatula and lysates were collected in a 1.5 ml Eppendorf tube. Following centrifugation at 13.000 x g for 5 mins at 4°C, the cell-free lysate was carefully collected, avoiding the pellet, into new microtubes and store at -80°C.

Primary keratinocytes were plated at 15.000 cells/well (24 well plates) in 0.5 ml supplemented KBM-2 media and grown to 70-80% confluence. Before stimulation the media was changed to KBM-2 without EGF and hydrocortisone supplementation. Cells were stimulated for 24h with IL17A and TNFα.

Primary keratinocytes were subsequently treated with the following drugs: betamethasone (10^{-4} M, 10^{-6} M, 10^{-8} M); calcipotriol (10^{-6} M, 10^{-7} M, 10^{-8} M); betamethasone (10^{-4} M, 10^{-6} M, 10^{-8} M) combined with calcipotriol (10^{-6} M, 10^{-7} M, 10^{-8} M); cyclosporine A (1 μg/ml, 10 μg/ml, 50 μg/ml), and MTX (0.1 μM, 1 μM, 10 μM).

Cells were incubated post-treatment for another 24 h.

Cell supernatants and lysates were collected the following day as described above and stored at -80°C.

2.6.4 Cytotoxicity assays (LDH, ToxiLight)

2.6.4.1 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme which is released following cell damage. The amount of LDH release can be quantified by a colorimetric reaction. LDH induces the formation of pyruvate from lactate by reducing Nicotinamide Adenine Dinucleotide to Nicotinamide Adenine Dinucleotide plus Hydrogen, which is then used by diaphorase enzyme to convert tetrazolium salt to formazan, a red product measureable at 490 nm [205, 206].

Pierce LDH cytotoxicity assay kit (Thermo Fisher Scientific) was used following the manufacturer's recommendations to measure LDH activity in HaCaT cell supernatants and lysates, in order to control for any cytotoxic effect of the drugs used in the experiments. 50 μl of each sample was transferred to a 96-well flat bottom plate (Thermo Fisher Scientific) and mixed with equal amount of reaction mixture. The plate was incubated at room temperature for 30 mins protected from light. Following incubation, the reaction was stopped by adding 50 μl of stop solution (provided) to each sample. The absorbance was measured at 490nm and corrected with the readings at 680nm. An LDH positive control was included at each plate. In addition, DMEM without supplementation was included as

control, and DMEM containing 1% FBS and 1% streptomycin/penicillin (used culture media in the experiments) was included as a blank. Both resulted in very low readings.

2.6.4.2 ToxiLight cytotoxicity assay

ToxiLight (Lonza, Nottingham, UK) is a non-destructive bioluminescent cytotoxicity assay which quantitatively measures adenylate kinase (AK) released from dead cells [207, 208]. The reaction is based on the detection of bioluminescent emission produced when ADP is converted to ATP in the presence of AK in the sample, using luciferase as a catalyzator. The ToxiLight assay was carried out following the manufacturer's recommendations. 20 µl of supernatants and cell lysates from HaCaT and primary keratinocyte experiments were loaded on a luminescence compatible 96 well white plate (Greiner Bio-One LUMITRAC™, Fisher Scientific). Initially a 1:2 dilution was used for cell lysates. However, the RIPA buffer used to obtain the cell lysate had an inhibitory effect on the expression of AK. Therefore a number of different dilutions with PBS were tried (1:1; 1:2; 1:4; 1:10; 1:20) in order to minimise the inhibitory effect of the samples' RIPA content. This effect was obtain with 1:20 dilution. After reconstitution, 100 µl of AK detection reagent (AKDR) was added to each well. After an incubation period of 5 mins at room temperature, plates were read by a luminometer (Optima, BMG Labtech).

2.7 Optical coherence tomography (OCT)

OCT imaging was obtained by placing the 'VivoSight' topical OCT probe (Michelson Diagnostics, Kent, UK) on the desired location, capturing a 4 mm area. The probe consists of four parallel Swept-Source OCT systems. The machine scans a 4 mm wide area, to a depth of 2 mm, by using a laser beam of a central wavelength of 1310 and 150 nm laser sweep, capturing 250 vertical B-scans, with an inter-frame spacing of 4 µm. In order to assess the depth of sampling by tape stripping in healthy individuals, a 4 mm area at the volar forearm was sampled and imaged using OCT in a healthy volunteer. The area scanned was marked and divided into two equal halves. The left half served as reference point, while the right half was taped with increasing number of adhesive discs. Repeated simultaneous OCT imaging of the two halves were obtained following: 10, 20, 30, 40, and 50 consecutive tapes. The obtained scans were saved as DICOM and TIFF files. OCT Analyse software (Michelson Diagnostics, Kent, UK) was used to process the scans and reconstruct the skin surface roughness.

2.8 Statistical analysis

The MS dataset was analysed following normalisation steps with Perseus software version 1.6.1.3. T-test was used to determine significant data points between the means of two groups, with a permutation based FDR 0.05 calculation. Results were visualised in the form of volcano plots.

Protein quantification data from tape stripping samples and RT-qPCR data from plucked hair follicles were analysed with GraphPad Prism software, version 7.00 (California, USA). One-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test was used to determine statistically significant differences between groups.

Receiver operating characteristic (ROC) curve was used in order to analyse and visualise the diagnostic sensitivity and specificity of different protein markers. The optimal cut off level was determined by Youden index analysis.

Data resulting from cell experiments were analysed using GraphPad Prism software, version 8.1.0. One-way ANOVA followed by the Dunnett multiple comparison test was used to determine statistical significance, comparing the means of each treatment group with the mean of the positive (stimulated but nontreated) control.

Results are depicted as mean ± SD (where SEM is depicted that is stated in the legend). Statistical significance was determined as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Chapter 3 Profiling Eczematous and Psoriatic Inflammation via Non-invasive Tape Stripping and Mass-spectrometry

3.1 Introduction

Skin inflammation results in disturbed protein expression in psoriasis and eczema, including modified expression of inflammatory and keratinocyte differentiation markers. Biomarker studies are generally based on protein and mRNA profiling from invasive skin biopsy material. Large protein profiling studies of the pathologically involved epidermis by non-invasive methods are scarce, and lack comparison between pathologies, focusing and evaluating differences in protein expression either between eczema and healthy, or psoriasis and healthy skin. However, in order to understand why certain pathophysiological changes lead to the development of the psoriatic instead of the eczematous phenotype, and to identify a true biomarker, with high disease specificity and sensitivity, direct comparison of disease protein profiles in addition to healthy controls is essential.

Therefore, psoriatic, eczematous and healthy skin was sampled using a non-invasive tape stripping method. Samples were taken from both lesional and non-lesional skin in the case of eczema, as well as from para-lesional skin in the case of psoriasis. Following protein extraction optimised for MS analysis, large scale protein profiling has been carried out using MS. The aim of this approach was to shed light to pathophysiological changes in psoriasis and eczema, to understand the underlying disease mechanisms and to detect disease specific diagnostic biomarkers.

3.2 Sample collection, processing and characteristics

In total, 140 samples were collected (for more details refer to Figure 3.1). From these, a number of samples were excluded during sample processing and MS data analysis due to insufficient total protein content (as a minimum of 50µg protein was necessary for optimal MS processing), poor MS processing or insufficient protein acquisition, as depicted in Figure 3.2. The final data analysis was performed on 102 tape stripping samples. The data file containing the full list of proteins detected by MS analysis is available as an attached electronic file entitled “MS LFQ data”.

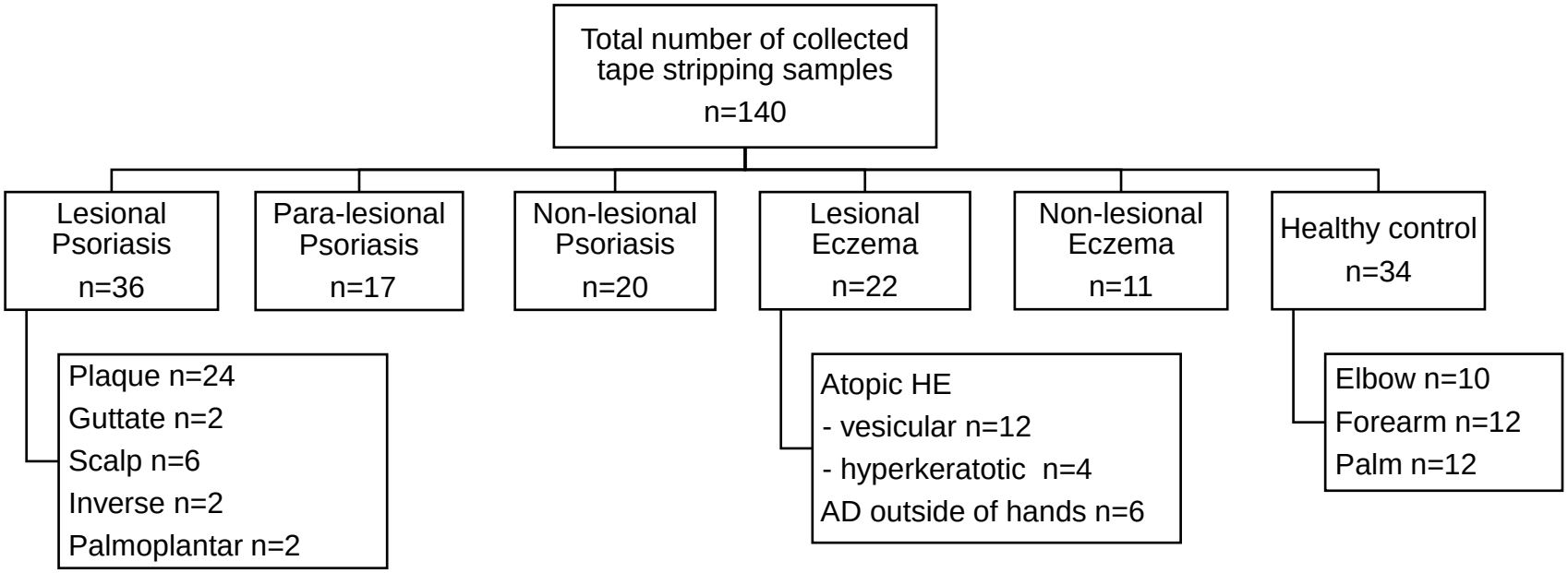

Figure 3.1 Characterisation of tape stripping samples collected for MS analysis.

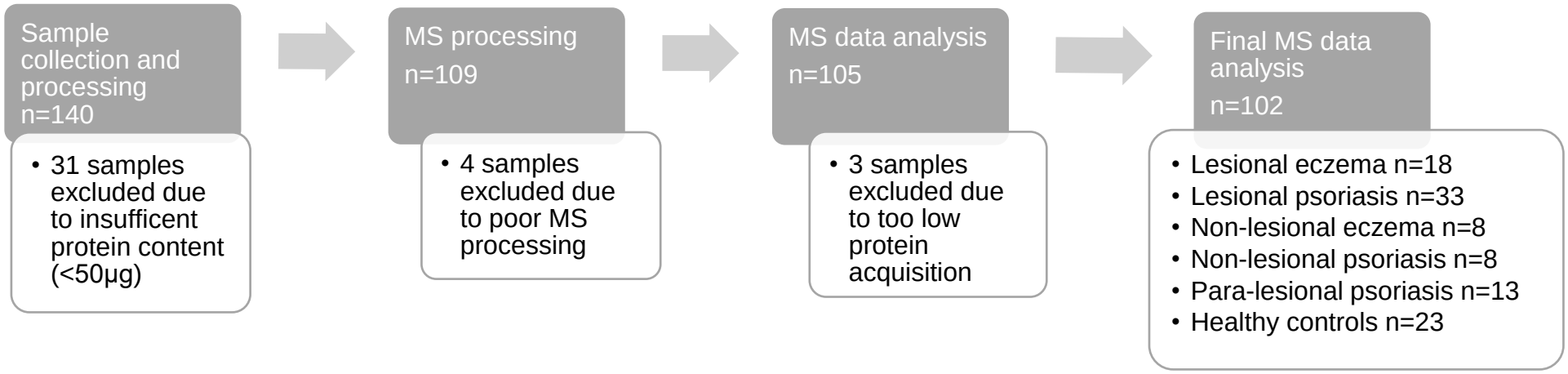

Figure 3.2 Tape stripping sample size following processing and data normalisation.

In total n=140 tape stripping samples were collected. From these the following 31 samples were excluded after sample processing due to insufficient total protein content: n=1 lesional, n=11 non-lesional and n=4 para-lesional psoriasis; n=1 lesional and n=3 non-lesional eczema; and n=11 healthy. Further 4 samples were excluded following MS analysis due to poor MS processing. Protein expression data was obtained for 105 samples. During MS data analysis and normalisation further 3 samples were excluded due to too low protein acquisition, including 1 lesional eczema sample with only 55 identified proteins (µ=345 proteins), 1 lesional psoriasis sample with 77 and 1 non-lesional psoriasis samples with 111 identified proteins (µ=365 proteins). The final data analysis was performed on a total of n=102 tape stripping sample.

3.3 Mass spectrometry data analysis

A total of 690 proteins were detected by LC-MS-MS, as described in the methodology section, and processed using MaxQuant software. Protein acquisition data was analysed in the Perseus software version 1.6.1.3. Following data filtering and normalisation, we analysed 644 proteins by t-test to determine significant expression levels between disease and control groups, with a permutation based FDR 0.05 calculation. Results were visualised in the form of volcano plots. For a summary of the most regulated proteins with special interest see Table 3.4.

3.4 Most abundant proteins detected via tape stripping

Sampling the superficial layers of the epidermis via tape stripping allowed for the detection of more than just structural cell components, such as cytokeratins. Numerous other proteins with a significant role in the skin barrier makeup were also abundant in tape strip material. The top ten most abundant proteins in each sample group are summarised in Table 3.1.

	Protein names	Gene names	Average protein abundance
Healthy	Keratin, type II cytoskeletal 1	KRT1	8.98E+09
	Keratin, type I cytoskeletal 10	KRT10	7.03E+09
	Zinc-alpha-2-glycoprotein	AZGP1	4.12E+09
	Caspase-14	CASP14	3.03E+09
	Dermcidin	DCD	2.58E+09
	Keratin, type I cytoskeletal 9	KRT9	2.58E+09
	Desmoplakin	DSP	2.15E+09
	Galectin-7	LGALS7	1.99E+09
	Arginase-1	ARG1	1.91E+09
	Keratin, type II cytoskeletal 5	KRT5	1.31E+09
Psoriasis L	Galectin-7	LGALS7	1.22E+10
	Keratin, type II cytoskeletal 1	KRT1	1.03E+10
	Keratin, type II cytoskeletal 6A	KRT6A	9.62E+09
	Fatty acid-binding protein, epidermal	FABP5	7.04E+09
	Keratin, type I cytoskeletal 10	KRT10	5.17E+09
	Protein S100-A9	S100A9	4.27E+09
	14-3-3 protein sigma	SFN	4.09E+09
	Heat shock protein beta-1	HSPB1	3.96E+09
	Keratin, type II cytoskeletal 5	KRT5	3.89E+09
	Histone H2B type 1	HIST1H2B	3.40E+09
AD L	Caspase-14	CASP14	1.23E+10
	Zinc-alpha-2-glycoprotein	AZGP1	1.19E+10
	Galectin-7	LGALS7	9.94E+09
	Ribonuclease 7	RNASE7	5.73E+09
	Keratin, type II cytoskeletal 1	KRT1	5.35E+09
	Desmoglein-1	DSG1	4.87E+09
	Histone H2B type 1	HIST1H2B	4.82E+09
	Complement factor H-related protein 1 and 5	CFHR1; CFHR5	3.70E+09
	Keratin, type I cytoskeletal 10	KRT10	3.56E+09
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	3.03E+09
Psoriasis PL	Complement factor H-related protein 1 and 5	CFHR1; CFHR5	2.26E+10
	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	8.29E+09
	Katanin p60 ATPase-containing subunit A1	KATNA1	5.09E+09
	Cystatin-S;Cystatin-SA	CST4; CST2	4.81E+09
	Pyrin domain-containing protein 1	PYDC1	3.17E+09

Table 3.1 The most abundant proteins detected via skin tape stripping in different sample groups.

	Protein names	Gene names	Average protein abundance
Psoriasis PL	Protein FAM83H	FAM83H	2.74E+09
	Serine protease inhibitor Kazal-type 9	SPINK9	2.15E+09
	Sorbitol dehydrogenase	SORD	2.05E+09
	26S protease regulatory subunit 10B	PSMC6	1.57E+09
	Serine/threonine-protein phosphatase 4 catalytic subunit	PPP4C	1.38E+09
Psoriasis NL	Keratin, type II cytoskeletal 1	KRT1	6.44E+09
	Keratin, type I cytoskeletal 10	KRT10	6.20E+09
	Galectin-7	LGALS7	6.11E+09
	Zinc-alpha-2-glycoprotein	AZGP1	4.39E+09
	Caspase-14	CASP14	2.97E+09
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.30E+09
	Desmoplakin	DSP	2.17E+09
	Cystatin-A;Cystatin-A, N-terminally processed	CSTA	1.36E+09
	Keratin, type II cytoskeletal 6A	KRT6A	1.09E+09
	Fatty acid-binding protein, epidermal	FABP5	8.32E+08
AD NL	Complement factor H-related protein 1 and 5	CFHR1; CFHR5	2.38E+10
	Ribonuclease 7	RNASE7	8.69E+09
	Galectin-7	LGALS7	8.49E+09
	Desmoglein-1	DSG1	6.23E+09
	Dermcidin	DCD	3.40E+09
	Pyruvate kinase PKM	PKM	3.20E+09
	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	1.74E+09
	Histone H2B type 1	HIST1H2B	1.26E+09
	Katanin p60 ATPase-containing subunit A1	KATNA1	1.09E+09
	Gasdermin-A	GSDMA	1.05E+09

Table 3.1 *(continued)* The most abundant proteins detected via skin tape stripping in different sample groups.

3.5 Differentially expressed proteins between lesional and healthy skin tape strips

From the 644 proteins identified by MS across samples, data analysis highlighted 90 significantly differentially expressed proteins between lesional psoriasis and healthy skin, with 30 being overexpressed in healthy while 60 in psoriatic skin (see Table 3.2). When compared eczematous lesional samples to healthy, 23 significantly different proteins were detected. From these, 6 were significantly upregulated in healthy while 17 in eczematous lesional skin (see Table 3.3).

Significant	FDR adjusted p value	Difference (Pso L vs H)	Protein names	Gene names
+	4.276E-19	72.102	**Protein S100-A9**	S100A9
+	3.521E-19	51.201	**14-3-3 protein sigma**	SFN
+	2.798E-18	41.235	**Beta-defensin 4A**	DEFB4A
+	4.977E-13	32.423	**Histone H2B type 1-M/N/H/D/L/C/E/F/G/I; Histone H2B type 2-F**	HIST1H2B-M/N/H/C/D/L; HIST2H2BF
+	6.448E-17	31.617	**Fatty acid-binding protein, epidermal**	FABP5
+	1.968E-15	22.923	**Elafin**	PI3
+	9.874E-16	22.124	**Heat shock protein beta-1**	HSPB1
+	2.458E-13	19.903	**Histone H3.3;Histone H3.2;Histone H3.1;Histone H3.3C;Histone H3.1t**	H3F3A;HIST2H3A;HIST1H3A;H3F3C ;HIST3H3
+	2.561E-14	16.510	**Fructose-bisphosphate aldolase A**	ALDOA
+	3.588E-16	16.050	**Histone H2A type 2-C/-A/-B; Histone H2A type 1-J/-H/-D; Histone H2A.J/X**	HIST2H2A-C/A/B; HIST1H2A-J/H/D;H2AFJ/X
+	4.266E-13	15.282	**Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed**	ACTG1
+	1.468E-12	14.944	**Peptidyl-prolyl cis-trans isomerase A**	PPIA
+	2.215E-11	10.903	**Alpha-enolase**	ENO1
+	8.992E-09	10.211	**Histone H4**	HIST1H4A
+	3.282E-09	9.373	**Triose phosphate isomerase**	TPI1
+	2.589E-11	9.328	**Serpin B3**	SERPINB3
+	9.960E-14	9.171	**Galectin-7**	LGALS7
+	2.908E-08	7.810	**Protein S100-A8;Protein S100-A8, N-terminally processed**	S100A8
+	2.421E-09	7.193	**Cofilin-1**	CFL1
+	4.800E-04	7.073	**Neutrophil defensin 1 and 3**	DEFA3;DEFA1
+	1.944E-08	6.602	**14-3-3 protein zeta/delta**	YWHAZ
+	5.375E-10	5.910	**Calmodulin-like protein 3**	CALML3
+	4.450E-05	5.305	**Putative elongation factor 1-alpha-like 3;Elongation factor 1-alpha 1-2**	EEF1A1P5;EEF1A1-A2
+	1.379E-05	5.213	**Histone H1.4**	HIST1H1E
+	1.843E-04	4.585	**Ig gamma-1 chain C region**	IGHG1
+	8.117E-06	4.548	**Ig alpha-1 chain C region**	IGHA1

Table 3.2 Significantly differential expression of proteins between lesional psoriasis and healthy tape strip samples.

Significant	FDR adjusted p value	Difference (Pso L vs H)	Protein names	Gene names
+	1.132E-08	4.464	**Peroxiredoxin-1**	PRDX1
+	6.110E-08	4.370	**Nucleoside diphosphate kinase B;Nucleoside diphosphate kinase A**	NME2;NME1
+	1.213E-04	4.314	**Prelamin-A/C;Lamin-A/C**	LMNA
+	2.434E-04	4.285	**Ig kappa chain C region**	IGKC
+	5.125E-06	4.259	**60S acidic ribosomal protein P1**	RPLP1
+	1.202E-03	3.861	**Retroviral-like aspartic protease 1**	ASPRV1
+	6.060E-06	3.762	**78 kDa glucose-regulated protein**	HSPA5
+	4.878E-10	3.717	**Phosphoglycerate mutase 1-2;Probable phosphoglycerate mutase 4**	PGAM1;PGAM2;PGAM4
+	8.313E-06	3.647	**Protein disulfide-isomerase**	P4HB
+	7.218E-05	3.490	**40S ribosomal protein S20**	RPS20
+	4.703E-05	3.432	**60S ribosomal protein L22**	RPL22
+	1.871E-04	3.415	**Beta-defensin 103**	DEFB103A
+	9.990E-09	3.303	**Elongation factor 2**	EEF2
+	7.438E-04	3.263	**Suprabasin**	SBSN
+	6.329E-06	3.095	**Interleukin-36 gamma**	IL36G
+	4.150E-06	3.077	**Heat shock cognate 71 kDa protein**	HSPA8
+	2.957E-03	2.930	**Serpin B4**	SERPINB4
+	3.953E-03	2.891	**Calmodulin-like protein 5**	CALML5
+	2.747E-04	2.870	**Tubulin beta-4B chain;Tubulin beta-4A chain**	TUBB4B;TUBB4A
+	5.201E-04	2.823	**Cellular retinoic acid-binding protein 2**	CRABP2
+	4.934E-04	2.566	**40S ribosomal protein S19**	RPS19
+	1.227E-03	2.514	**Epiplakin**	EPPK1
+	4.136E-03	2.302	**Ig gamma-2 chain C region**	IGHG2
+	1.369E-03	2.287	**Histone H1.5**	HIST1H1B
+	3.934E-03	2.241	**Tropomyosin alpha-3 chain**	TPM3
+	7.911E-04	2.174	**Protein disulfide-isomerase A3**	PDIA3
+	7.754E-05	2.155	**60S ribosomal protein L12**	RPL12
+	1.813E-03	2.136	**Thymidine phosphorylase**	TYMP

Table 3.2 *(continued)* Significantly differential expression of proteins between lesional psoriasis and healthy tape strip samples.

Significant	FDR adjusted p value	Difference (Pso L vs H)	Protein names	Gene names
+	9.925E-04	2.113	**Myosin-9**	MYH9
+	2.012E-03	2.019	**Heterogeneous nuclear ribonucleoprotein A1**	HNRNPA1
+	1.371E-02	1.965	**60S ribosomal protein L31**	RPL31
+	3.806E-03	1.909	**40S ribosomal protein S7**	RPS7
+	1.257E-02	1.890	**Periplakin**	PPL
+	7.448E-03	1.865	**Protein S100-A12;Calcitermin**	S100A12
+	6.267E-03	1.746	**Phosphoglycerate kinase 1**	PGK1
+	6.312E-03	-1.640	**GTP-binding nuclear protein Ran**	RAN
+	6.096E-03	-1.710	**Glia maturation factor beta**	GMFB
+	7.819E-03	-1.713	**Oligoribonuclease, mitochondrial**	REXO2
+	2.769E-03	-1.734	**Interleukin-37**	IL37
+	7.855E-03	-1.790	**Serpin B7**	SERPINB7
+	7.824E-03	-1.840	**Deoxyribonuclease-2-alpha**	DNASE2
+	5.922E-03	-1.846	**Cyclic nucleotide-gated cation channel beta-1**	CNGB1
+	7.861E-03	-1.880	**Antileukoproteinase**	SLPI
+	8.684E-04	-1.926	**Phenylalanine--tRNA ligase beta subunit**	FARSB
+	1.725E-03	-1.952	**Serpin B8**	SERPINB8
+	2.139E-03	-2.010	**Transthyretin**	TTR
+	1.101E-03	-2.208	**Desmocollin-3**	DSC3
+	3.378E-03	-2.214	**Gasdermin-A**	GSDMA
+	2.215E-04	-2.287	**Cystatin-M**	CST6
+	7.423E-04	-2.363	**Proactivator polypeptide-like 1**	PSAPL1
+	7.945E-04	-2.380	**Dipeptidyl peptidase 1**	CTSC
+	1.712E-05	-2.412	**Plectin**	PLEC
+	1.004E-04	-2.538	**Prolactin-inducible protein**	PIP
+	4.284E-04	-2.583	**Serpin A12**	SERPINA12
+	2.963E-04	-2.615	**Serpin B12**	SERPINB12
+	2.849E-05	-2.928	**Gamma-glutamylcyclotransferase**	GGCT

Table 3.2 *(continued)* Significantly differential expression of proteins between lesional psoriasis and healthy tape strip samples.

Significant	FDR adjusted p value	Difference (Pso L vs H)	Protein names	Gene names
+	2.616E-04	-3.015	**Desmocollin-1**	DSC1
+	1.803E-03	-3.022	**WAP four-disulfide core domain protein 12**	WFDC12
+	8.152E-04	-3.158	**Zinc-alpha-2-glycoprotein**	AZGP1
+	4.685E-06	-3.234	**Arginase-1**	ARG1
+	2.974E-06	-3.838	**Bleomycin hydrolase**	BLMH
+	2.091E-06	-3.979	**Cathepsin L2**	CTSV
+	8.922E-07	-5.463	**Kallikrein-7**	KLK7
+	4.268E-05	-5.701	**Secreted Ly-6/uPAR-related protein 1**	SLURP1
+	2.457E-06	-5.720	**Superoxide dismutase [Cu-Zn]**	SOD1
+	1.130E-09	-6.779	**Kallikrein-5**	KLK5
+	3.989E-13	-7.237	**Desmoglein-1**	DSG1
+	3.708E-11	-11.051	**Catalase**	CAT
+	2.781E-09	-14.513	**Dermcidin;Survival-promoting peptide;DCD-1**	DCD

Table 3.2 *(continued)* Significantly differential expression of proteins between lesional psoriasis and healthy tape strip samples.

Significant	FDR adjusted p value	Difference (Ecz L vs H)	Protein names	Gene names
+	6.113E-12	32.121	**14-3-3 protein sigma**	SFN
+	1.157E-09	30.016	**Histone H2B type 1- C/D/E/F/G/H/I/M/N/L; Histone H2B type 2-F;**	HIST1H2B-C/D/E/F/G/H/I/M/N/L; HIST2H2BF;
+	1.376E-10	23.641	**Protein S100-A9**	S100A9
+	9.897E-09	19.032	**Fatty acid-binding protein, epidermal**	FABP5
+	2.244E-12	17.739	**Histone H3.3;Histone H3.2;Histone H3.1;Histone H3.3C;Histone H3.1t**	H3F3A;HIST2H3A;HIST1H3A;H3F3C;HIST3H3
+	1.095E-07	11.988	**Heat shock protein beta-1**	HSPB1
+	4.019E-11	10.892	**Histone H2A type 2-A/B/C; Histone H2A type 1-D/G/H/J; Histone H2AX**	HIST2H2A-A/B/C; HIST1H2A-D/G/H/J; H2AFX
+	5.389E-08	8.370	**Galectin-7**	LGALS7
+	1.818E-07	8.283	**Histone H4**	HIST1H4A
+	8.836E-06	7.522	**Alpha-enolase**	ENO1
+	7.124E-06	6.200	**Actin, cytoplasmic 2**	ACTG1
+	1.221E-07	5.536	**14-3-3 protein zeta/delta**	YWHAZ
+	3.384E-06	5.096	**Peptidyl-prolyl cis-trans isomerase A**	PPIA
+	1.506E-04	4.698	**Fructose-bisphosphate aldolase A**	ALDOA
+	1.451E-04	4.481	**Beta-defensin 4A**	DEFB4A
+	1.304E-03	4.277	**Ig alpha-1 chain C region**	IGHA1
+	4.561E-03	4.148	**Ig kappa chain C region**	IGKC
+	2.245E-04	4.101	**Serpin B3**	SERPINB3
+	4.732E-04	3.831	**Haptoglobin**	HP
+	3.765E-03	3.449	**Apolipoprotein A-I**	APOA1
+	9.659E-04	3.070	**Calmodulin-like protein 3**	CALML3
+	1.475E-03	2.366	**Tubulin beta-4B chain;Tubulin beta-4A chain**	TUBB4B;TUBB4A
+	3.382E-04	2.134	**Phosphoglycerate mutase 1-2;Probable phosphoglycerate mutase 4**	PGAM1;PGAM2;PGAM4
+	4.244E-05	-3.961	**Kallikrein-5**	KLK5

Table 3.3 Significantly differential expression of proteins between lesional eczema and healthy tape strip samples.

Significant	FDR adjusted p value	Difference (Ecz L vs H)	Protein names	Gene names
+	1.407E-04	-4.248	**Bleomycin hydrolase**	BLMH
+	1.270E-04	-4.566	**Arginase-1**	ARG1
+	1.082E-06	-5.016	**Desmoglein-1**	DSG1
+	6.024E-05	-5.651	**Catalase**	CAT
+	1.278E-03	-5.671	**Dermcidin;Survival-promoting peptide;DCD-1**	DCD

Table 3.3 *(continued)* Significantly differential expression of proteins between lesional eczema and healthy tape strip samples.

3.5.1 Presence of plasma proteins

Compared to healthy samples, significantly high amounts of plasma-associated substances were present in lesional skin, such as **Ig alpha-1 chain C region**, **Ig kappa chain C region**, and **haptoglobin** in eczema and **Ig alpha-1 chain C region**, **Ig gamma-1 chain C region**, **Ig gamma-2 chain C region**, **Ig kappa chain C region** in psoriasis, reflecting the inflammatory status and perturbed barrier function in both diseases.

3.5.2 Stratum corneum barrier constituents, proteins and enzymes involved in intercellular cohesion and desquamation

Changes in adhesion molecule expression is a physiologic progress in epidermal turnover. Enzymatic degradation of intercellular adhesion molecules is an important process in the desquamation of corneocytes.

Desmoglein 1 (**DSG1**) expression was significantly lower in both eczema (5.01 fold) and psoriasis (7.24 fold) when compared to healthy. DSG1 is crucial in interkeratinocyte adhesion and is mainly expressed in the upper part of the epidermis. Its dysfunction or destruction by autoimmune processes is known to lead to blister formation (e.g. pemphigus) in the upper epidermal layers [209].

Other desmosome constituents, including **desmoglein 3** (**DSG3**), **corneodesmosin**, **desmocollin 1** and **3**, and members of the plakin family (**desmoplakin**, **envoplakin**, **periplakin**, **epiplakin**, **plectin**) did not present significantly differential expression between eczema and healthy samples. However, in psoriasis other barrier constituents also showed significantly decreased levels compared to healthy, including **desmocollin 1** (by 3.01 fold) and **3** (by 2.23 fold), and **plectin** (by 2.41 fold), known to link desmosomes and hemidesmosomes to the intermediate filament network [210]. On the other hand, **epiplakin**, involved in keratin filament reorganization with a protective role against cellular stress [211], was significantly upregulated in psoriasis samples, by 2.51 fold.

Seemingly, in lesional samples there was a significant decrease in the expression of enzymes involved in the lysis of intercellular adhesion molecules.

Zinc-alpha-2-glycoprotein (**ZAG**), a protein with enzymatic activity known to have an effect on epidermal differentiation [212], was found to be significantly decreased in lesional psoriasis (by 3.16 fold) compared to healthy and also in eczema (by 2.40 fold), although without significance in the latter.

Cathepsin L2, a relatively newly discovered cysteine protease with a role in corneodesmosome degradation, was also significantly downregulated in psoriatic

lesional stratum corneum compared to healthy controls, by 3.98 fold, while in lesional eczema this downregulation was less pronounced. **Dipeptidyl peptidase 1** (**DPP1**), another cysteine protease known to activate serine proteases such as neutrophil derived elastase and cathepsin G, showed similar decrease in lesional psoriasis, by 2.38 fold, compared to healthy skin. At the same time, **Cystatin-M**, a cysteine protease inhibitor, was also significantly decreased in the lesional psoriasis stratum corneum, by 2.29 fold. In addition, MS identified other members of the cystatin family at the level of the stratum corneum, including **cystatin A, B, C, SA** and **SN**, although these did not show significant differences in their expression levels between lesional and healthy skin.

Serine proteases expressed in the outermost epidermal layers, **kallikrein 5** (**KLK5**), and **7** (**KLK7**), also play crucial role in desquamation by cleaving corneodesmosomal proteins responsible for cellular adhesion [213]. MS identified in total 8 kallikrein family members in tape stripping material (**kallikrein 5-11** and **13**). Lesional psoriasis samples showed a significant downregulation in serine protease levels compared to healthy samples, with a 6.78 fold decrease in **KLK5** and a 5.46 fold decrease in **KLK7** expression. Beside their role in desquamation, **KLK7** expression is also correlated with keratinocyte terminal differentiation and cornification (Ovaere et al., 2009). **KLK5** (having an activator role for KLK7) [213] but not KLK7, was found to be significantly decreased in eczematous skin by 3.96 fold, compared to healthy.

Parallel to the low expression of serine proteases in lesional compared to healthy skin, especially pronounced in lesional psoriasis, the upregulation of **serine protease inhibitors** in lesional tape stripping samples was remarkable.

Elafin (**PI3/SKALP**) showed significantly increased levels in both lesional psoriasis (by 22.92 fold) and eczema (by 3.03 fold) compared to healthy controls. Elafin is known to have a protective role against tissue-damaging proteases during inflammatory processes. It exhibits weak KLK7 but strong neutrophil elastase and proteinase 3 inhibition [213]. MS data analysis showed significant upregulation of another KLK7 inhibitor in psoriatic versus healthy skin, **secretory leukocyte protease inhibitor** (**SLPI**), also known as **antileukoproteinase**, by 1.88 fold. SLPI also inhibits neutrophil elastase and cathepsin G, complementing elafin activity [213], and it has been shown to have antimicrobial properties against *Pseudomonas aeruginosa* and *S. aureus* [214].

In addition, MS detected the presence of 12 **serpin family members**, with the role of serine protease inhibition. **Serpin B8**, **A12**, **B12** were significantly overexpressed in healthy compared to psoriatic skin. On the other hand, **serpin B4** and especially **B3** showed much higher levels in psoriatic lesional skin

compared to healthy, by 2.93 and 9.33 fold respectively. Serpin B3 was also significantly upregulated in lesional eczema samples relative to healthy, by 4.10 fold. Other serpin family members, **serpin B1** (also known as **leukocyte elastase inhibitor**), **serpin B6**, **serpin B7**, **serpin B13** showed a tendency to be higher in healthy skin compared to lesional samples, except for **serpin B5**, but at a non-significant level.

One enzyme on the other hand, **retroviral-like aspartic protease 1** (**ASPRV1**), speculated to play a role in keratinocyte differentiation and desquamation [215], had significantly higher expression in lesional psoriatic skin compared to healthy, by 3.86 fold.

Caspase 14 and **bleomycin hydrolase** (**BH**) are both proteases present in the stratum corneum, playing an important role in profilaggrin [216] and filaggrin processing [217], respectively. **BH** was significantly downregulated in lesional eczema samples compared to healthy skin, by 4.25 fold, as well as in lesional psoriasis samples, by 3.84 fold. **Caspase 14** also showed lower expression levels in both eczematous and psoriatic skin, however, this was not significant.

In both psoriasis and eczema there was a significant downregulation of **arginase 1** (**ARG1**), involved in urea synthesis [218], by 3.23 and 4.57 fold respectively.

Filaggrin was inconsistently detected across samples, therefore it was excluded from the final analysis during data normalisation.

An overview of the regulation of the differentially expressed proteases and protease inhibitors mentioned above are depicted in Figure 3.3.

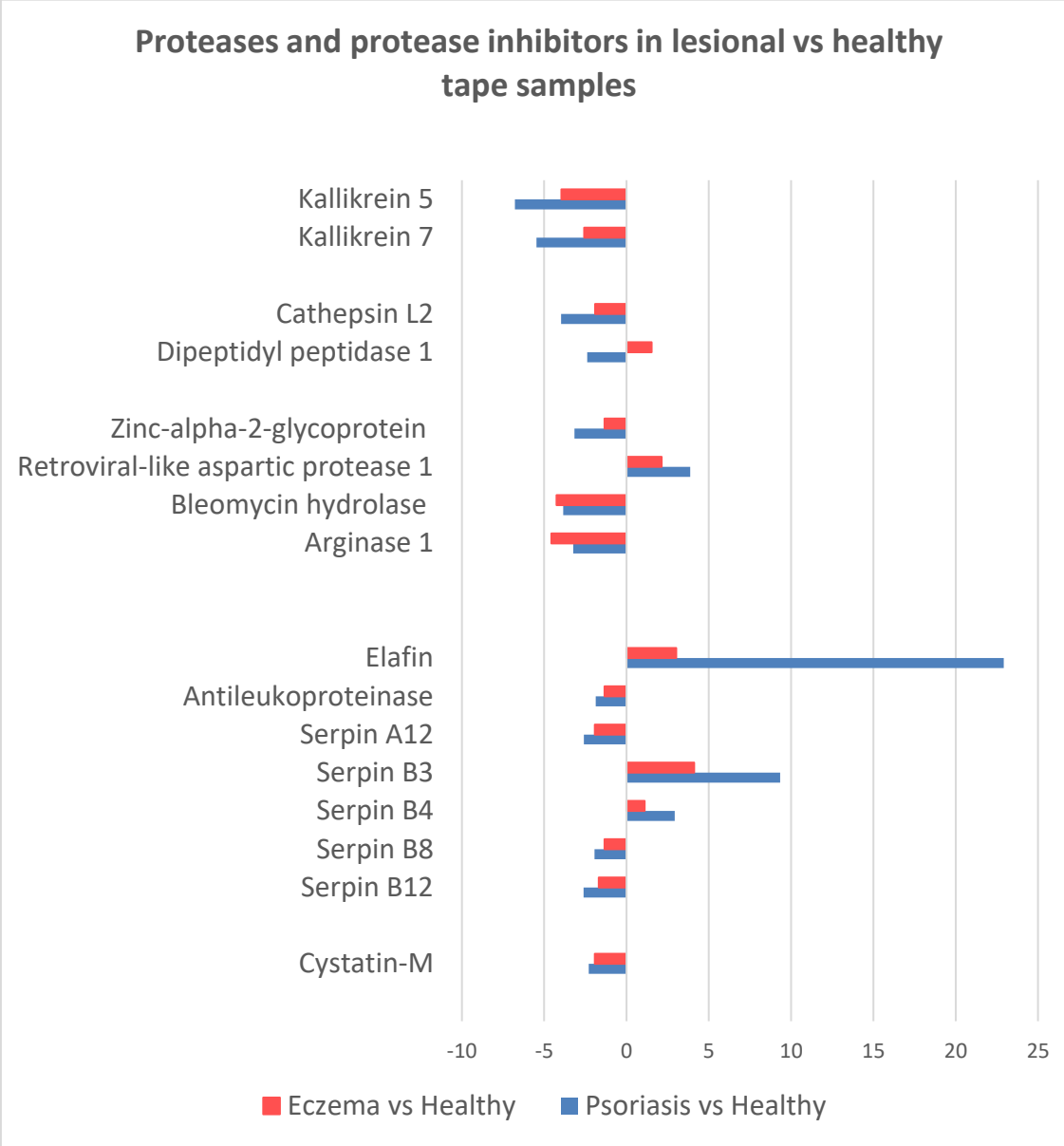

Figure 3.3 Proteases and protease inhibitors in lesional vs healthy tape samples.
An overview of the most regulated proteases and protease inhibitors detected by MS in lesional psoriasis vs healthy and lesional eczema vs healthy tape strip samples are as follows: serine proteases (KLK5, KLK7), cysteine proteases (Cathepsin L2, DPP1), proteolytic enzymes with a role in desquamation, keratinocyte differentiation, filaggrin processing and epidermal hydration (ZAG, ASPRV1, BH, ARG1), neutrophil and serine protease inhibitors (elafin, antileukoproteinase, serpin A12, B3, B4, B8, B12) and cysteine protease inhibitor (cystatin-M). Neutrophil protease inhibitor elafin showed the most remarkable upregulation in lesional psoriasis samples by 22.92 fold.

3.5.3 Stratum corneum differentiation markers and proapoptotic proteins

No significant difference was detected between the expression of differentiation markers involucrin and loricrin in healthy and lesional skin.

However, **suprabasin**, a novel epidermal differentiation marker, with similar structure to involucrin and loricrin and a potential cornified envelop precursor [219], was significantly upregulated in psoriasis samples by 3.26 fold compared to healthy. Another newly described late keratinocyte differentiation marker, **calmodulin-like protein 5**, also known as **calmodulin-like skin protein**, was also significantly upregulated in psoriatic skin compared to healthy by 2.89 fold, and similarly in eczema, but not at a significant level. On the other hand, **calmodulin-like protein 3**, had significantly increased levels in both eczematous (by 3.07 fold) and psoriatic lesional skin (by 5.91 fold) compared to healthy.

Compared to healthy there was a slight, but non-significant decrease in lesional eczema of **secreted Ly-6/uPAR-related protein 1** (**SLURP1**), a proapoptotic protein with role in the terminal differentiation of keratinocytes, and of **gasdermin A**, another proapoptotic protein with bactericidal properties, with both significantly downregulated in lesional psoriasis by 5.70 and 2.21 fold respectively.

On the other hand, **galectin 7**, a protein implicated in intercellular and cell-matrix interactions also with proapoptotic properties [220], showed significant overexpression in lesional skin, by 9.17 fold in psoriasis and by 8.37 fold in eczema.

3.5.4 Antimicrobial peptides

Dermcidin, a constitutively expressed antimicrobial peptide produced by eccrine sweat glands and secreted into the sweat reaching the epidermal surface [221], was significantly lower in both eczema (5.67 fold) and psoriasis (14.51 fold) when compared to healthy.

WAP four-disulfide core domain protein 12 (**WFDC12**), a protein with both serine proteinase inhibitor and antimicrobial properties, was also significantly decreased in lesional psoriasis compared to healthy by 3.02 fold, but with a non-significant decrease in eczema.

In contrast, **hBD2** was significantly overexpressed in both eczematous (by 4.48 fold) and psoriatic lesions (by 41.24 fold) compared to healthy skin. Other members of the defensin family, **hBD3** as well as **α-defensins**, **human neutrophil peptides** (**HNP**) **1–3**, were also found increased in eczematous lesional skin compared to healthy, but not at a significant level. In contrast,

comparison of their expression between psoriasis and healthy samples revealed significant increase of **hBD3** (by 3.41 fold) as well as of neutrophil secreted **HNP 1-3** (by 7.07 fold) in psoriasis.

From the 11 **S100 family members** known to be present in the human epidermis [159], 8 members were detected by MS in tape stripping skin samples. Members of the S100 family with recognised antimicrobial activity [222] showed significantly elevated expression in psoriatic lesional skin compared to healthy, **S100A8** by 7.81 fold, **S100A9** by 72.10 fold. S100A8/9 is also referred to as calprotectin, an important marker for disease activity in inflammatory bowel diseases [223]. In psoriasis **S100A12** also showed a 1.86 fold increased expression compared to healthy. In eczematous skin, only **S100A9** presented with significantly higher expression by 23.64 fold, in contrast to healthy.

Other members, including **S100A7** (**psoriasin**), **S100A10**, **S100A11**, **S100A14**, **S100A16** showed only modest changes between healthy and lesional tape samples.

3.5.5 Proteins involved in cell metabolism, signalling pathways and cytoskeletal changes

Fatty acid binding protein 5 (**FABP5**), involved in the uptake, transport, and metabolism of fatty acids, was significantly upregulated in both lesional psoriasis (by 31.62 fold) and lesional eczema samples (by 19.03 fold) compared to healthy.

Apolipoprotein A-I (**APOA1**), another protein involved in lipid metabolism, was significantly upregulated in lesional eczema samples, by 3.45 fold, compared to healthy, but not in lesional psoriasis. MS also detected the presence of 4 other apolipoprotein members, although no significant changes have been detected in their expression levels across samples: **apolipoprotein A-II**, **apolipoprotein A-IV**, **apolipoprotein C-III** and **apolipoprotein D**.

Proactivator polypeptide-like 1 (**PSAPL1**), thought to play a role in sphingolipid metabolism, had significantly lower expression levels in lesional psoriasis compared to healthy skin (2.36 fold).

Three enzymes with glycolytic activity were significantly upregulated in lesional skin compared to healthy: **alpha-enolase**, by 10.90 fold in psoriasis and by 7.52 fold in eczema and **fructose-bisphosphate aldolase A** (**ALDOA**) by 16.51 fold in psoriasis and by 4.70 fold in eczema, and **phosphoglycerate mutase** (**PGAM**) by 3.72 fold in psoriasis and by 2.13 fold in eczema. Furthermore, **triose-phosphate isomerase** (**TPI1**) and **phosphoglycerate kinase 1**, also involved in gluconeogenesis, similarly had significantly higher expression levels in lesional

psoriasis compared to healthy, by 9.37 and 1.75 fold respectively. Although these enzymes were also increased in eczema, this was not at a significant level.

Furthermore, three enzymes with important antioxidant activity were downregulated in lesional skin versus healthy. **Superoxide dismutase [Cu-Zn]** (**SOD1**) expression was significantly decreased in lesional psoriasis (by 5.72 fold) and also in lesional eczema (by 2.62 fold) compared to healthy stratum corneum, although in the latter at a non-significant level. An even bigger decrease was noted in the expression of **catalase** (**CAT**) in both lesional psoriasis (by 11.05 fold) and eczema (by 5.65 fold). In addition **gamma-glutamylcyclotransferase** (**GGCT**), a major enzyme involved in glutathione metabolism and an antioxidant [224], was also significantly downregulated (by 2.93 fold) in lesional psoriasis compared to healthy skin. Less difference was noted between its expression in healthy versus lesional eczema. **Peroxiredoxin 1** however, another antioxidant enzyme, was significantly upregulated in psoriatic lesional stratum corneum compared to healthy, by 4.46 fold, while the other three members of the peroxiredoxin family identified by MS (**peroxiredoxin 2**, **5**, **6**) showed no differential expression.

Heat shock proteins (HSPs) are expressed by cells following exposure to stress. There was a significantly high expression in both lesional eczema and psoriasis of **HSP beta-1**, also known as **HSP27**, by 11.99 and 22.12 fold respectively. In addition, in psoriasis there was a significant increase of **HSP 70 kDa protein 5 (HSPA5)** by 3.76 fold, and of **heat shock cognate 71 kDa protein** (**HSP73**) by 3.08 fold. MS also identified other members of the heat shock family, however, beside slight differences, these were similarly expressed between groups: **HSP10**; **HSP60**; **HSP70 protein 1A/1B**, **protein 2**, **protein 6**, **protein 7**; **HSP 90-alpha**, **-beta**.

Transthyretin (**TTR**), a transport protein for thyroxine (thyroid hormone) and retinol-binding protein [225], had significantly higher levels (by 2.01 fold) in healthy compared to psoriatic skin. On the other hand **cellular retinoic acid-binding protein 2** (**CRABP2**) was significantly lower in healthy compared to psoriatic lesional samples (by 2.82 fold).

From the 7 isoforms of the **14-3-3 protein family**, playing an important role in signalling pathways [226], MS identified 6 in the tape stripping material: **14-3-3 protein beta/alpha**, **gamma**, **zeta/delta**, **eta**, **epsilon** and **sigma**. From these, two were significantly upregulated in both eczema and psoriatic lesional samples compared to healthy: **14-3-3 protein zeta/delta** by 5.54 and 6.60 fold and **14-3-3 sigma** by 32.12 and 51.20 fold respectively.

Cofilin 1 (**CFL1**), a regulator of actin dynamics and cell migration, was significantly upregulated in tape samples of lesional psoriasis by 7.19 fold compared to healthy. CFL1 levels were also increased in lesional eczema samples compared to healthy, but at a non-significant level.

3.5.6 Pro- and anti-inflammatory proteins

IL36γ expression was significantly upregulated in psoriatic lesional skin versus healthy, by 3.09 fold. In lesional eczema samples, IL36γ also showed a tendency to be increased (by 1.31 fold) compared to healthy, however at a non-significant level. Interestingly, expression levels of the counter regulatory **IL36RA** were similar in lesional psoriasis and healthy samples, with only subtle elevation in healthy skin, but it was increased in lesional eczema samples compared to healthy, even though non-significantly (by 1.30 fold).

MS also detected the presence of other IL1 family members with anti-inflammatory effect in the tape stripping material. In psoriasis **IL37** expression was significantly decreased by 1.73 fold compared to healthy skin. A non-significant decrease was also detected in eczema versus healthy skin, by 1.29 fold. Relative to healthy, IL1 receptor antagonist (**IL1RN)** levels were slightly increased in both lesional psoriasis and eczema samples, although non-significantly.

Peptidyl-prolyl cis-trans isomerase A, also known as **cyclophilin A**, with regulatory roles in intracellular signalling, transcription, inflammation, and apoptosis [227], was significantly upregulated by 14.94 fold in lesional psoriasis and by 5.10 fold in lesional eczema compared to healthy skin.

3.5.7 Other cellular components

There was a marked increase in the detection of **histones** (chromatin structural proteins), other **nuclear components** (Prelamin-A/C, Lamin-A/C, Nucleoside diphosphate kinase A/B), **ribosomal proteins** (including subunits, elongation factors, heterogeneous nuclear ribonucleoproteins), **endoplasmic reticulum enzymes** (protein disulfide-isomerase, 78 kDa glucose-regulated protein, protein disulfide-isomerase A3) and **components of the cytoskeleton** (tubulins, actins, tropomyosin alpha-3, thymidine phosphorylase, myosin9) in lesional psoriatic skin compared to healthy. At the same time, **deoxyribonuclease-2-alpha** (**DNase2**), involved in nuclear DNA degradation, had significantly decreased levels in lesional psoriasis compared to healthy skin, by 1.84 fold, and it also showed decrease in lesional eczema, by 1.76 fold.

3.6 Differentially expressed proteins between eczema and psoriasis

As presented, expression of a range of proteins were significantly changed compared to healthy skin in both lesional eczema and psoriasis. As most of these proteins overlap in their expression levels in both conditions compared to normal controls, they mark the presence of general inflammation, rather than indicate disease specific changes. Therefore, the direct comparison between different diseases such as lesional psoriasis with lesional eczema samples is indispensable in the search of a specific biomarker, which could distinguish accurately between the two conditions.

MS data analysis identified 16 significantly differentially expressed proteins when comparing lesional psoriasis with lesional eczema. From these, 4 were significantly increased in lesional eczema and 12 in lesional psoriasis (see Figure 3.4).

3.6.1 Proteins increased in lesional psoriasis versus eczema

From the 12 upregulated proteins in lesional psoriasis, 3 were cell organelle and nuclear components: **histone H1.4**, **protein disulfide-isomerase** and **40S ribosomal protein S20**, reflecting the accelerated turnover of psoriatic keratinocytes, reaching the stratum corneum without complete terminal differentiation and degradation of cellular components.

The most striking upregulation was noticed in the level of the antimicrobial peptide **hBD2** expression, which was by 9.20 fold higher in lesional psoriasis compared to eczema. Similarly high expression was detected in psoriasis for **elafin**, upregulated by 7.57 fold and for **S100A8**, increased by 4.58 fold compared to lesional eczema. Just as hBD2, S100A8 has antimicrobial potential, but it may also indicate infiltration by neutrophils which highly express S100A8 [228]. Elafin also seems to reflect increased neutrophil activity in psoriatic lesional skin compared to lesional eczema, as its main function is to protect against neutrophil derived protease activity [229].

From the three glycolytic enzymes previously presented to be significantly upregulated in both lesional eczema and psoriasis compared to healthy skin, only **ALDOA** showed significantly increased expression in psoriasis when directly compared to eczema, by 3.51 fold.

Cofilin-1, with a role in cell compaction and inflammation, was increased by 3.21 fold in lesional psoriasis. **CRABP2**, involved in keratinocyte differentiation by regulating the intracellular metabolism of retinoic acid [230], was overexpressed

in lesional psoriasis by 2.99 fold compared to eczema samples, in which CRABP2 expression was similar to healthy skin.

Cyclophilin A, the binding protein for the immunosuppressant drug cyclosporine A, although significantly increased in both eczema and psoriasis compared to healthy controls, it was by 2.93 fold upregulated in lesional psoriasis compared to eczema.

Although most antioxidative proteins were downregulated in lesional skin, the expression of **peroxiredoxin-1** was increased in lesional psoriasis compared to lesional eczema, by 2.53 fold.

The only pro-inflammatory cytokine detected by MS in the tape harvested skin layers, **IL36γ**, showed 2.36 fold increased levels in lesional psoriasis compared to lesional eczema.

Other upregulated proteins in lesional psoriasis compared to eczema, which however did not reach the significance level are worth mentioning: **S100A9**, **triosephosphate isomerase** (with role in gluconeogenesis), **serpin B3** and **B4** (serine protease inhibitors), **suprabasin** (epidermal differentiation marker), **hBD3** (antimicrobial peptide), as well as further **cell organelle** (ribosomal proteins, elongation factors) and **cytoskeletal components** (actin, profilin1).

3.6.2 Proteins increased in lesional eczema versus psoriasis

The significantly increased expression of two serum components, **serotransferrin** and **haptoglobin** in lesional eczema compared to lesional psoriasis, by 4.55 and 3.24 fold respectively, potentially reflect the severely impaired barrier function associated with serum leakage into the epidermal compartment in eczema. This reflects the hallmark histopathological feature of spongiosis for eczematous reactions.

APOA1, involved in lipid metabolism, was overexpressed by 4.15 fold in lesional eczema samples compared to psoriasis and by 3.45 fold when it was compared to healthy. It is worth noting that APOA1 expression was not significantly different between lesional psoriasis and normal controls.

DPP1, better known as **cathepsin C**, a serine protease activator, was also significantly increased in lesional eczema samples compared to psoriasis by 3.63 fold, but was not significantly upregulated compared to healthy skin. Psoriasis samples showed reduced Cathepsin C levels compared to healthy skin.

Proteins with a more than 2 fold, although non-significant increase in lesional eczema compared to psoriasis were: **SLURP1** (proapoptotic protein), **dermcidin** (antimicrobial protein expressed in sweat), **SOD1** (antioxidative enzyme), **KLK7**

(serine protease) and **cathepsin L2** (corneodesmosomal protein degrading enzyme).

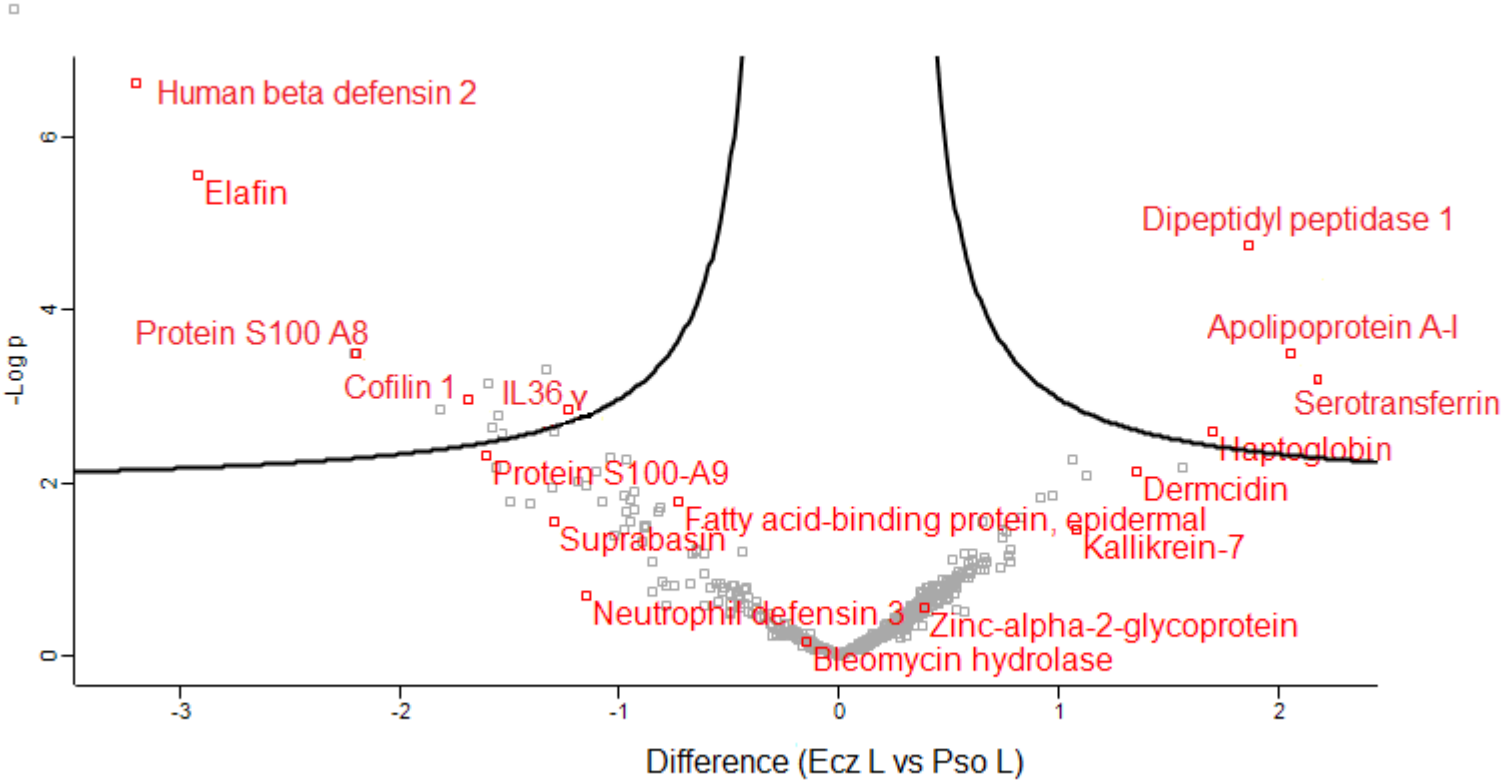

Figure 3.4 Differentially expressed proteins between lesional eczema and psoriasis.

Volcano plot analysis depicting the differential expression of proteins between lesional eczema (Ecz L, on the right) and psoriasis (Pso L, on the left). The lines demark the level of significance, calculated on a permutation based false discovery rate of 0.05. Proteins were graphed by log 2 fold change (Difference) and significance (-Log p).

3.7 Differentially expressed proteins between healthy looking, lesional and healthy skin

There is an identified clinical need to diagnose inflammatory conditions as early as possible to allow for disease modifying interventions and to prevent co-morbidities. So far, we have insufficient tools to identify patients “at risk” of developing e.g. psoriatic disease including PsA and to diagnose patients with subtle lesions. Such tools however could facilitate the initiation of early targeted therapy, potentially preventing full onset of a chronic condition. We have therefore also included non-lesional and para-lesional skin samples into our analysis. There is also a possibility to gain further insight into pathogenetic processes from samples not over-dominated by a fully established inflammatory response.

3.7.1 Protein expression in the non-lesional and para-lesional skin of psoriatic patients versus healthy

When comparing protein expression in the non-lesional skin of psoriatic patients with the expression profile in healthy skin, a wide range of proteins were upregulated in the non-involved psoriatic skin. However, none of these changes on their own were marked enough to allow differentiation from normal controls.

Only one protein showed significantly increased levels in non-lesional skin, protein **S100-A9**, which was upregulated by 6.09 fold. Non-significant upregulation was observed for **elafin** by 3.51 fold, **serine protease inhibitor Kazal-type 5** (**SPINK5**) (by 2.98 fold) and **serpin A12** (by 2.46 fold). Interestingly, **α-defensins** (mainly secreted by neutrophils) also showed a 2.90 fold increase. All these changes might suggest subclinical inflammation and an increased presence of neutrophils even in healthy looking psoriatic skin without macroscopic morphological changes.

When looking at the inflammatory cytokine **IL36γ**, there is a very slight increase in its expression in non-lesional psoriatic compared to healthy skin, associated with a more than 1 fold increase in the level of anti-inflammatory **IL36RN**, **IL37** and **IL1RN** expression, although, none of these changes reach significance level.

When comparing the protein expression in the para-lesional skin versus healthy, the modest differences already seen in the non-involved psoriatic samples present more markedly. A significantly increased expression of anti-inflammatory cytokines **IL36RN** (by 2.71 fold), **IL1RN** (by 2.48 fold)**,** and of **IL37** (by 1.77 fold) along with a significant increase of the pro-inflammatory **IL36γ** (by 1.76 fold) are noticeable changes in para-lesional psoriatic skin versus healthy. When directly comparing para-lesional and lesional psoriatic skin, it is obvious that there is a

significant decrease in these anti-inflammatory cytokines in lesional compared to para-lesional skin, by 3.07 fold in the case of **IL37**, by 2.74 fold in the case of **IL36RN** and by 2.20 fold for **IL1RN**. These results indicate that the level of anti-inflammatory cytokines **IL37**, **IL36RN** and **IL1RN** starts to increase in the non-involved skin of psoriasis patients, being significantly upregulated in the para-lesional skin, while their levels fall considerably once the inflammation is established in the lesional psoriatic skin, in parallel with the significant increase of **IL36γ** expression.

As mentioned, many proteins are somewhat but non-significantly upregulated in non-lesional psoriatic skin compared to healthy and these differences seem more pronounced when comparing para-lesional with non-lesional psoriatic skin. However, when comparing healthy skin expression data with that of the para-lesional psoriatic skin, the majority of proteins are significantly upregulated, more precisely 577 of the 644 identified by MS. For the most relevant proteins with a significant overexpression in para-lesional skin refer to Table 3.4. The only proteins which were significantly downregulated in para-lesional skin compared to normal controls were barrier constituents, **desmoglein 1** and **desmoplakin**, **keratin type II cytoskeletal 1** and **arginase 1**, involved in the synthesis of ornithine and urea.

3.7.2 Protein expression in the para-lesional compared to the lesional skin of psoriatic patients

While almost all identified proteins by MS were upregulated in para-lesional skin compared to healthy, protein expression was also considerably different between lesional and para-lesional psoriatic skin. 33 proteins already significantly increased in para-lesional psoriasis were further upregulated in lesional psoriasis, however, the majority of proteins showed significant downregulation in lesional compared to para-lesional skin.

The 33 most upregulated proteins in lesional psoriasis were also the ones already described to be upregulated compared to healthy skin.

520 proteins were significantly downregulated in lesional compared to para-lesional psoriatic samples, with the antimicrobial **dermcidin** expressed in sweat being the most downregulated by 24.07 fold. Its level increased from healthy to para-lesional skin and then significantly decreased in the lesional psoriatic samples. A similar trend was noticed for a number of other proteins (see Table 3.4).

3.7.3 Differentially expressed proteins between non-lesional eczema and non-lesional psoriasis

The protein expression in tape samples taken from the non-involved sites of both diseases (forearm) were directly compared. However, no significantly different expression was identified between the two groups. A 3.78 fold increase has been found in the expression of **S100A9** in non-lesional psoriasis, with a few others also showing a more than 2 fold increase (the full list of differentially expressed proteins is included into the electronic file “Volcano plot analysis outcomes”).

3.7.4 Protein expression in the non-lesional compared to the lesional skin of eczema patients and healthy

No significant differences were found comparing eczematous non-lesional skin to healthy controls. Protein expression in the non-lesional versus lesional skin tape strip samples are comparable to what was seen for healthy.

Main function	Protein name	Gene name	Pso NL (vs. Healthy)	Pso PL (vs. Healthy)	Pso L (vs. Healthy)	Ecz NL (vs. Healthy)	Ecz L (vs. Healthy)
Pro-inflammatory cytokines	**IL36γ**	**IL36G**	~	↑*	↑*	~	~
Anti-inflammatory cytokines	**IL36 receptor antagonist**	**IL36RN**	↑	↑*	~	↑	~
	IL37	**IL37**	↑	↑*	↓*	~	~
	IL1 receptor antagonist	**IL1RN**	~	↑*	~	↑	~
S100 family members	**S100A7**	**S100A7**	↑	↑*	~	↑	~
	S100A8	**S100A8**	↑	↑*	↑↑↑*	~	↑
	S100A9	**S100A9**	↑↑*	↑↑*	↑↑↑↑↑↑*	↑	↑↑↑↑*
	S100A10	**S100A10**	↑	↑*	↑	↑	~
	S100A11	**S100A11**	↑	↑*	↑	~	~
	S100A12	**S100A12**	↑	↑*	↑*	~	~
	S100A14	**S100A14**	↑	↑*	~	↑	~
	S100A16	**S100A16**	↑	↑*	~	↑	~
Serine protease inhibitors	**Elafin**	**PI3**	↑	↑↑*	↑↑↑↑*	↑	↑*
	Antileukoproteinase	**SLPI**	↑	↑	↓*	~	~
	Serpin A1	**SerpinA1**	↑	↑*	~	↑	~
	Serpin A3	**SerpinA3**	~	↑*	~	↑	~
	Serpin A12	**SerpinA12**	↑	↑*	↓*	~	↓
	Serpin B1	**SerpinB1**	~	↑↑*	↓	~	~
	Serpin B3	**SerpinB3**	~	↑*	↑↑↑*	↓	↑↑*
	Serpin B4	**SerpinB4**	↑	↑	↑*	~	~
	Serpin B5	**SerpinB5**	↑	↑*	↑	↑	↑
	Serpin B6	**SerpinB6**	↑	↑*	~	↑	~

Table 3.4 Differential protein expression in tape stripping samples of healthy versus non-lesional, para-lesional and lesional psoriasis, as well as non-lesional and lesional eczema.

~ indicates similar expression as to that in healthy, with lower than 0.5 fold difference; arrows indicate more than 0.5 fold increase ↑, or decrease ↓ in the expression of proteins at different stages of inflammation compared to healthy, by each arrow representing approximately 1 (log 2) fold change; * indicate significant difference.

Main function	Protein name	Gene name	Pso NL (vs. Healthy)	Pso PL (vs. Healthy)	Pso L (vs. Healthy)	Ecz NL (vs. Healthy)	Ecz L (vs. Healthy)
Serine protease inhibitors	Serpin B7	SerpinB7	~	↑*	↓	~	~
	Serpin B8	SerpinB8	↑	↑*	↓*	~	~
	Serpin B12	SerpinB12	↑	↑*	↓*	~	↓
	Serpin B13	SerpinB13	↑	↑*	~	↑	~
	Serine protease inhibitor Kazal-type 5	SPINK5	↑	↑↑*	↓	↑	~
	Serine protease inhibitor Kazal-type 9	SPINK9	↑	↑*	↓	~	~
Serine proteases	Kallikrein 5	KLK5	↑	~	↓↓*	↓	↓↓*
	Kallikrein 6	KLK6	↑	↑*	~	~	~
	Kallikrein 7	KLK7	~	~	↓↓*	~	↓
	Kallikrein 8	KLK8	~	↑*	↓	~	~
	Kallikrein 9	KLK9	↑	↑*	~	↑	~
	Kallikrein 10	KLK10	↑	↑*	~	~	~
	Kallikrein 11	KLK11	~	↑*	↓	~	~
	Kallikrein 13	KLK13	↑	↑*	~	↑	~
Corneodesmosome degradation enzymes / enzymes involved in desquamation	Zinc-alpha-2-glycoprotein	AZGP1	↑	~	↓*	~	↓
	Cathepsin L2	CTSV	↑	↑	↓↓*	~	↓
	Dipeptidyl peptidase 1	CTSC	~	↑	↓*	~	↑
	Cystatin-M	CST6	~	↑	↓*	~	↓
	Retroviral-like aspartic protease 1	ASPRV1	~	~	↑*	~	↑
Lysosomal proteases	Cathepsin B	CTSB	↑	↑*	~	↑	~
	Cathepsin D	CTSD	↑	↑*	~	~	~
	Cathepsin L1	CTSL	↑	↑*	~	↑	~
Neutrophil proteases	Cathepsin G	CTSG	↑	↑*	~	~	~
	Myeloperoxidase	MPO	↑	↑*	~	↑	~
	Neutrophil elastase	ELANE	↑	↑*	~	~	~

Table 3.4 *(continued)* Differential protein expression in tape stripping samples of healthy versus non-lesional, para-lesional and lesional psoriasis, as well as non-lesional and lesional eczema.

Main function	Protein name	Gene name	Pso NL (vs. Healthy)	Pso PL (vs. Healthy)	Pso L (vs. Healthy)	Ecz NL (vs. Healthy)	Ecz L (vs. Healthy)
Neutrophil proteases	Cathelicidin	CAMP	↑	↑*	↓	~	~
	Lysozyme C	LYZ	~	↑	~	~	~
	Lactotransferrin	LTF	↑	↑*	↑	~	~
	Neutrophil gelatinase-associated lipocalin	LCN2	~	↑*	~	~	~
Antimicrobial peptides	Dermcidin	DCD	↑	↑*	↓↓↓*	~	↓↓*
	WAP four-disulfide core domain protein 12	WFDC12	↓	↓	↓*	↓↓	↓
	hBD2	DEFB4A	↑	↑*	↑↑↑↑↑*	~	↑↑*
	hBD3	DEFB103A	~	↑*	↑*	↑	↑
	Human neutrophil peptides 1-3	DEFA3;DEFA1	↑	↑	↑↑*	↑	↑
Sweat associated peptides	Prolactin-inducible protein	PIP	~	~	↓*	~	↓
Lipid metabolism	Fatty acid binding protein 5	FABP5	↑	↑	↑↑↑↑↑*	↑	↑↑↑↑*
	Apolipoprotein A-I	APOA1	~	↑*	~	~	↑*
	Apolipoprotein A-II	APOA2	↑	↑*	~	~	↑
	Apolipoprotein A-IV	APOA4	↑	↑*	↓	↑	~
	Apolipoprotein C-III	APOC3	↑	↑*	~	↑	~
	Apolipoprotein D	APOD	~	↑*	↓	~	~
	Proactivator polypeptide-like 1	PSAPL1	~	~	↓*	~	↓
Glycolysis, neoglucogenesis	Alpha-enolase	ENO1	~	~	↑↑↑*	~	↑↑↑*
	Fructose-bisphosphate aldolase A	ALDOA	~	↑*	↑↑↑↑*	↑	↑↑*
	Phosphoglycerate mutase	PGAM	↑	↑*	↑↑*	~	↑*
	Triose-phosphate isomerase	TPI1	~	↑	↑↑↑*	~	↑
	Phosphoglycerate kinase 1	PGK1	↑	↑	↑*	~	~
Retinoic acid regulation	Transthyretin	TTR	~	↑	↓*	~	↓
	Cellular retinoic acid-binding protein 2	CRABP2	↑	↑*	↑*	~	~
Antioxidant activity	Superoxide dismutase [Cu-Zn]	SOD1	~	~	↓↓*	↓	↓

Table 3.4 *(continued)* Differential protein expression in tape stripping samples of healthy versus non-lesional, para-lesional and lesional psoriasis, as well as non-lesional and lesional eczema.

Main function	Protein name	Gene name	Pso NL (vs. Healthy)	Pso PL (vs. Healthy)	Pso L (vs. Healthy)	Ecz NL (vs. Healthy)	Ecz L (vs. Healthy)
Antioxidant activity	Catalase	CAT	~	~	↓↓↓*	↓	↓↓*
	Gamma-glutamylcyclotransferase	GGCT	~	↑	↓*	~	↓
	Peroxiredoxin 1	PRDX1	↑	↑*	↑↑*	~	↑
	Peroxiredoxin 2	PRDX2	~	↑	~	↑	~
	Peroxiredoxin 5	PRDX5	↑	↑*	~	↑	~
	Peroxiredoxin 6	PRDX6	↑	↑*	~	↑	~
	Heat shock protein 10kDa, mitochondrial	HSPE1	↑	↑*	~	~	~
	Heat shock protein beta 1 (HSP27)	HSPB1	~	~	↑↑↑↑*	↓	↑↑↑*
	Heat shock protein 60kDa, mitochondrial	HSPD1	↑	↑*	~	~	~
	Heat shock protein 70kDa 1B and 1A (HSP72)	HSPA1B;HSPA1A	↑	↑*	~	~	↑
	Heat shock protein 70kDa protein 2	HSPA2	↑	↑*	~	↑	~
	Heat shock protein 70kDa protein 5	HSPA5	↑	↑*	↑↑*	↑	↑
	Heat shock protein 70kDa protein 6 and 7	HSPA6;HSPA7	~	↑*	~	~	~
	Heat shock cognate 71kDa protein (HSP73)	HSPA8	↑	↑*	↑*	↑	↑
	Heat shock protein 70kDa protein, mitochondrial	HSPA9	↑	↑*	~	↑	~
	Heat shock cognate 90kDa protein alpha	HSP90AA1	↑	↑*	~	↑	~
	Heat shock cognate 90kDa protein beta	HSP90AB1	↑	↑*	~	~	~
	Heat shock protein 90kDa beta member 1	HSP90B1	↑	↑*	~	~	~
Proapoptotic proteins, intercellular interactions	Galectin1	LGALS1	↑	↑*	~	~	~
	Galectin3	LGALS3	↑	↑*	~	↑	↑
	Galectin-3-binding protein	LGALS3BP	↑	↑*	↓	↑	~
	Galectin7	LGALS7	↑	↑	↑↑↑*	~	↑↑↑*
	Galectin-related protein	LGALSL	↑	↑*	~	↑	~
	Secreted Ly-6/uPAR-related protein 1	SLURP1	↓	↓	↓↓*	↓	↓
	Gasdermin A	GSDMA	↑	↑	↓*	↑	↓
Cell proliferation	Granulin	GRN	↑	↑*	↓	~	↓

Table 3.4 *(continued)* Differential protein expression in tape stripping samples of healthy versus non-lesional, para-lesional and lesional psoriasis, as well as non-lesional and lesional eczema.

Main function	Protein name	Gene name	Pso NL (vs. Healthy)	Pso PL (vs. Healthy)	Pso L (vs. Healthy)	Ecz NL (vs. Healthy)	Ecz L (vs. Healthy)
Cell migration	Cofilin 1	CFL1	↑	↑*	↑↑*	↑	↑
Signalling pathways	14-3-3 protein beta/alpha	YWHAB	↑	↑*	~	~	~
	14-3-3 protein gamma	YWHAG	↑	↑*	~	~	~
	14-3-3 protein zeta/delta	YWHAZ	↑	↑*	↑↑*	↑	↑↑*
	14-3-3 protein epsilon	YWHAE	↑	↑*	~	~	~
	14-3-3 protein eta	YWHAH	↑	↑*	↓	~	~
	14-3-3 protein sigma	SFN	~	↑	↑↑↑↑*	~	↑↑↑↑*
Intracellular signalling, transcription, inflammation, and apoptosis	Cyclophilin A	PPIA	↑	↑*	↑↑↑*	↑	↑↑*
Neovascularisation	Thymidine phosphorylase	TYMP	↑	↑*	↑*	↑	~
Barrier constituents	Desmoglein 1	DSG1	↓	↑*	↓↓*	↓	↓↓*
	Desmoglein 3	DSG3	↑	↑*	~	↑	~
Barrier constituents	Desmocollin 1	DSC1	↑	~	↓*	~	↓
	Desmocollin 3	DSC3	↑	↑*	↓*	~	~
	Corneodesmosin	CDSN	↑	↑*	↓	↑	~
	Plectin	PLEC	↑	~	↓*	~	↓
	Epiplakin	EPPK1	~	↑	↑*	↓	↑
Keratinocyte differentiation markers	Suprabasin	SBSN	↑	↑*	↑*	~	~
	Calmodulin-like protein 3	CALML3	~	↑*	↑↑*	~	↑*
	Calmodulin-like protein 5	CALML5	↓	↓	↑*	↓	↑
Profilaggrin/filaggrin processing	Caspase 14	CASP14	↑	~	↓	~	↓
	Bleomycin hydrolase	BLMH	↓	~	↓*	~	↓↓*
Urea synthesis	Arginase1	ARG1	↓	↓*	↓*	↓	↓↓*

Table 3.4 *(continued)* Differential protein expression in tape stripping samples of healthy versus non-lesional, para-lesional and lesional psoriasis, as well as non-lesional and lesional eczema.

3.8 Overview of expected changes detected via MS explained by known morphopathological features

Many changes detected via MS in tape strip material of inflammatory skin were expected and reflect the morphopathological features of psoriasis and AD, also validating the reliability of tape stripping (see Table 3.5).

Morphopathological feature	Changes reflected by MS data
Psoriasis	
Keratinocyte hyperproliferation and incomplete terminal differentiation with the persistence of cell organelles and nuclei in the upper skin layers (parakeratosis).	Overexpression of nuclear (HSPs, retinoid receptors, histones, prelamins and lamins) and cell organelle (ribosomal, endoplasmatic and cytoskeletal) proteins. Modulation of metabolic pathways (increased glycolysis, lipid metabolism), cellular stress (antioxidative molecules).
Increased keratinocyte turn over with excessive scaling.	Regulation of corneodesmosome degradation enzymes / enzymes involved in desquamation and tight junction proteins.
Neutrophil infiltration.	Increased neutrophil protease levels in para-lesional psoriatic samples (e.g. α-defensins, MPO, cathelicidine); neutrophil derived proteins (HNPs, S100 family members) and neutrophil protease inhibitors (elafin) in lesional psoriasis.
Neovascularisation.	Proteins involved in angiogenesis (thymidine phosphorylase).

Table 3.5 Morphopathological changes in psoriasis and AD reflected in the tape strip MS protein expression data of inflammatory skin.

HSP – heat shock protein; MPO – myeloperoxidase; HNP - human neutrophil peptides.

Morphopathological feature	Changes reflected by MS data
Atopic Dermatitis	
Epidermal barrier disruption, impairment of filaggrin / natural moisturising factor.	Depletion of enzymes with a key role in profilaggrin/filaggrin processing (bleomycin hydrolase) and urea synthesis (arginase1).
Spongiosis (intercellular oedema, increased apoptosis).	Disregulation of corneodesmosome degradation enzymes and tight junction proteins.
	Overexpression of proapoptotic proteins (galectin 7); high presence of serum components (serotransferrin, haptoglobin) in the epidermal compartment.

Table 3.5 *(continued)* Morphopathological changes in psoriasis and AD reflected in the tape strip MS protein expression data of inflammatory skin.

3.9 Discussion

The MS data findings describe 644 proteins and their differential expression in psoriatic lesional, non-lesional and para-lesional samples compared to eczematous lesional and non-lesional as well as to healthy control tape strip samples. It was unexpected that this non-invasive, epidermal sampling technique combined with state of the art MS proteomics would identify such a large amount of differentially expressed proteins, including non-structural molecules involved in a range of biological processes. The findings described in this chapter not only present the different protein expression between lesional and normal skin, but also shed light on immune response dynamics at a point when pro-inflammatory processes overwhelm counterregulatory mechanisms as suggested by comparing healthy, non-involved, para-lesional and lesional samples. Comparing two different inflammatory diseases with strong epidermal involvement allows us to comment on the disease biomarker potential of identified differentially expressed proteins.

3.9.1 Pro- and anti-inflammatory cytokines

One of the potential biomarkers for psoriatic inflammation detected in this study by MS was **IL36γ**. IL36γ expression was significantly upregulated both in para-lesional and lesional psoriatic skin compared to healthy but also to lesional eczema. IL36γ was also the only “classical” pro-inflammatory cytokine identified

by MS in tape harvested epidermal material. This is likely due to the abundance of IL36γ in the upper epidermal layers [144]. The absence of other pro-inflammatory cytokines/chemokines in the MS data indicate their too low abundance relative to other proteins detected.

A novel, yet unpublished finding relates to the differential expression of anti-inflammatory cytokines **IL36RN**, **IL1RN** and **IL37** throughout the different stages of inflammation of psoriatic skin. Their expression levels tend to be increased in non-lesional skin and further peaked in para-lesional psoriatic skin (significant increase compared to healthy); however were near healthy skin levels in lesional skin, except for IL37 which showed significant downregulation. The decrease of these regulator molecules in lesional psoriatic skin was significant for all three compared to para-lesional skin. It can be speculated that their levels increase in subclinical inflammation in an effort to limit the inflammatory psoriatic response. It is unclear why low levels are found at sites of florid psoriatic inflammation and it would certainly be of high interest to explore this further. These dynamic changes were not so obvious in eczematous skin.

The above mentioned mediators are all members of the IL1 family. **IL1RN** binds to IL1R1, acting as an antagonist for IL1α and IL1β [231]. IL1RN loss of function gene mutation, called DIRA, leads to an autoinflammatory disease with a constellation of symptoms including pustulosis [232]. **IL36RN** regulates the activity of IL36 and its loss of function mutation, called DITRA, leads to the development of severe pustular psoriasis [43].

IL37 expression was the most downregulated IL1 family member in lesional psoriasis. This finding is supported by previous studies based on skin biopsy RNA sequencing [233, 234], although the authors did not examine IL37 expression in para-lesional skin. It has been demonstrated that IL37 has an indirect inhibitory effect on TLR-, IL1-, IL18-, IL33-, and IL36-mediated inflammation [235] and it can decrease keratinocyte production of IL8, IL6, and S100A7 [236].

3.9.2 Signs of subclinical inflammation (S100A8/A9), degradation enzymes and their inhibition

In the non-lesional skin of psoriatic patients there was a trend for increased expression of proteins suggestive of early epidermal inflammation, including **elafin** and other **serine protease inhibitors**, such as **SPINK5** and **serpin A12**, as well as neutrophil secreted **α-defensins**. The only protein significantly increased in non-lesional skin compared to healthy was **S100A9**, which together with **S100A8** (a complex known as **calprotectin**) is known for its antimicrobial effect and to regulate inflammation by leukocyte recruitment, stimulating

neutrophil migration [237, 238]. In lesional psoriatic skin **S100A8**, but especially **S100A9** had impressively upregulated levels compared to healthy. Faecal calprotectin is already used as a diagnostic screening test and as an activity marker for inflammatory bowel disease (IBD), being able to predict a flare with 80% accuracy [223]. Although calprotectin is an indicator of general inflammation rather than a specific disease marker, its outstandingly high levels found in active IBD allow discrimination from other bowel diseases [223]. Based on its successful application in the gastroenterology field and on our results indicating that its levels are highly upregulated in active inflammation, it is plausible that calprotectin could be used as an objective disease activity marker for inflammatory skin conditions such as psoriasis, potentially replacing or supplementing less reliable clinical assessment tools based on clinical judgement (such as the PASI score) in a research environment.

Elafin, a neutrophil serine protease inhibitor, was specifically and highly upregulated in psoriasis lesional samples. Elafin has been previously shown to be upregulated in the upper layers of psoriatic epidermis in skin biopsies, and its protective role against tissue damage produced by the proteolytic activity of infiltrating neutrophils has been discussed [229, 239]. IL17 [240], IL1β and TNFα seem to upregulate elafin expression in keratinocytes [241]. As IL17 and TNFα are known to be highly expressed in psoriasis and to act synergistically on keratinocytes, this could explain the preferential and very high upregulation of elafin in psoriasis as compared to moderate increase in eczema lesions.

Elafin was not the only upregulated serine protease inhibitor detected. **Serpin B3** and **B4** were significantly upregulated in psoriatic lesional tape strip material, with serpin B3 also being significantly higher in lesional eczema compared to healthy. Both have been used previously as squamous carcinoma markers, however, there is increasing evidence that they are involved in the pathogenesis of a number of inflammatory diseases, including AD and psoriasis. Th2 cytokines (IL4, IL13) as well as Th17 cytokines (IL17, IL22) can upregulate serpin B3 and B4 in keratinocytes and airway epithelial cells [138]. Our results indicate that they are not discriminatory between the two inflammatory conditions, which is also supported by previous data showing elevated serum levels in both eczema and psoriasis compared to healthy [138]. Nevertheless, their levels could correlate with disease activity. On the other hand, other serpin family members, **serpin A12**, **B8**, and **B12**, were found to be significantly downregulated in psoriatic skin. **Serpin A12**, also known as **vaspin**, has been linked to keratinocyte differentiation and seems to have an anti-inflammatory, regulatory effect on skin inflammation in psoriasis [242]. The two key psoriatic cytokines, IL17 and TNFα, synergistically inhibit serpin A12 expression by keratinocytes in *in vitro* experiments, while low

serpin A12 levels result in upregulation of pro-inflammatory cytokines TNFα, IL1β, IL6, IL8, and monocyte chemoattractant protein 1 by other immune cells, and vice versa [242].

In parallel to altered serine protease inhibitor activity, there was significant downregulation of serine proteases. **KLK5** and **KLK7**, together with other kallikrein family members, play crucial role in desquamation by degrading intercellular corneodesmosomal connections. **KLK5** acts on **KLK7** activation which is also involved in keratinocyte terminal differentiation and cornification [213]. The hereditary deficiency of their counter regulatory serine protease inhibitor SPINK5, also known as LEKTI, leads to severe barrier disruption manifesting as potentially life threatening ichthyosiform erythroderma and atopic features, known as Netherton syndrome [243]. Increased kallikrein activity has also been previously linked to AD [244, 245] and to psoriasis [246], although with limited data available for the latter. The existing data seems to contradict our findings of low kallikrein levels in lesional skin. However, it also indicates that kallikrein activity rather than quantity is the main pathomechanistic factor in inflammatory disease development, underlying the importance of the delicate balance of regulatory molecules. Other enzymes involved in corneodesmosome degradation were also significantly decreased in lesional psoriasis further highlighting the disturbed desquamation process in this pathology, including **ZAG**, **cathepsin L2** and **DPP1**, also known as **cathepsin C**. Although **ZAG** is an adipokine, it has been shown to have enzymatic activity, playing a role in normal cell cohesion and desquamation, and in the terminal differentiation of keratinocytes by participating in nuclear destruction [212]. ZAG became known as a marker for well differentiated carcinomas [247-250] and for its potential in the treatment of obesity [251, 252]. A recent study investigating the role of ZAG in AD found that filaggrin is downregulated in parallel with TSLP upregulation following ZAG knockdown in normal human epidermal keratinocytes, while topical application of ZAG to AD induced mice resulted in the reduction of pro-inflammatory cytokines (IL4, IL17 and IFNγ) and in the improvement of AD severity. The authors also highlight ZAG as a potential disease biomarker for AD [253]. However, our results show that ZAG downregulation is even more pronounced in psoriasis. The role of ZAG in psoriasis has not yet been investigated, although our results indicate that similarly to AD, ZAG could play an important part in the pathogenesis of psoriasis.

DPP1 is a cysteine protease, which is known to activate serine proteases, including neutrophil elastase and cathepsin G. Although the role of DPP1 in inflammatory skin diseases is yet to be elucidated, it has been investigated for other pathologies known to be driven by neutrophilic inflammation, such as

COPD, asthma [254] and recently for the prevention of acute respiratory distress syndrome associated with COVID-19 [255]. DPP1 inhibition with the oral drug AZD7986 successfully reduced whole blood neutrophil elastase activity in healthy individuals [256]. Furthermore, a phase 2 study recently announced the successful use of the first oral DPP1 inhibitor (INS1007) for the treatment of non-cystic fibrosis with bronchiectasis [257]. DPP1 inhibition might be of therapeutic effect in a number of other diseases where neutrophils are key players, including dermatological conditions amongst them with psoriasis, especially in its pustular form. Our finding that DPP1 levels are decreased in tape strips of lesional psoriasis might be due to the deeper localisation of DPP1 in the thickened stratum corneum of the psoriatic plaque. Both clinical trials investigating the therapeutic effect of DPP1 inhibition reported skin related side effects, including dry, scaly patches, fissures, hyperkeratosis over the small joints of the hands, elbows and exfoliation on palmar and plantar areas [256, 257]. These skin related side effects are not surprising as DPP1 deficiency has been linked to palmoplantar hyperkeratosis (Haim-Munk and Papillon-Lefevre syndromes), however, the pathomechanistic effect behind it is unclear [258]. As the side effects described resemble skin findings we would expect to see in psoriasis, it is plausible that DPP1 is in fact downregulated in plaque psoriasis, as it is suggested by our results, and it contributes to the psoriatic pathology by other mechanistic effects in addition to neutrophil serine protease activation.

Cystatin M inhibits cysteine proteinases protecting keratinocytes against uncontrolled proteolytic activity [259]. Cystatin M was first identified as being downregulated in breast cancer, acting as a tumour suppressor [260]. This has been suggested to be true also for lung and squamous cell carcinoma of the skin [261]. Its level was significantly decreased in our lesional psoriasis tape samples, a finding supported by the literature describing cystatin M downregulation not only in lesional psoriasis but also in eczema [262]. Mice experiments showed that cystatin M is necessary for normal cornified layer formation with depleted levels leading to altered barrier function and increased TEWL [263, 264]. Furthermore, patients with hereditary cystatin M deficiency present with dry skin and hypotrichosis, associated with altered keratinocyte proliferation and differentiation on biopsies [265], histopathology changes which are also typically seen in psoriasis. Therefore, it is likely that just as in carcinomas where cystatin M acts as a tumour suppressor, it also regulates keratinocyte proliferation and differentiation in psoriasis.

3.9.3 Neutrophil proteases and antimicrobial peptides (AMP)

Neutrophil infiltration and corneal accumulation is a common sign on psoriasis histopathology [71]. In para-lesional psoriatic tape samples this early infiltration of neutrophils was already reflected by the significant increase of neutrophil proteases with antimicrobial activity, including expression of **cathepsin G**; **myeloperoxidase**; **neutrophil elastase**; **cathelicidin/LL37**; **lactotransferrin** and **neutrophil gelatinase-associated lipocalin**.

A further increased expression was detected for the antimicrobial **defensins**, **hBD2** and **hBD3**. In lesional eczema only **hBD2** expression was significantly upregulated. Beta defensins are expressed mainly in the upper layers of the stratum corneum by keratinocytes. Their production is induced by microbial encounter and by inflammatory cytokines [266]. **HBD2** is mainly active against Gram negative (e.g. *P. aeruginosa*, *Escherichia coli*), while **hBD3** against Gram positive (e.g. *S. aureus*) bacteria. HBDs are now also well recognised to have “cytokine/chemokine” function and to influence inflammatory responses by keratinocytes [267]. Our results of increased antimicrobial expression of hBDs and S100 family members in lesional psoriatic compared to eczematous skin are well supported by the literature and the clinical observation of increased *S. aureus* colonisation and infection in AD patients [268-271], as there are virtually no skin infections present in psoriatic plaques. This finding is also supported by previous microarray and immunohistochemistry studies [160, 266]. However, the relative absence of antimicrobial peptides in AD is not the only factor contributing to *S. aureus* colonisation. A constellation of other important host (skin barrier dysfunction, decreased filaggrin levels, altered skin pH, decreased microbial diversity and altered lipid profiles) and microbial (superantigens, proteases and toxins) factors also need to be present and explain for the high infection rate in AD [269, 270].

However, we also found some AMP decreased in both lesional eczema, and lesional psoriasis, including: **dermcidin**. Both **dermcidin** and **PIP**, another protein found significantly downregulated in psoriasis, are constitutively expressed by sweat glands and reach the upper layers of the epidermis by being secreted into sweat [221, 272]. Dermcidin exhibits a broad spectrum of antimicrobial activity, both against Gram negative and positive bacteria, and plays a role in the innate immune response [221]. Its reduced expression in lesional skin may be due to blockade of sweat gland ducts due to hyperproliferation and dramatic morphological changes. Temperature regulation and sweating is known to be disturbed in inflamed skin. The exact role of PIP in the skin has not been widely investigated. It has been suggested that due to the disturbed barrier function in AD skin, PIP penetrates deeper into the epidermal layers than in

normal skin, where it is mainly found on the surface. It has been proposed that PIP has proteolytic activity and can induce keratinocyte proliferation in *in vitro* skin models. Its deeper penetrance in AD epidermis might explain partly the distribution pattern of eczema to skin folds, due to the expressed sweat accumulation in these areas [272]. However, this would not be true for psoriasis, which localises mainly to extensor surfaces, with lower amounts of sweat production. In our theory, both in eczema, but especially in psoriasis there is perturbed secretion of sweat to the epidermal surface, which would explain why both dermcidin and PIP have lower detection levels in lesional compared to healthy skin. Indeed, abnormality of the sweat ducts in psoriasis, which manifests in hypohidrosis due to the blockage of the superficial sweat ducts, has been previously reported in the literature [273]. The type of sweat dysfunction in eczema is debated, but while it seems like there is a decrease in sweat excretion to the surface of the eczematous skin, paradoxical hyperhidrosis can be also triggered by various factors [274, 275]. Indeed, sweating is reported as a trigger factor for AD by most patients, while there is evidence that high sodium chloride concentration can impact on the polarisation of naïve $CD4^+$ T cells into Th17 cells [276].

Apart from the presence of pro- and anti-inflammatory cytokines, neutrophil infiltration, modified enzyme activity, defence response and corneodesmosome degradation, MS data analysis revealed that a number of other pathophysiological pathways were also impacted in both eczema and psoriasis, including lipid metabolism, glycolysis, retinoid metabolism, oxidative stress, regulation of apoptosis, keratinocyte differentiation and signalling pathways.

3.9.4 Metabolic pathways

One of the most upregulated proteins in both lesional psoriasis and eczema, connected to the lipid metabolism pathway, was **FABP5**. FABP5 was first identified as being overexpressed in psoriatic epidermis, therefore initially it was called psoriasis associated fatty acid binding protein. It plays a role in fatty acid transport and metabolism. Its increase in psoriasis is postulated to be due to the altered metabolic status in psoriasis, with the increase of lipids in the epidermis [277]. It has also been suggested that FABP5 might play a role in keratinocyte differentiation [278]. The finding that FABP5 is significantly overexpressed in the stratum corneum of both lesional psoriasis and eczema versus healthy is supported by other tape stripping studies [279, 280]. FABP5 was even proposed as having a biomarker potential for AD, when compared to healthy skin [279]. However, the direct comparison between lesional eczema and psoriasis presented in this work, confirming that FABP5 has similar expression profile in

both skin diseases, has not been previously reported. Interestingly, new research also suggests that high fat diet leads to the increased expression of FABP5, which can consequently activate inflammasomes, with the release of IL1β and IL18, exacerbating the inflammatory cascade leading to Th1 and Th17 polarisation and to the formation of the inflammatory skin lesions seen in psoriasis [281]. Maybe this is the link for the reported improvement of psoriasis in the context of weight loss [282].

All members of the apolipoprotein family were detected upregulated in para-lesional but not in lesional psoriatic skin. However, in lesional eczema samples, **APOA1**, a major constituent of high-density lipoprotein (HDL), showed increased levels not only compared to healthy but also compared to lesional psoriasis. There are only a few reports investigating the role of APOA1 in correlation with inflammatory diseases. Previous studies assessing if the level of plasma APOA1 could be correlated with the development of cardiovascular diseases in psoriasis and PsA are conflicting [283]. High serum APOA1 levels have also been reported in asthma [284]. Research indicates that APOA1 attenuates immune cell function during cholesterol extraction from the cell and it reduces neutrophil and eosinophil activity in the airways by decreasing the production of granulocyte-colony stimulating factor. High APOA1 serum levels seem to indicate less severe asthma [285]. Interesting as it sounds, the role of APOA1 in skin inflammation has not been investigated yet. Our results, in accordance with a previous tape stripping study finding that APOA1 expression is higher in lesional eczema compared to healthy [279], indicate that APOA1 may have an important role in atopic inflammation. We also found significantly higher expression in eczema compared to psoriatic lesional samples, and it could be therefore further investigated for its suitability as a diagnostic biomarker.

A marked overexpression of glycolytic enzymes: **alpha-enolase, ALDOA, phosphoglycerate mutase** (**PGAM**), **phosphoglycerate kinase** and **triosephosphate isomerase** (**TPI1**), was detected by MS in lesional skin when compared to healthy, especially in lesional psoriasis. These glycolytic enzymes enable cells to obtain the necessary energy via glycolysis, called the **Warburg effect**, even in a hypoxic environment, promoting their survival in a stressful microenvironment. It is known that tumour cells use more glucose in order to generate ATP even when normal oxygen supply is available [286]. The overexpression of glycolytic enzymes has been associated with tumourigenesis as they support cell survival, proliferation, tumour migration and invasion, being negative prognostic markers in cancer [286-290]. The exact role of these glycolytic enzymes in inflammatory skin diseases, such as eczema and psoriasis, is yet unknown and evidence is lacking. As glucose is a preferential source of

energy for proliferating cells and as there is a marked increase in keratinocyte proliferation rate in psoriasis, it is plausible that just as in tumour cells, the activation of the anaerobic glycolytic pathway supports cell proliferation and survival in the stressful microenvironment created by the increased energy need triggered by cell proliferation and inflammation. Our findings are also supported by metabolic studies, revealing an increased glycolytic activity in psoriasis [291, 292], with the production of lactic acid, which in turn promotes alternatively activated (M2) macrophages with an anti-inflammatory/immunosuppressive profile [293]. It has been also shown that dimethyl fumarate, a commonly used antipsoriatic drug, exhibits its anti-inflammatory effects by inactivating the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, leading to reduced aerobic glycolysis, resulting in an immune switch from an inflammatory phenotype favouring classically activated (M1) macrophages and effector TH1 and TH17 lymphocytes to an anti-inflammatory profile with the promotion of M2 macrophage and regulatory T cell differentiation [294].

The findings of this thesis also highlighted the modified expression of proteins involved in retinoic acid transport and metabolism. **Transthyretin** (**TTR**) transports retinol bound to the retinol-binding protein [225]. Our results showed high levels of transthyretin in healthy skin compared to psoriasis. The beneficial role of retinoids, such as acitretin or alitretinoin, in the treatment of psoriasis and other hyperkeratotic skin diseases is well known. The role of transthyretin in the skin has not been widely investigated. It is conceivable that reduced transthyretin levels in psoriatic skin with decreased retinol delivery might contribute to the formation of hyperkeratosis. At the same time **cellular retinoic acid-binding protein 2** (**CRABP2**) was upregulated in psoriatic lesional skin versus healthy. CRABP2 is known to be involved in the intracellular metabolism and transport of retinoic acid (from the cytosol to the nucleus) and it is a factor in keratinocyte differentiation. It has been shown that reduced CRABP2 levels contribute to early aging, as absence of CRABP2 in knock–out mouse led to decrease in the thickness of hypodermal, dermal and epidermal layers, with reduced keratinocyte proliferation and differentiation [230]. It is possible that the increase in CRABP2 detected by our results tend to be a counter regulatory effect, aiming to increase keratinocyte differentiation and to reduce proliferation. We could not detect CRABP1 expression in our samples via MS. CRABP1 also contributes to the intracellular metabolism of retinoic acid. Immunohistochemistry revealed that CRABP1 is highly expressed in all layers of the psoriatic epidermis compared to healthy, except in the stratum corneum [295]. The lack of CRABP1 in our results seems in line with predominant stratum corneum sampling.

3.9.5 Antioxidative molecules

In lesional skin, there was also modified expression of proteins with key roles in response to oxidative stress, with decrease of **gamma-glutamylcyclotransferase** (**GGCT**), **catalase** (**CAT**) and **superoxide dismutase [Cu-Zn]** (**SOD1**) but increase of several **HSP**s and **peroxiredoxin 1**. The role of oxidative stress has been already highlighted in skin diseases [296], including psoriasis [297-299]. Reactive oxygen species (ROS) can lead to cell death, DNA damage and to the release of pro-inflammatory cytokines [296]. Antioxidative proteins have cell protective function but their role in skin inflammation remains to be clearly understood. ROS have an important role in the development of cardiovascular diseases as they lead to the abnormal oxidation of low-density lipoprotein (LDL) and to lipid peroxidation [300, 301]. Increased ROS activity might be one of the reasons why there is an increased cardiovascular risk seen in psoriasis patients [302, 303]. It has been shown that ROS have an essential role in the ethipathogenesis of contact allergies by breaking down hyaluronic acid (component of the extracellular matrix) into immunogenic fragments [304]. Topical application of SOD in AD is currently under investigation [305].

3.9.6 Keratinocyte differentiation/ proliferation/ apoptosis/ signalling pathways

Another pathway which was modified in lesional skin was related to apoptosis. **Galectin 7** was similarly upregulated in lesional eczema and psoriasis. It was reported that galectin 7 expression increases with the decrease of keratinocyte proliferation [220]. The absence of galectin 7 leads to increased proliferation with reduced differentiation in *in vitro* experiments, which also supports this inverse relationship [306]. It has been also shown that galectin 7 exhibits proapoptotic properties [220]. Therefore, our results of high galectin 7 levels in the stratum corneum of AD and psoriasis seem to be controversial. However, our findings are supported by other tape stripping studies, reporting increased galectin 7 levels in AD, as an indicator of barrier disruption and disease severity [307], and psoriasis [280], although these do not compare its expression levels between the two pathologies. The exact role of galectin 7 in inflammatory dermatosis yet needs to be established, but research suggests that it might act as an alarmin [169], which would explain its high expression in both psoriasis and AD.

Another protein with important role in apoptosis, **SLURP1** was significantly downregulated in lesional psoriasis samples compared to healthy. SLURP1 has been shown to play an important role in keratinocyte terminal differentiation, being a marker of late differentiation, and in programed cell-death, acting through

nicotinic acetylcholine receptors [308]. Interestingly, mutation in the SLURP1 gene, causing depletion or absence of the protein, has been identified as the causative agent for the development of Mal de Meleda, a hereditary palmoplantar keratoderma [309]. It has been shown that SLURP1 expression is increased in human psoriasis by immunostaining [310] and in the mouse imiquimode psoriasis model. It is upregulated by IL22 which has major impact on epithelia innate immune responses [311], but not directly by IL17 and TNFα. SLURP1 seems to also have potent antimicrobial activity against *S. aureus* [312]. Our results suggest that SLURP1 is significantly decreased at the level of the stratum corneum in psoriatic lesional skin compared to controls. This might be due to its consumption by the time it reaches the top layers of the epidermis. Unfortunately, there is not enough data available yet to understand the full extent of how SLURP1 may contribute to the pathophysiology of psoriasis or other inflammatory skin diseases.

Cofilin1 (CFL1), involved in actin modulation and cell migration, has been previously shown to correlate with negative prognosis in melanoma, due to an increased risk of tumour invasion/metastasis [313]. LIM kinase 1 (LIMK1) has an inhibitory effect on cofilin1 expression. LIMK1 is a serine-threonine specific kinase and is mainly expressed in the upper granular layers of the epidermis. While LIMK1 inhibits the Stat3 pathway, leading to cell compaction, cofilin1 seems to activate Stat3 and block cell compaction. Downregulation of LIMK1 in psoriatic epidermis, leading to the upregulation of cofilin1 and consequently to the inhibition of cell compaction and activation of Stat3 pathways, has been described [314]. These results are in line with our MS data, which also found significantly increased expression of cofilin1 in lesional psoriasis.

14-3-3 family members seem to play important role in cell signalling. MS identified 6 isoforms out of 7 of the **14-3-3 protein family** in tape stripping material. This is in accordance with previous results based on biopsy material, confirming that except of the tau isoform, all other members of the 14-3-3 protein family are present in the normal epidermis [315]. We found a more than 32 fold upregulation of the sigma isoform and a more than 5 fold increase of the zeta/delta isoform in both lesional psoriasis and eczema. The upregulation of 14-3-3 sigma in lesional psoriatic skin has been reported previously [316], including in tape stripping material [280]. The expression of both of these 2 isoforms increases towards the upper layers of the epidermis, with keratinocyte differentiation [315]. **14-3-3 sigma** expression is specific for the epidermis. Its production is induced by p53 in keratinocytes following DNA damage, it has an influence on the cell cycle, keratinocyte proliferation and differentiation, and its inactivation leads to keratinocyte immortalisation [316]. The evidence regarding the role of

14-3-3 zeta/delta in the epidermis and in inflammatory skin diseases is currently lacking. This isoform has been mainly investigated in tumourigenesis, as it has an antiapoptotic effect [317] and promotes cell survival against various stress factors (e.g. hypoxia, hypoglycaemia) [318]. Although according to our results neither of these 2 molecules could act as a biomarker in distinguishing psoriasis from eczema, as they have similar expression profiles, they are still of significant interest in the understanding of the pathogenesis of inflammation. As keratinocyte hyperproliferation with incomplete differentiation is present in both eczema, but especially in psoriasis, 14-3-3 protein zeta/delta could play a crucial role in sustaining keratinocyte survival and it might be suitable as a new treatment target.

Cyclophilin A is a protein with regulatory effects on intracellular signalling, transcription, inflammation, and apoptosis [227] and importantly is a binding partner for the immunosuppressant drug cyclosporine A. Cyclosporine A is widely used to control both psoriasis and eczema flares [319, 320]. A previous study showed that cyclophilin A expression does not differ between psoriatic and normal skin [321], however, in our study we did find a significant upregulation of cyclophilin A in both lesional psoriatic (more pronounced) and eczematous stratum corneum samples compared to normal controls. Our findings are supported by a previous report showing that cyclophilin A expression is upregulated with keratinocyte differentiation [322]. The common view is that cyclosporine A mainly acts through the inhibition of T cell proliferation and cytokine production [323]. Our results further highlight that cyclopsorine A may act to a marked extent directly on keratinocytes. Further investigations regarding cyclophilin A expression and its direct effect on keratinocytes in the subcorneal layers of the epidermis in hypeproliferative skin diseases should represent a future research focus as cyclophilin A clearly has clinical-translational significance.

3.9.7 Neoangiogenesis

Increased angiogenesis in lesional psoriatic dermis, with the formation of tortuous blood vessels, is one of the characteristic histopathologic findings in psoriasis. **Thymidine phosphorylase** (**TYMP**) regulates intracellular thymidine levels by catalysing its breakdown into thymine and 2-deoxy-alpha-D-ribose 1-phosphate, playing an important role in DNA maintenance. However, due to its almost identical structure to the platelet-derived endothelial cell growth factor (PD-ECGF) it also exhibits potent chemotactic activity on endothelial cells, promotes endothelial proliferation and neovascularisation [324]. Its presence in the epidermis has been confirmed to be increased with keratinocyte differentiation,

in various skin tumours [324], but also in lesional psoriasis [325]. This is also supported by our findings, which indicate that TYMP is significantly increased in the lesional psoriatic stratum corneum compared to healthy, but not in lesional eczema. TYMP produced in higher quantities by psoriatic keratinocytes is likely to act on neoangiogenesis [325].

3.9.8 Barrier constituents and keratinocyte differentiation markers

Cell adhesion molecules seemed to be generally downregulated in lesional skin, especially in psoriasis. **Desmoglein 1** showed the most significantly decreased expression compared to healthy samples, in both lesional eczema and psoriasis. DSG1 is a known target in the autoimmune bullous disease, pemphigus foliaceus and in the bacterial toxin induced staphylococcal scalded skin syndrome. Homozygous mutations in DSG1 gene, resulting in deficient expression of DSG1, has been recently described in severe dermatitis, multiple allergies and metabolic wasting syndrome, leading to perturbed epidermal integrity and barrier dysfunction with acantholysis and subcorneal blister formation. On the other hand, heterozygous mutations in the DSG1 gene result in palmoplantar keratoderma [209]. Spongiosis, with the formation of intraepidermal vesicles is one of the key histopathologic features of eczema lesions. It has been shown that by increasing the DSG3/DSG1 ratio in the epidermis of transgenic mice, mimicking that of mucous membranes, abnormal scaling occurs at the stratum corneum due to premature loss of corneocyte cohesion, leading to excessive transepidermal water loss [326]. Harmon et al demonstrated how DSG1 promotes epidermal keratinocyte stratification and differentiation via the scaffolding protein erbin [327]. According to the results of this thesis, reduced DSG1 expression seems to play a role in the perturbed maturation and differentiation process described in psoriasis and eczema.

In contrast to other cell adhesion molecules identified in this analysis, **epiplakin**, a member of the plakin family, was slightly increased in lesional eczema, but showed significant upregulation in psoriasis lesional samples compared to healthy controls. **Epiplakin** is known to increase in response to mechanical stress, such as in wound healing, facilitating tissue remodelling by decreasing keratinocyte motility and migration [328, 329]. At the same time it has been suggested that epiplakin might play a role in cell proliferation and differentiation in the corneal epithelium [330] and it has been shown that its expression increases in keratinocytes undergoing differentiation [331]. It has been proposed that epiplakin has a protective role against keratin filament disruption in response to stress [211]. The role of epiplakin in diseases such as eczema and psoriasis has not been investigated. Based on the MS data analysis and on the data

suggested in the current literature, we speculate that epiplakin is increased as a counter regulatory mechanism protecting the keratin filament network in keratinocytes undergoing intense hyperproliferation and rapid turn-over.

Suprabasin, is a novel epidermal differentiation marker, with similar structure to involucrin and loricrin, and it is a potential cornified envelop precursor, expressed in the suprabasal layers of the epidermis [219, 332]. Our results show that suprabasin expression is significantly upregulated in psoriasis lesional samples compared to healthy controls. The role of suprabasin has been investigated in various epithelial [333, 334] and non-epithelial carcinomas [335, 336] and it is recognised as a novel oncoprotein. It has been shown to play a role in tumour cell proliferation [334], to moderately increase cell proliferation of vein outer wall cells *in vitro* [337] and to induce tumour neoangiogenesis [333]. Suprabasin also seems to play a role in keratinocyte differentiation [219]. Both hyperproliferation and neoangiogenesis are characteristic features of psoriasis, however the role of suprabasin in the aethiopathogenesis of psoriasis has not so far been investigated. One recently published tape stripping study comparing suprabasin expression in the stratum corneum of AD patients and healthy individuals by MS found a decrease in suprabasin expression in AD samples [332]. Our results did not show significantly different expression levels between eczematous and healthy skin. Nevertheless, the above mentioned study was based on very small numbers, by comparing samples from n=6 AD and n=3 healthy controls, and it used cellophane tapes which penetrance might differ compared to D-squame tapes. Aoshima et al also found significant decrease in suprabasin levels in the serum of AD patients compared to healthy controls but not in the serum of patients with psoriasis. They also demonstrated that suprabasin has an antiapoptotic effect on keratinocytes [332]. Further research clarifying the role of suprabasin in psoriasis would be of interest.

Calmodulin-like protein 5 (**CALML5**) and **3** (**CALML3**), other markers of keratinocyte differentiation, were both significantly increased in psoriatic lesional skin, however, only **CALML3** showed significantly higher levels not only in psoriasis but also in eczema compared to healthy. **CALML5** has been previously found to be increased in psoriatic lesional samples compared to normal skin. It is described as a calcium-binding protein, with a role in late keratinocyte differentiation. Its increased expression in psoriatic skin has been related to its decreased degradation associated with elevated calcium levels [168]. There is no published literature on the role of **CALML3** in the skin and our study is the first to highlight its significant increase in both eczema and psoriasis by using non-invasive tape stripping. Most probably, similarly to **CALML5**, it has a role in keratinocyte differentiation, however, further research is needed to establish its

exact role. Interestingly, one study examining the efficacy of etanercept treatment in psoriasis listed the gene of CALML3 amongst the down-regulated genes in etanercept responder patients [338].

Other barrier related enzymes identified by MS were **bleomycin hydrolase** (**BH**) and **caspase 14**, both involved in profilaggrin/filaggrin processing. BH co-localises with filaggrin in the granular layer and stratum corneum, having an essential role in the degradation of filaggrin into free amino acids acting as natural moisturising factors (NMF) [217]. Both in the absence of caspase 14 [216] and BH [217] there is deficient NMF formation, leading to dehydration and further barrier dysfunction. Significantly decreased BH levels have been found in AD skin biopsy material [339]. It has been also suggested that BH and caspase 14 play important role in the terminal differentiation of keratinocytes, with an upregulation in orthokeratotic and hyperkeratotic skin diseases, such as erythrokeratoderma and lichen planus, and downregulation in parakeratotic pathologies, such as pityriasis rubra pilaris and psoriasis [340]. Another view is that rather than directly altering keratinocyte differentiation causing psoriasis, caspase 14 or BH deficiency aggravates psoriatic lesions by leading to a disrupted barrier function [216].

Th1 cytokine, INFγ seems to have potent downregulatory effect on BH and Th2 cytokine, IL4 can indirectly reduce its expression [340]. These results support our MS findings, where BH levels were significantly decreased in both lesional psoriasis and eczema compared to healthy tape strip samples.

Mutations in the filaggrin gene account for 25-50% of AD [10], it is however recognised that perturbed filaggrin processing also plays an important role in its pathogenesis [341]. In this study **filaggrin** was unreliably detected in tape strip material by MS, therefore it was excluded from the data analysis. The presence of multiple filaggrin mutations could account for the unreliable detection. Another possibility is that the majority of filaggrin is already completely degraded in our samples as the applied 10 tapes only collect the upper portion of the stratum corneum, with the need of at least 30 consecutively applied tapes for its complete removal in healthy skin [342, 343]. Filaggrin breakdown starts at the base of the stratum corneum, leading to the formation of the NMF assuring adequate skin hydration [344]. The degradation of filaggrin in our samples is also reflected by the reliable detection of histidine, one of its main degradation product, in the tape material.

MS also identified the perturbed expression of another protein playing important role in the formation of the skin barrier system. **Arginase 1** (**ARG1**), a cytosolic enzyme responsible for the synthesis of ornithine and urea, was significantly

downregulated in psoriatic, but especially in eczematous lesional skin compared to healthy. **ARG1** competes with nitric oxide (NO), known to inhibit keratinocyte proliferation and facilitate keratinocyte differentiation, for the same substrate, L-arginine. ARG1 mRNA has been previously shown to be upregulated in psoriatic lesional skin biopsies compared to normal skin, and it has been suggested that ARG1 leads to keratinocyte hyperproliferation by reducing NO production [345]. The same study demonstrated *in vitro* reduction of ARG1 mRNA expression upon stimulation of normal keratinocytes with Th1 cytokines, and suggested that ARG1 overexpression seen in lesional psoriasis biopsies is probably an abnormal expression pattern to the presence of abundant Th1 pro-inflammatory cytokines [345]. ARG1 expression seem to be upregulated by Th2 cytokines (IL4, IL13) [218]. Interestingly, findings similar to ours have been shown in the serum of AD patients on a protein level [346] and in another tape stripping study comparing AD tape samples with healthy [199]. In addition, a recent study suggested lower ARG1 activity in lesional psoriasis, despite the previously seen high ARG1 levels [347]. Low levels or activity of ARG1 can potentially lead to low urea content and decreased hydration of the epidermis and could play part in the pronounced barrier function deficiency in AD.

3.9.9 Persistence of cell organelles due to incomplete differentiation

Finally, in lesional psoriasis, MS identified a significant increase in different **cell organelle** and **nuclear proteins** paralleled with a decrease in **DNase2** levels compared to the stratum corneum samples of healthy skin. This could reflect the retention of cell organelles and nucleus (parakeratosis) due to the perturbation of the degradation process during cornification, a consequence of the increased keratinocyte proliferation rate, rapid cell turn-over leading to the incomplete terminal differentiation observed in psoriasis.

3.9.10 Further proteins of potential interest

Some of the proteins of interest have not been mentioned or discussed in detail as the evidence of their role in normal as well as in inflammatory skin is scarce. A brief summary of their known function and pathological involvement regarding psoriasis and AD is included in Table 3.6.

	Pso	AD	Function	Pathologic involvement	References
Proteases / degradation enzymes					
Cathepsin L2 (SCTP)	↓		cysteine protease degradation of corneodesmosomal proteins → desquamation	↓ keratinocyte hyperproliferation; perturbed hair follicle cycle	[6, 348, 349]
ASPRV1	↑		aspartic protease - predominantly expressed in the skin keratinocyte terminal differentiation and desquamation	↑ differentiated SCC; not expressed in undifferentiated tumours	[215, 350]
Serine protease inhibitors					
WFDC12	↓		serine protease inhibitor antibacterial properties	TNFα, IL1β in lesional psoriatic skin → ↑WFDC12 → pro-inflammatory role?	[351, 352]

Table 3.6 Proteins of potential interest with limited skin related research regarding their functional involvement in the pathology of inflammatory skin diseases.

Arrows indicate significant increase ↑, or decrease ↓ in the expression of proteins in lesional psoriasis (Pso) and lesional atopic dermatitis (AD) compared to healthy. NK - not known - the protein's role in the pathogenesis of skin diseases, particularly of AD and psoriasis, is not known; WFDC12 - WAP four-disulfide core domain protein 12; DAMPs - damage-associated molecular patterns; SCTP - stratum corneum thiol protease; ASPRV1 - retroviral-like aspartic protease 1; PSAPL1 - proactivator polypeptide-like 1; GGCT - gamma-glutamylcyclotransferase; CAT - catalase; SOD1 - superoxide dismutase [Cu-Zn]; HSP - heat shock protein; ROS - reactive oxygen species.

	Pso	AD	Function	Pathologic involvement	References
Serine protease inhibitors					
Serpin B8	↓		serine protease inhibitor cell-cell adhesion	↓ / absent - exfoliative ichthyosis	[353]
Serpin B12	↓		serine protease inhibitor epithelial cell protection from proteolytic enzymes → maintains barrier integrity	NK	[354]
Proapoptotic protein					
Gasdermin A	↓	↓	pore-forming activity (autophagy, necrotic cell death) bactericidal	DAMPs→ ↑ inflammatory caspases → activation of gasdermin D → pyroptosis (inflammatory cell death) → release of inflammatory cytokines e.g. IL1β, IL18	[355, 356]
Methabolic pathways					
PSAPL1 (prosaposin like1)	↓		sphingolipid metabolism	prosaposin (almost identical counterpart) - ↓ psoriasis and AD → depleted ceramide generation → dysregulation of the skin's barrier function ?	[357-359]

Table 3.6 *(continued)* Proteins of potential interest with limited skin related research regarding their functional involvement in the pathology of inflammatory skin diseases.

	Pso	AD	Function	Pathologic involvement	References
Antioxidative molecules					
GGCT	↓		glutathione metabolism potent antioxidant	ROS → cell death → DNA damage → ↑ pro-inflammatory cytokines involved in psoriasis pathogenesis antioxidant molecules → neutralisation of ROS	[224, 298, 299]
CAT	↓	↓	potent antioxidant		
SOD1	↓	↓	potent antioxidant		
Peroxiredoxin 1	↑		potent antioxidant	Lipopolysaccharide, TNFα → release from cells	[360]
HSP27 **HSP27**	↑ ↑	↑ ↑	potent antioxidant anti- and pro-apoptotic activities keratinocyte differentiation	↑ UV irradiation (psoriatic skin) → protects against cell damage anti-inflammatory → ↓ IL8 and prostaglandin E2 acts as self-antigen in streptococcal induced psoriasis → pathogenic T cell response	[361]
HSP70 family members (HSPA5, HSP73)	↑		potent antioxidant anti-apoptotic	↑ keratinocytes surrounding dendritic APCs in early psoriasis ↓ IL4, IL13, IL17F but ↑ IL17A, IL22 mRNA expression (mouse psoriasis imiquimod model) enhances antigen uptake in keratinocytes	[361-363]

Table 3.6 *(continued)* Proteins of potential interest with limited skin related research regarding their functional involvement in the pathology of inflammatory skin diseases.

Brief summary

In summary, much of the MS data are supported by previous research based on immunohistochemistry, microarray and mRNA analysis of full thickness biopsy material. However, the data obtained brings novelty as it was based on samples obtained by a non-invasive procedure, and allowed for a non-biased, objective study of proteins expressed in inflammation. Importantly it not only compared pathologically involved with normal skin, but also compared changes between two different pathologies, allowing for the identification of potential disease markers and perturbed physiological pathways.

This chapter successfully demonstrates that the use of MS in conjunction with tape strip sampling can be used as a powerful strategy to identify potential biomarkers and to provide new insight in to the mechanisms of inflammatory skin disease.

Chapter 4 Quantification of disease markers in tape stripping of psoriasis and eczema patients

4.1 Introduction

The large scale protein profiling by MS performed in Chapter 3 provided an unbiased approach that identified multiple potential psoriasis and eczema biomarkers. In addition, it allowed insight into disease mechanisms. The next step was to validate the potential biomarkers by more high-throughput methods, including ELISA and Multiplex bead-based quantification assays, in order to assess their true potential (specificity, sensitivity, reliability and reproducibility) of detected proteins. The presence of other cytokines and chemokines, known to play an important role in the ethiopathogenesis of psoriasis and eczema, were also tested.

Although no reliable biomarker was identified for the diagnosis of lesional eczema, two very promising protein biomarkers were identified for psoriatic lesional skin: IL36γ and elafin. Despite the lack of commercially available ELISAs for some proteins preventing the follow up of all potential biomarker proteins identified by MS, future development may allow for the diagnosis of early psoriasis and eczema from apparently healthy looking skin.

4.2 RNA isolation from tapes

Previous publications have suggested that tape stripping provides a potential method for RNA sampling [201, 364, 365].

In order to prevent RNA degradation following sample collection three different ways of sample transport and storage have been tried. The 10 collected tapes were either transferred to an empty container, transported on dry ice and stored at -80°C; treated with RNAlater and then transported in an empty container to be stored at -80°C; or completely submerged and transported in RNAlater to be stored at 4°C. RNeasy Mini extraction Kit (QIAGEN) was used for RNA extraction, and the quality of the obtained RNA was assessed by Agilent bioanalyser. This allocates an RNA Integrity Number (RIN) by scoring the RNA from 1 (totally degraded RNA) to 10 (perfectly preserved RNA). The minimal RIN number should be at least 5 in order to obtain reliable RT-qPCR quantification [366].

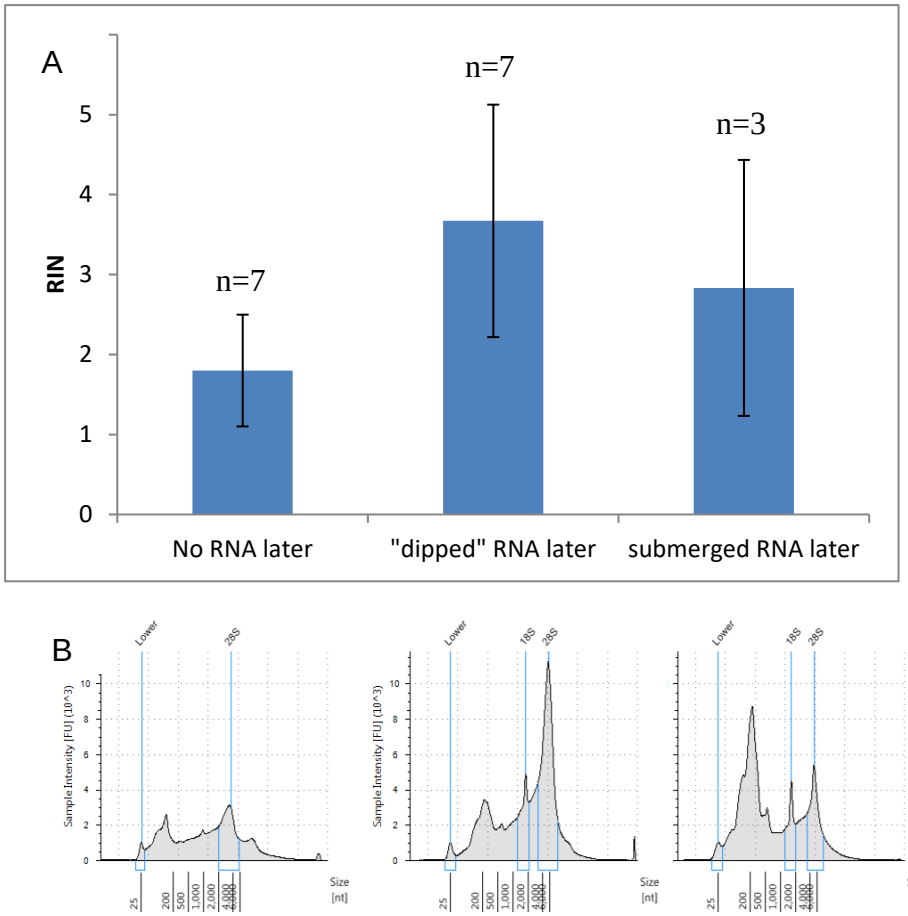

Figure 4.1 RNA quality obtained from tapes with different transport and storage techniques.

(A) The quality of RNA as determined by the Agilent assay was below the recommended minimal RIN of 5 even after protecting samples from RNA degradation by using RNAlater. (B) Ribosomal peak electropherogram depicting the quality of RNA with each transport method used. Figure adapted from the master thesis work of Anne Latcko.

However, using our methodical approach, the mRNA quality derived from tapes was insufficient, with low average RIN number (RIN under 2 for the samples transported only on dry ice and around 3-4 for the samples protected with RNAlater; see Figure 4.1). Therefore, this approach was declined due to a potentially high number of false negative results caused by RNA degradation (work carried out by Anne Latzko, personal communication).

4.3 Selection of markers by ELISA based approaches

As psoriasis and eczema are considered to be predominantly Th1/Th17 and Th2 mediated diseases, respectively, cytokine panels were screened along with mediators known to be involved in the pathogenesis of eczema and psoriasis by ELISA based approaches, while others were selected based on the results of the MS analysis of tape samples, as follows:

- **Th1 associated response:** CXCL9 (also known as monokine induced by gamma interferon - MIG), CXCL10 (also known as interferon gamma-induced protein 10 - IP10), CXCL11 (also known as interferon-inducible T-cell alpha chemoattractant - I-TAC), IFNα, IL2, IL12p40, IL12p70, IL18, IL27;
- **Th2 associated response:** CCL11 (Eotaxin-1), CCL17 (TARC), TSLP, IL4, IL5, IL9, IL13, IL21, IL33;
- **Th17 associated response:** IL11, IL15, IL17, IL22, IL23, CCL20 (MIP3A);
- **Neutrophil infiltration associated proteins:** elafin (IP3/SKALP), IL36γ, IL8, CXCL1 (GROα), CXCL5 (ENA78);
- **Antimicrobial peptides:** hBD2, hBD3;
- **Others, with multiple functions:** S100A8/A9, TNFα, IL1α, IL1β, IL6, IL10, CCL2 (MCP-1), CCL3 (MIP1a), CCL4 (MIP1b), CCL5 (RANTES), CCL27 (CTACK), RNase7, Caspase14, Cathepsine S, granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF, and fibroblast growth factor (FGF).

Despite the documented involvement of the above mediators in psoriasis and eczema many of these were rejected early in the project due to their too low or unreliable detection levels, including lymphokines (IFNγ, IL4, IL13, IL5, IL22, IL17), chemokines (MCP-1, MIP1a, MIP1b, RANTES, eotaxin-1, MIG, ENA-78, TSLP), or because they showed similar expression levels in both psoriasis and eczema, therefore did not have biomarker potential (IFNα, TNFα, IL12p70, IL6, IL9, IL10, IL12p70, IL21, IL27, IL33, GM-CSF, VEGF, FGF).

4.4 Sampling depth

As we could not detect a number of mediators implicated in the pathogenesis of psoriasis and eczema, it was critical to know which level of the epidermis we sample and how deep we sample by applying 10 consecutively placed D-Squame tapes. The available literature show that in the case of healthy skin, the application of at least 30 consecutive D-Squame tapes is necessary to completely remove the stratum corneum [342, 343]. It is also known to be an inverse relationship between the amount of scales collected and the number of tapes applied, in parallel with the increase of corneocyte adhesion towards the deeper layers of the epidermis [367].

We were able to confirm this by using OCT allowing for visualisation of the tape stripped skin surface roughness and epidermal thickness in a healthy control (see Figure 4.2). An area which equalled the surface of the tape was marked on the volar forearm. Half of this area was consecutively tape stripped, while the other

half remained intact. OCT imaging showed that removal of the stratum corneum is incomplete even following 30 consecutively applied tapes.

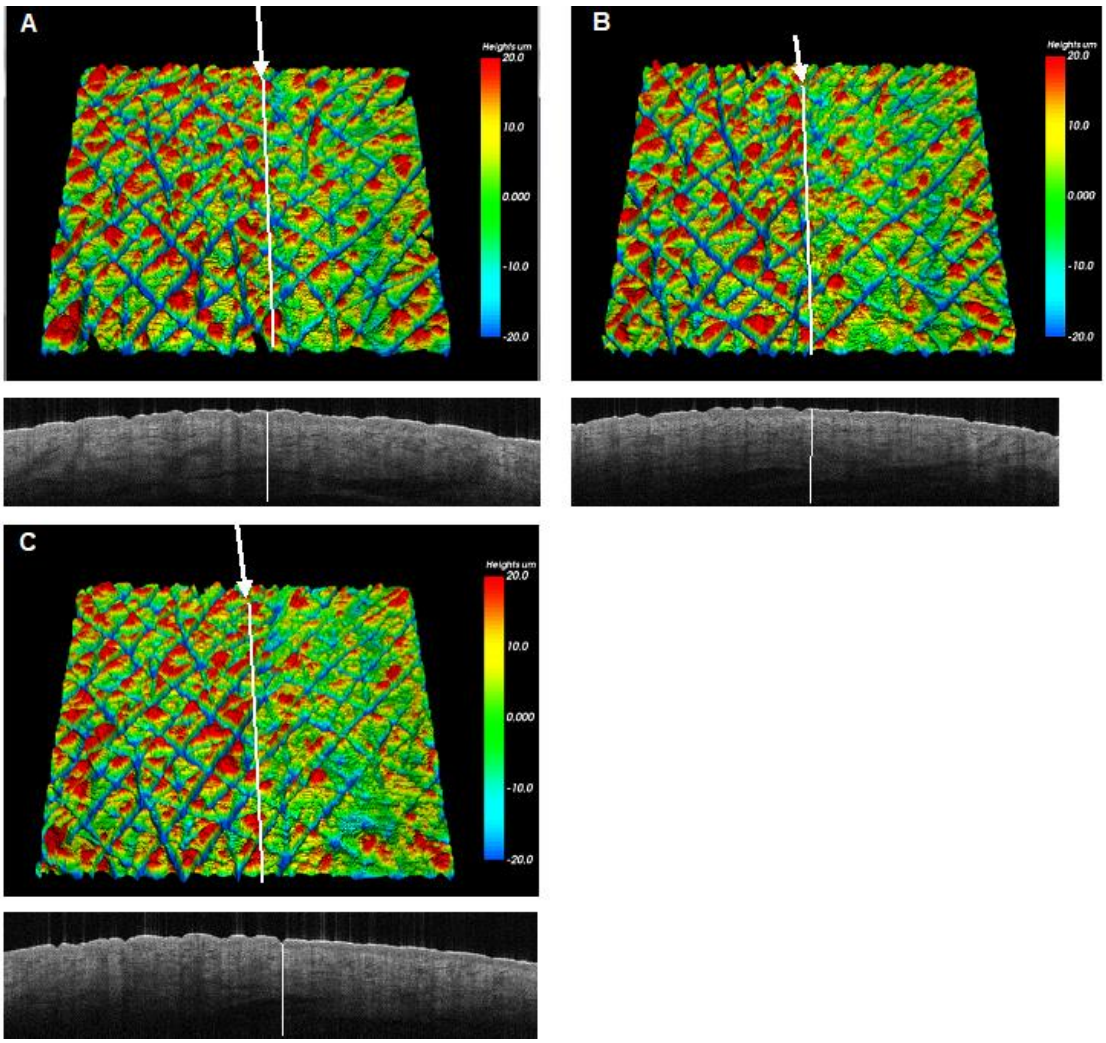

Figure 4.2 OCT imaging of skin surface roughness and epidermal thickness before and after sampling by tape stripping.

The skin of the volar forearm of a healthy volunteer was scanned before tape stripping. Then the area was divided into 2 equal parts. Consecutive D-Squame adhesive discs were applied on one half of this area while the other half remained intact. OCT images showing smoothening of the skin surface in the “taped” area (right hand side of each image), following: (A) 10 consecutive tapes; (B) 20 consecutive tapes; and (C) 30 consecutive tapes.

According to the *in vivo* OCT measurements, the amount of samples obtained by tape stripping also varies depending on the anatomical site of the sample. On the volar forearm, where the epidermal thickness was measured to be approximately 0.11 mm, the change in epidermal thickness following the application of 10 tapes was hardly noticeable, but it was reduced by 0.02-0.03 mm following 30 tapes. The application of further tapes did not seem to have greater impact. On the palmar aspect of the hands, where the stratum corneum is known to be the thickest, the epidermal thickness before tape stripping application was measured

to be approximately 0.33 mm, while this was reduced by 0.02 mm following the first 10 tapes.

This demonstrates that the sampling depth varies even in healthy skin depending on the anatomical location. As this variance can be even greater in the pathologically involved skin, due to the underlying inflammatory changes, some normalisation of samples in order to avoid sampling bias is critical.

4.5 Normalisation of protein expression

As visualised by OCT (see Figure 4.2), sampling by tape stripping remains well on top of the epidermis in healthy skin and is confined to the cornified layer. However, due to the potential heterogeneity of sample location, skin morphology, structure and cellular composition within different sampling sites, no suitable “housekeeping protein” for normalisation of cytokine expression can be used. One potential candidate to inform on the degree of inflammation in all inflammatory skin responses is **S100A8/A9** (**calprotectin**) [144, 368]. Although increased expression compared to non-lesional skin was found in both eczema and psoriasis, S100A8/A9 was overall higher expressed in psoriatic lesions, as previously showed by MS (see Figure 4.3). This was not correlated to the severity of clinical inflammatory symptoms (mean lesion score in psoriasis: 2.31; in eczema: 2.23).

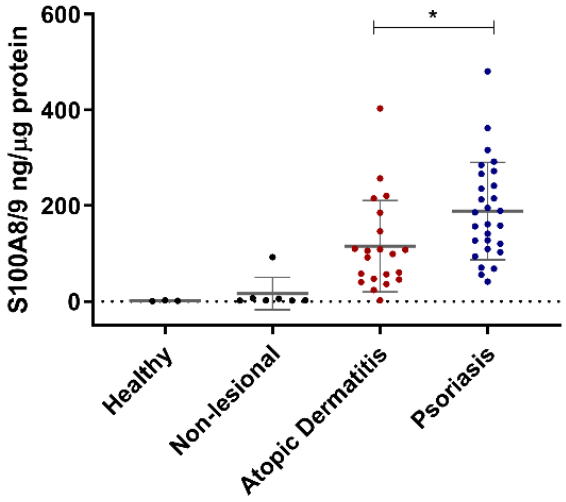

Figure 4.3 S100A8/A9 expression in tape stripping samples.

The expression of S100A8/A9 was significantly increased in lesional psoriasis (mean, 115.1 ng/µg) compared to lesional eczema (mean, 188.6 ng/µg), indicating that it could not be used as a “housekeeping protein” for the normalisation of tape stripping results (healthy n=3; non-lesional n=7; AD n=21; psoriasis n=28). ****p<0.0001.

However, as the aim of the study was to identify robust diagnostic markers in an easily obtainable sample suitable for routine clinical settings, all readings throughout the study were normalised to the total protein content of the individual sample. Protein content as measured by BCA assay ranged from 23 to 942 µg/ml (average 149 µg/ml); the average protein yield in the eczema group was 146 µg/ml for lesional and 68 µg/ml for non-lesional skin, in the psoriasis group 266 µg/ml for lesional, 70 µg/ml for para-lesional and 81 µg/ml for non-lesional skin. The mean for healthy skin samples was 85 µg/ml.

Tape stripping was performed as described in the methodology chapter from healthy volunteers or patients with clinically diagnosed AD or psoriasis. All non-lesional samples were taken from the ventral lower arm region. The number of “healthy”, “non-lesional”, and “para-lesional” samples are lower than in the patient cohort as total protein content was too low to quantitate in a number of samples and samples with a low protein content (< 50 µg/ml) were excluded from the analysis. No difference was found in protein content of non-lesional samples across all groups.

4.6 Inflammatory changes in lesional eczema and psoriasis

4.6.1 Comparison of chemokine expression (IL8, CXCL1, CCL20) in tape samples

Both neutrophils and pro-inflammatory lymphokines IL17/IL22 are known to play a key role in the pathogenesis of psoriasis [28]. The epidermis is known to be a rich source of neutrophil attractant chemokines, including CXCL1 (GROα) and IL8 [369], as well as CCL20 with potent chemotactic role for IL17/IL22 producing lymphocytes [59]. Their expression levels were significantly upregulated in psoriatic compared to eczematous lesional tape strip material, while they had undetectable or very low levels in healthy and non-lesional samples. However, when assessing their biomarker potential to discriminate psoriatic from eczematous inflammation, ROC curve analysis indicated that although they show good sensitivity and specificity for psoriatic inflammation, other markers might have better biomarker potential (see Figure 4.4).

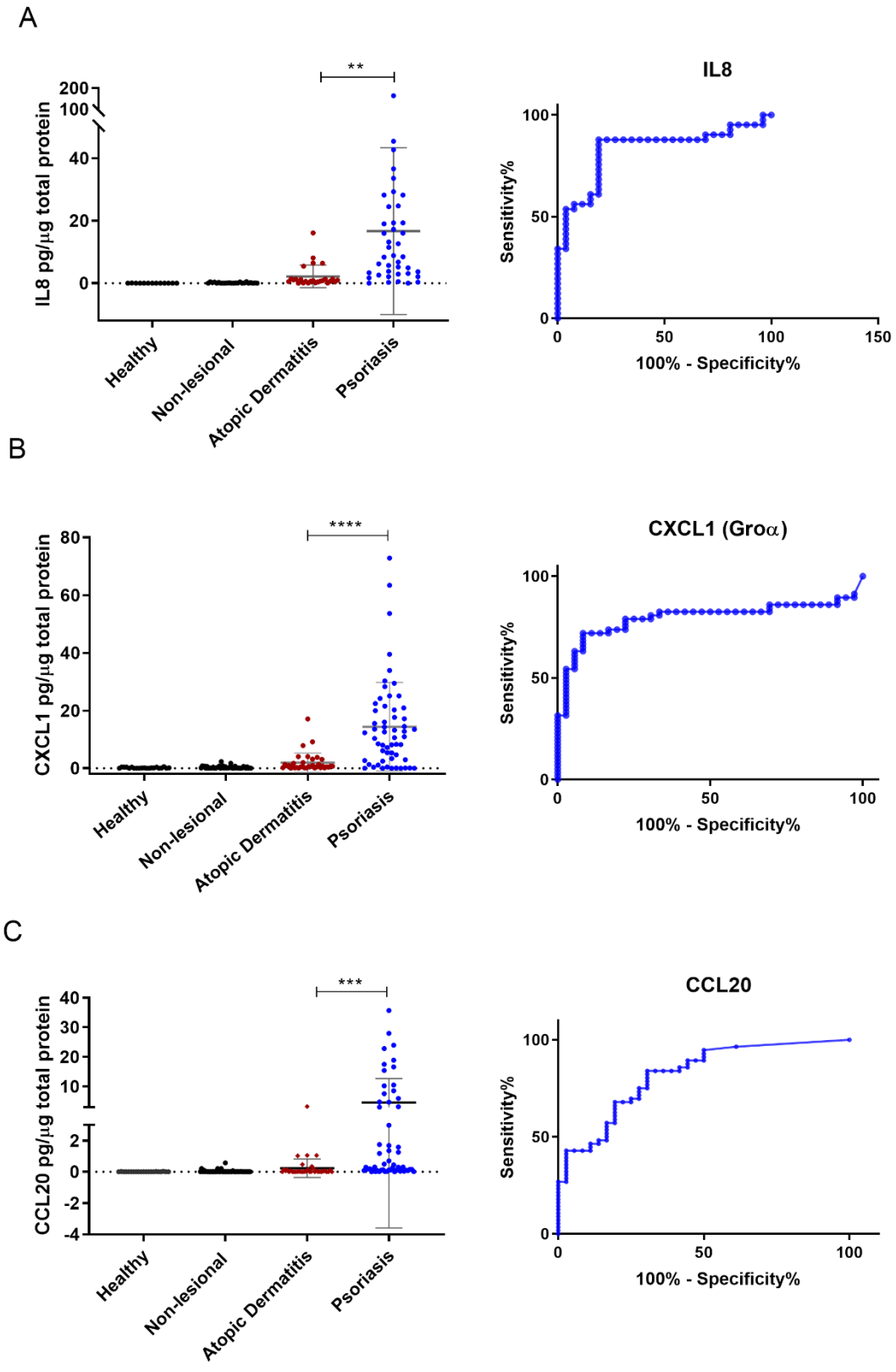

Figure 4.4 Expression of neutrophil recruiting CXCL1 (GROα), IL8 and IL17/22 lineage cell recruiting CCL20 are significantly higher in psoriatic inflammation than in eczema.

Figure 4.4 ***(continued)*** (A) IL8: AUC 0.83, SE 0.0523, 95% CI0.726 to 0.931 (healthy n=13, mean 0.010 pg/µg; non-lesional psoriasis & eczema n=24, mean 0.124 pg/µg; AD n=26, mean 2.182 pg/µg; plaque psoriasis n=41, mean 16.65 pg/µg); (B) CXCL1: AUC 0.796, SE 0.049 , 95% CI 0.7 to 0.891 (healthy n=21, mean 0.155 pg/µg; non-lesional psoriasis & eczema n=44, mean 0.329 pg/µg; AD n=36, mean 1.935 pg/µg; plaque psoriasis n=57, mean 14.40 pg/µg); (C) CCL20: AUC 0.82, SE 0.045, 95% CI 0.731 to 0.905 (healthy n=21, mean 0.004 pg/µg; non-lesional psoriasis & eczema n=45, mean 0.032 pg/µg; AD n=36, mean 0.231 pg/µg; plaque psoriasis n=56, mean 4.545 pg/µg); **p<0.01; ***p<0.001; ****p<0.0001.

4.6.2 Antimicrobial protein expression in tape samples

A number of reports investigating biopsies from AD and psoriasis patients have shown higher expression of the antimicrobial peptide hBD2 in psoriatic individuals [370]. Moreover, cohort studies have shown a significant association between the risk of developing psoriasis and higher genomic copy numbers for hBD2 [161]. hBD3 has also gained interest due to its increased antimicrobial activity against the AD-associated *S. aureus* over hBD2 [267]. Our MS data indicated that both hBD2 and hBD3 were significantly upregulated in lesional psoriasis, while only hBD2 showed significantly increased levels in lesional eczema compared to healthy.

Protein expression of both defensins were analysed by ELISA. While hBD2 and hBD3 were both upregulated in lesional skin when compared to healthy, only hBD3 showed significantly higher expression in psoriatic compared to AD samples (see Figure 4.5). However, because of a marked overlap between its expression in AD and psoriasis, it is unlikely to be suitable as a diagnostic marker (ROC curve analysis: AUC 0.855, SE 0.088, 95% CI 0.68 to 1.03).

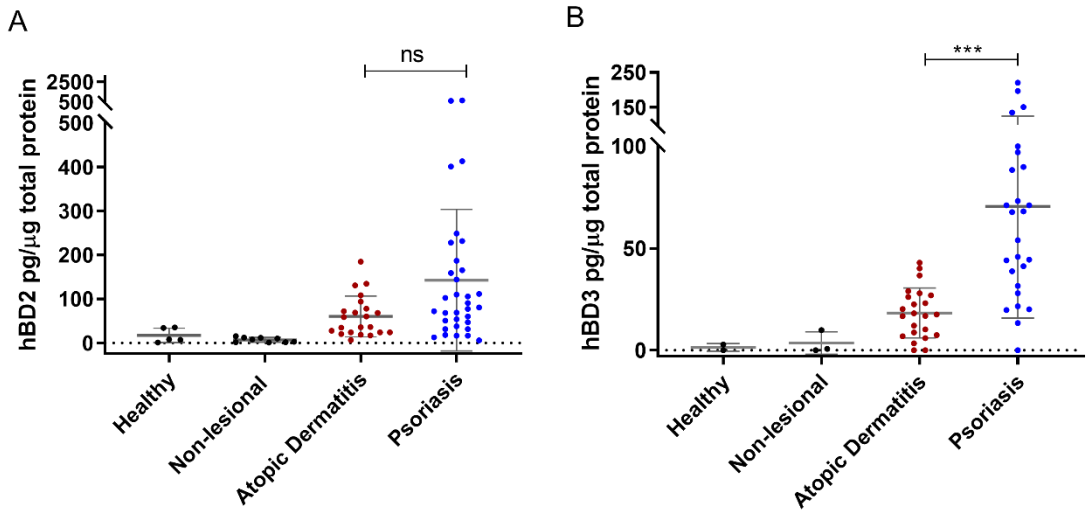

Figure 4.5 Epidermal hBD3 and not hBD2 is significantly higher expressed in psoriasis compared to AD as detected by tape stripping.

(A) hBD2 expression was higher in both lesional eczema and psoriasis compared to healthy and non-lesional tape samples (mean, 60.24/142.24/16.98/7.013 pg/µg), without showing differential expression between the two conditions (healthy n=5; non-lesional psoriasis & eczema n=9; AD n=22; plaque psoriasis n=33). (B) hBD3 expression was significantly higher in lesional psoriasis (mean, 70.51 pg/µg) compared to eczema (mean, 18.18 pg/µg) (healthy n=2; non-lesional psoriasis & eczema n=3; AD n=23; plaque psoriasis n=26). Patient cohorts overlap with those depicted in previous figures, not all the samples have been measured for hBD content. ns – non-significant; ***p<0.001.

4.6.3 Potential markers for atopic inflammation

Most proteins identified as overexpressed in AD in the MS data analysis were serum proteins (serotransferrin, haptoglobin).

Serum **CCL17** (**TARC**) [125] and **CCL27** (**CTACK**) [371] have been shown to correlate with disease activity and severity in AD patients, being considered one of the best markers available for AD even at tissue level [126, 174, 372]. CCL17 acts on CCR4 and thus is involved in the chemotaxis of IL4/IL13 producing lymphocytes, while CCL27 acts via CCR10 and attracts memory T cells to the skin [373].

As depicted in Figure 4.6, **CCL27** was similarly increased in both lesional eczema and psoriasis in epidermal tape stripping samples, and it did not show differential expression between the two conditions. **CCL17** was not detectable in a significant number of eczema (25%) as well as psoriatic (57.4%) samples, however, when present it seems to be specific for atopic inflammation. Although it could not be used as a tape stripping biomarker to identify eczematous inflammation, these results suggest that it might be of value in disease endotyping and potentially as a predictor marker for therapeutic response.

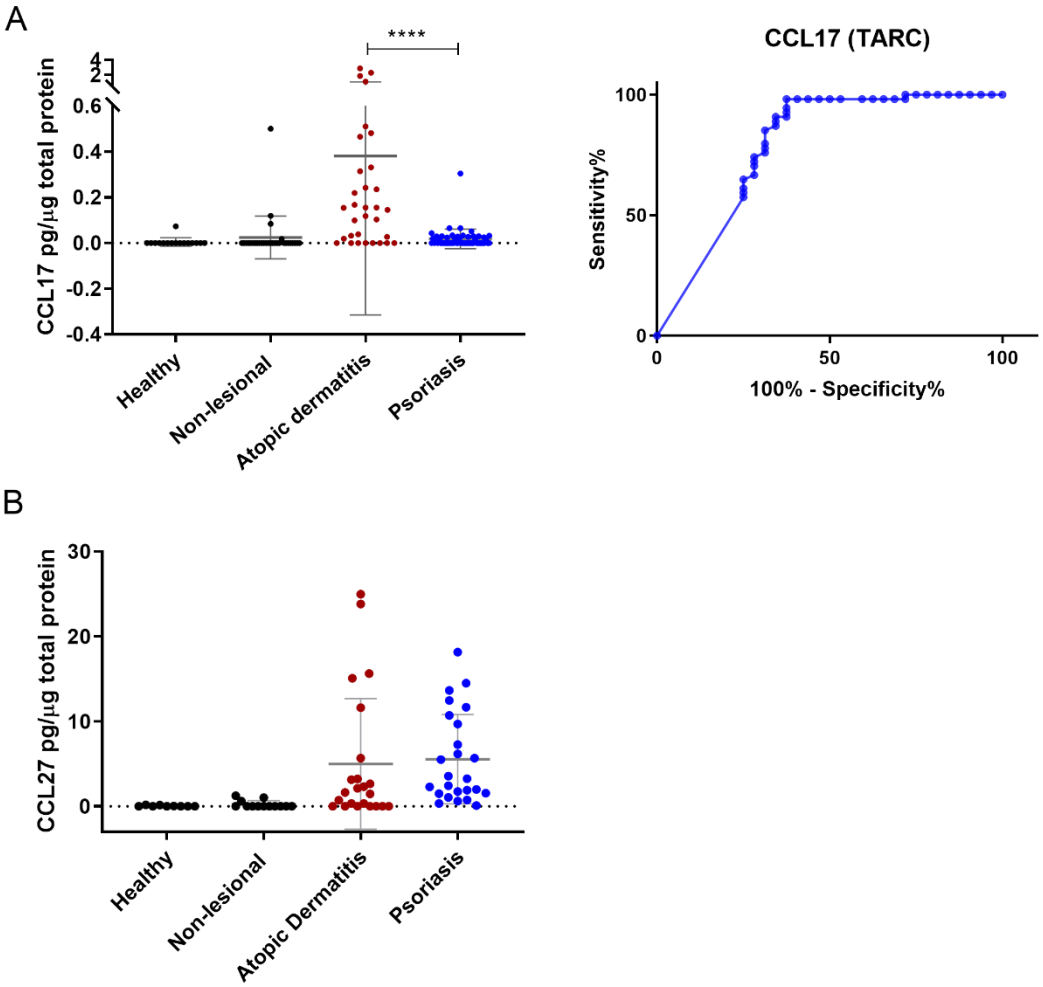

Figure 4.6 CCL17 but not CCL27 is preferentially expressed in eczema lesions.

(A) CCL17 expression is significantly higher in lesional AD compared to psoriasis (mean 0.381/0.018 pg/µg), when detectable. ROC curve analysis: AUC 0.789, SE 0.057, 95% CI 0.676 to 0.903 (healthy n=16; non-lesional psoriasis & eczema n=30; AD n=32; plaque psoriasis n=54). (B) CCL27 showed no differential expression between lesional eczema and psoriasis (mean, 4.991/5.543 pg/µg); (healthy n=9; non-lesional psoriasis & eczema n=13; AD n=23; plaque psoriasis n=25). ****p<0.0001.

4.6.4 Potential markers for psoriatic inflammation

Based on the MS data analysis, a number of markers seemed to be promising as having a biomarker potential for distinguishing psoriatic from eczematous inflammation in epidermal samples. From these, based on the available literature and commercially available reagents, IL36γ and elafin seemed to be the most promising candidates.

4.6.4.1 IL36γ

Upregulation of IL36γ mRNA and protein has previously been shown in lesional psoriasis [144, 374]. Our MS data analysis highlighted that IL36γ has a good biomarker potential to distinguish between eczema and psoriasis. As no commercial IL36γ ELISA was available at the time, protein quantification was performed by a newly developed, in-house sandwich ELISA (see methodology chapter). As the MS data indicated, IL36γ expression did not differ between healthy (mean, 13.5 pg/µg) and non-lesional (mean, 7.5 pg/µg) tape stripping samples, but it was slightly elevated in para-lesional psoriasis (mean, 33.5 pg/µg). IL36γ was increased in lesional eczema (mean, 71 pg/µg) compared to healthy and non-lesional samples, especially in the case of chronic, lichenified lesions, however, this change was not significant. On the other hand, lesional psoriasis (mean, 719 pg/µg) showed significant upregulation in IL36γ expression, not only compared to healthy controls but also to lesional eczema. ROC curve analysis showed that IL36γ has excellent biomarker potential to distinguish psoriasis from eczema with a specificity of 100% (95% CI, 90% to 100%), and sensitivity of 94.44% (95% CI, 84.61% to 98.84%), at an optimal cut off level of 214 pg/µg total protein (Youden index, 0.944) (see Figure 4.7).

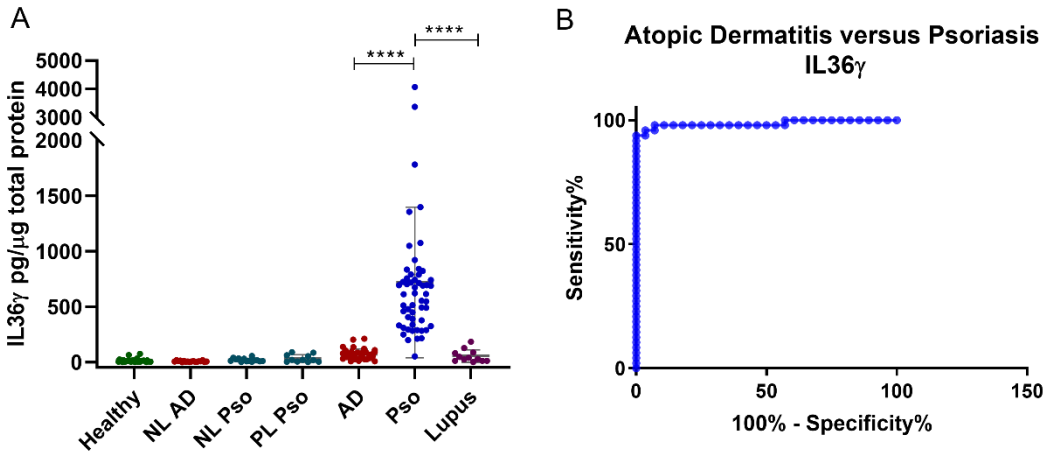

Figure 4.7 IL36γ as biomarker for differentiating psoriatic from eczematous inflammation.

(A) IL36γ expression was significantly higher in plaque psoriasis compared to AD, but also to other inflammatory skin diseases, such as lupus erythematosus (healthy n=34; non-lesional eczema n=18; non-lesional psoriasis n=14; para-lesional psoriasis n=11; AD n=35; plaque psoriasis n=54; lupus erythematosus n=12). (B) ROC curve analysis depicting the high specificity and sensitivity of IL36γ expression in lesional psoriasis compared to eczema (AUC 0.987, SE 0.011, 95% CI 0.965 to 1.000). ****p<0.0001.

In order to show its specificity for identifying psoriatic inflammation, IL36γ expression was also measured in other skin pathologies which occasionally present similar clinical features to eczema and psoriasis, such as in fungal infection (tinea), lichen planus, systemic-, subacute cutaneous- and chronic discoid lupus erythematosus. Although the number of samples included in the different disease categories were limited, our preliminary data shows that IL36γ expression levels in these pathologies fall below the diagnostic cut off level of 214 pg/µg.

The expression of IL36γ was also compared against the neutrophil recruiter CXCL1, which also presented biomarker potential as described above. The result showed that the expression of IL36γ is much more consistent and superior to that of CXCL1 across psoriasis patients (see Figure 4.8).

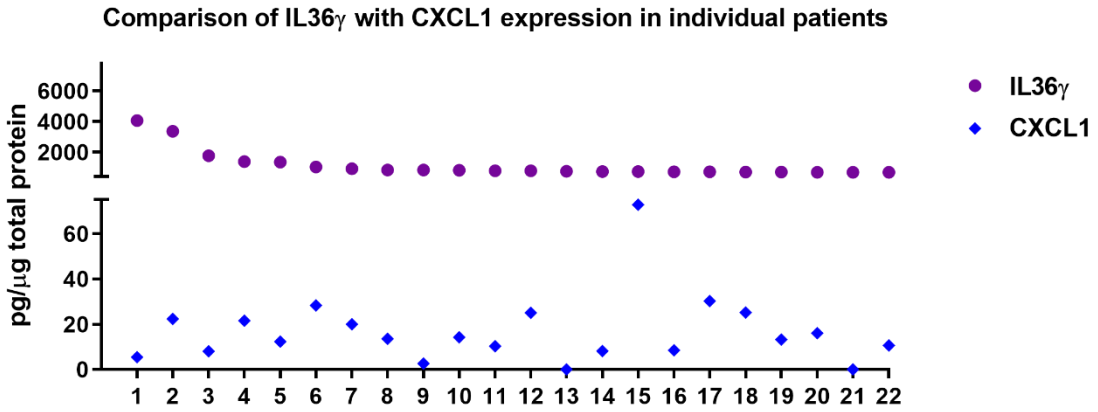

Figure 4.8 Comparison of IL36γ with CXCL1 expression in individual plaque psoriasis samples.

CXCL1 expression was matched to the patients with high IL36γ expression, with the latter being sorted from higher to lower expression levels. IL36γ expression is more consistent across patient samples when compared to CXCL1.

4.6.4.2 Elafin

In the MS data, elafin expression was found to be upregulated by almost 3 fold in lesional psoriasis samples compared to eczema. As this is a neutrophil elastase inhibitor [229], and it is known that neutrophils play a key role in psoriatic inflammation, its biomarker potential was promising.

When quantified, elafin expression showed a tendency for increased levels in lesional atopic skin (mean, 871.2 pg/µg) compared to healthy (mean, 20 pg/µg), non-lesional eczematous (mean, 28.2 pg/µg), non-lesional psoriatic (mean, 58.5 pg/µg) and para-lesional psoriatic (mean, 112.8 pg/µg) skin. Interestingly, just as suggested by MS analysis, elafin expression seems to increase already in subclinical psoriatic inflammation, reaching significantly high levels in lesional psoriasis (mean, 9668 pg/µg), not only compared to non-involved skin but also compared to lesional atopic inflammation. ROC curve analysis showed that elafin levels over 3240 pg/µg could be used as a perfect tape stripping biomarker to differentiate psoriatic from eczematous inflammation (see Figure 4.9). This suggests that elafin has an un-matched potential as a diagnostic marker for psoriatic inflammation, even when compared to IL36γ.

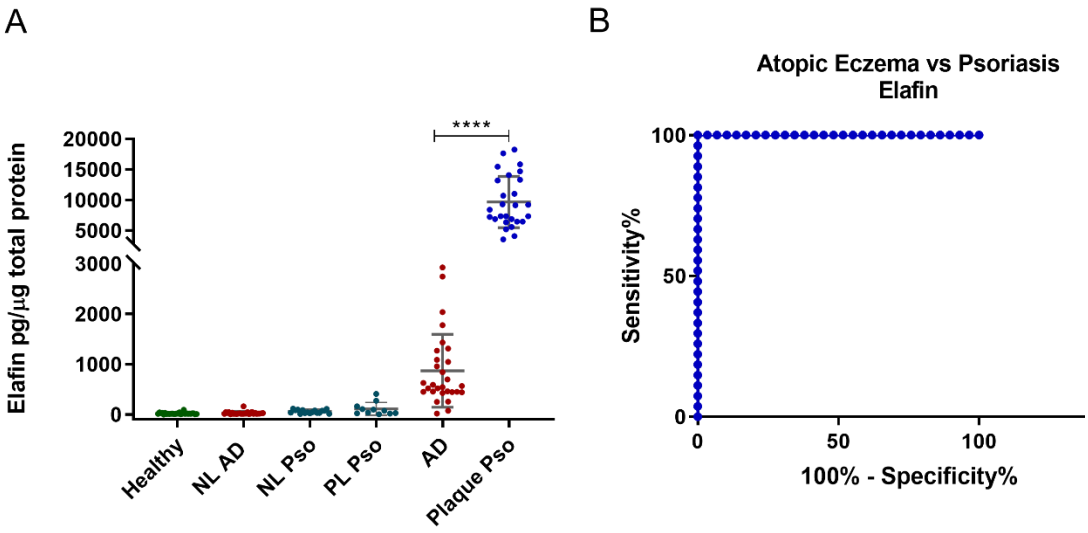

Figure 4.9 Elafin expression as a potential tape stripping biomarker for psoriatic inflammation.

(A) Elafin expression was significantly higher in plaque psoriasis compared to AD (healthy n=28; non-lesional eczema n=27; non-lesional psoriasis n=17; para-lesional psoriasis n=11; AD n=29; plaque psoriasis n=27). (B) ROC curve analysis depicting the overwhelmingly high specificity and sensitivity of elafin expression in lesional psoriasis compared to eczema (AUC 1, SE 0.000, 95% CI 1.000 to 1.000). ****p<0.0001.

4.6.4.3 The development of a composite biomarker

A disease biomarker needs to be specific, sensitive and reliable [182]. Based on our data, elafin has an unequalled biomarker potential to distinguish psoriatic from eczematous inflammation in lesional plaque psoriasis tape stripping samples.

However, as presented in the introduction, psoriatic inflammation can manifest with multiple disease phenotypes. Therefore, the development of a composite biomarker, to specifically identify psoriatic inflammation even in the most atypical presentations, might be more reliable than the use of one single marker. Although the number of patients sampled with other psoriasis subtypes is limited, preliminary data suggests that both IL36γ and elafin are upregulated not only in plaque psoriasis but also in other psoriatic phenotypes (see Figure 4.10).

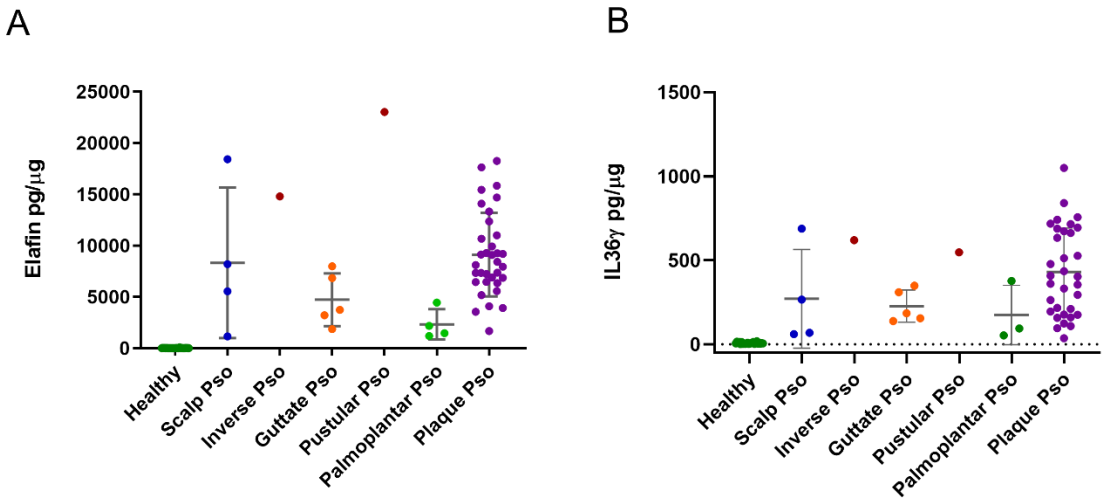

Figure 4.10 Elafin and IL36γ expression in different psoriasis phenotypes.
The same samples were used for the illustration of elafin and IL36γ in different psoriasis subtypes (healthy n=28; scalp psoriasis n=4; inverse psoriasis n=1; guttate psoriasis n=5; pustular psoriasis n=1; palmoplantar psoriasis n=4).

4.6.4.4 IL36γ and elafin expression in eczematous inflammation

Although both IL36γ and elafin were significantly overexpressed in psoriatic lesional skin compared to eczema, they also showed a tendency of being upregulated in lesional eczema compared to healthy and non-lesional skin. Therefore, it was examined if their expression levels are influenced by the atopy status of patients or in the case of hand eczema, by the predominant disease subtype (hyperkeratotic/vesicular). The same disease cohort was used to examine the expression of both markers. However, neither the expression levels of IL36γ nor that of elafin showed correlation with atopy status or with hand eczema phenotype (see Figure 4.11).

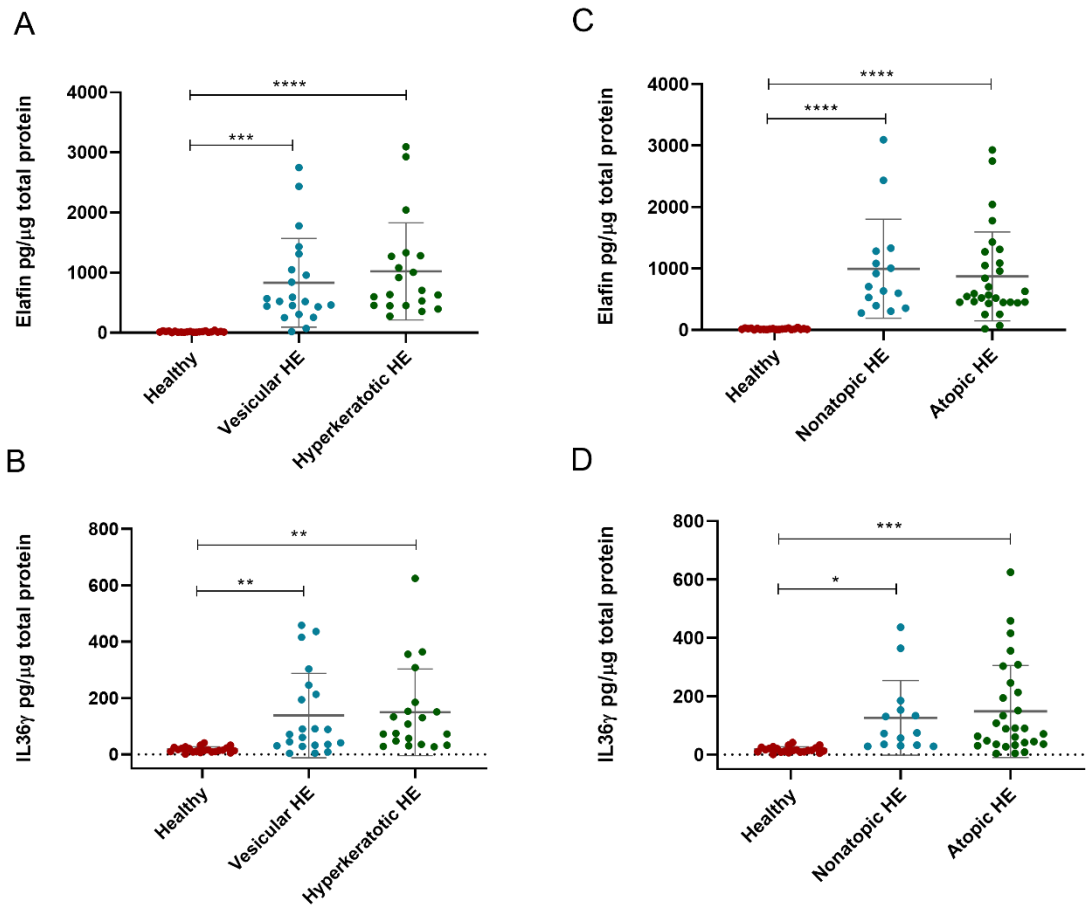

Figure 4.11 Comparison of IL36γ and elafin expression in different types of eczema.

(A) Elafin and (B) IL36γ expression levels are upregulated in a similar manner in both vesicular and hyperkeratotic subtypes of hand eczema compared to healthy controls (healthy n=22; vesicular HE n=21; hyperkeratotic HE n=20). (C) Elafin and (D) IL36γ levels are not influenced by atopy status and are significantly upregulated compared to healthy in both non-atopic and atopic eczema (healthy n=22; non-atopic HE n=15; atopic HE n=29). *<0.05; **<0.01; ***p<0.001; ****p<0.0001.

4.6.5 Other IL1 family members

The expression levels of other IL1 family members in tape stripping material was also examined, especially as most of these cytokines are also produced by keratinocytes, with potent pro-inflammatory role.

4.6.5.1 IL18

IL18 expression has been previously found to be upregulated in psoriatic lesional biopsy materials and scales compared to normal controls [375], while IL18 serum levels have been correlated with psoriatic disease severity [140]. In our tape stripping measurements, IL18 was also significantly upregulated in psoriasis (mean, 12.65 pg/µg) compared to healthy (mean, 0.023 pg/µg) and non-lesional (mean, 0.028 pg/µg) samples, but also compared to lesional eczema (mean,

3.823 pg/µg) and lupus erythematosus (mean, 0.583 pg/µg). However, as the ROC curve analysis demonstrates, the biomarker potential of IL18 is considerably inferior to that of IL36γ and elafin (see Figure 4.12).

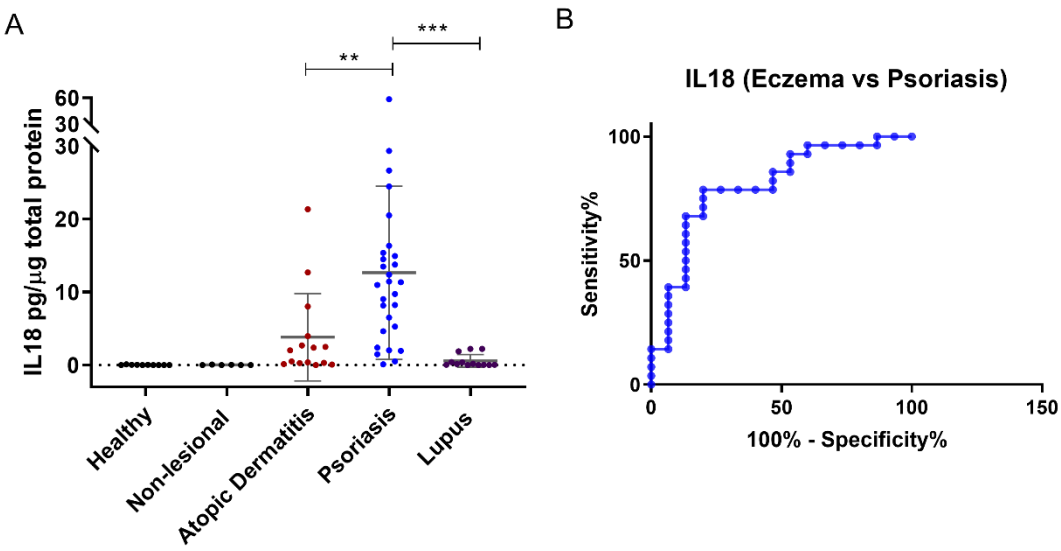

Figure 4.12 IL18 expression is significantly upregulated in psoriatic compared to eczematous tape stripping material.

(A) IL18 levels are significantly increased in psoriatic compared to atopic and lupus tape stripping samples as well as compared to healthy and non-lesional controls (healthy n=10; non-lesional n=6; AD n=15; psoriasis n=28; lupus erythematosus n=13); (B) ROC curve analysis depicting the diagnostic ability of IL18 for differentiating psoriatic from eczematous inflammation in tape strip samples (AUC 0.800, SE 0.073, 95% CI 0.655 to 0.944). **p<0.01; ***p<0.001.

4.6.5.2 IL1α and IL1β

While other IL1 family members were significantly overexpressed in psoriasis and, although to a lesser extent, also in eczema compared to healthy and non-lesional skin, the opposite was truth for **IL1α**. IL1α levels were significantly downregulated in both psoriasis and eczema (mean, 2.036/1.118 pg/µg) compared to healthy and non-lesional skin (mean, 16.03/11.95pg/µg), as well as to lupus erythematosus (mean, 17.30 pg/µg). No significant differences in the expression of **IL1β** were found between healthy and lesional skin, although there was a tendency for IL1β to be increased in inflammatory samples (see Figure 4.13).

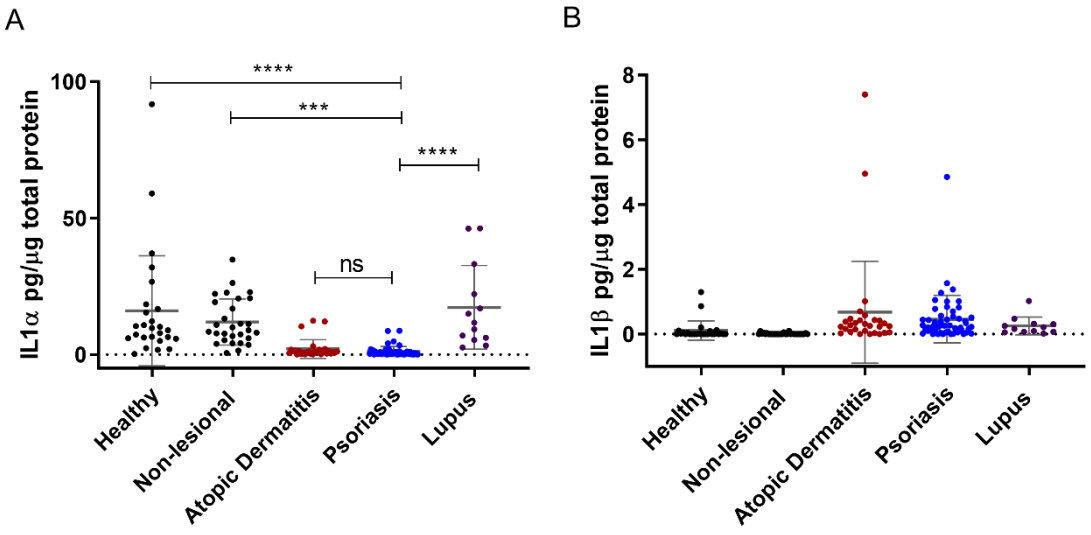

Figure 4.13 IL1α and IL1β expression in tape stripping samples.
(A) Unlike other members of the IL1 family, IL1α expression was significantly downregulated in lesional eczema and psoriasis compared to healthy and non-lesional tape samples, as well as to lupus; (B) No significant difference was noted in the expression of IL1β between healthy, non-lesional and inflammatory samples, although its levels were slightly increased in lesional tape stripping material. ns – non-significant; ***p<0.001; ****p<0.0001.

4.6.6 Markers of subclinical inflammation

One important research aim was to identify a marker which could not only differentiate subclinical inflammation from healthy skin, but also distinguish the underlying eczematous and psoriatic inflammation, predicting disease development and potentially associated co-morbidities, such as PsA, in healthy looking individuals. Although most markers showed a tendency to be slightly upregulated in non-lesional and para-lesional skin, as also suggested by the MS data analysis, this increase remained discrete and non-significant compared to expression levels in healthy samples. Except for the above mentioned inflammatory cytokine IL1α, which was significantly downregulated in lesional skin compared to both healthy controls and non-involved skin of patients, no other pro-inflammatory cytokine or chemokine showed significantly differential expression in non-lesional skin. One of the potential markers identified following the MS data analysis was **RNase7**, a ribonuclease with potent antimicrobial activity [376], produced by keratinocytes and accumulated on the skin surface. RNase7 production is increased by pro-inflammatory cytokines in both eczema and psoriasis [377].

Protein quantification showed that RNase7 is significantly upregulated in para-lesional psoriasis (mean, 389.0 pg/µg) samples compared to healthy (mean, 152.2 pg/µg), but also to lesional psoriasis (mean, 164.1 pg/µg) and eczema (mean, 184.4 pg/µg). Nevertheless, although it had higher levels in both non-

lesional and para-lesional psoriasis (mean, 303.3/389.0 pg/µg) compared to non-lesional eczema (mean, 247.7 pg/µg), this difference was not significant. Therefore it is not a promising candidate for early disease diagnostic, but it could be used as an indicator of an underlying inflammation in healthy looking skin, prompting further investigations or a closer clinical follow up.

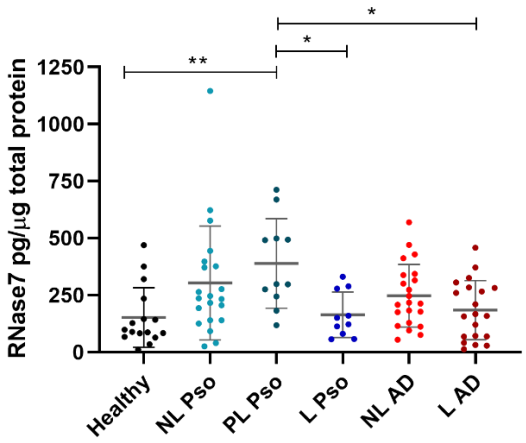

Figure 4.14 Increased expression of RNase7 in subclinical inflammation compared to healthy and lesional skin.

RNase7 expression was significantly increased in para-lesional psoriatic samples compared to healthy controls and lesional psoriasis and eczema. However, the expression of RNase7 was non-differential between samples of subclinical inflammation (healthy n=16; non-lesional psoriasis n=21; para-lesional psoriasis n=11; plaque psoriasis n=10; non-lesional AD; n=22; AD n=21); ** $p<0.01$, * $p<0.05$.

4.7 Hair-plucking as a non-invasive diagnostic alternative for the identification of psoriatic inflammation of the scalp

Clinical features of scalp psoriasis are often mistaken with that of seborrheic dermatitis (a form of eczema described in the introduction), and vice versa [68], potentially delaying the recognition and essential early treatment of comorbidities such as PsA. Therefore differentiating these two disease entities at the scalp level when no other anatomical site is involved is essential.

However, the hairy scalp is not suitable for tape stripping. An alternative method to obtain a non-invasive sample in order to avoid the need of an invasive biopsy (resulting in scarring and loss of hair in that area) is hair-plucking. Plucked hair follicles contain living epithelial cells. Their examination can be informative regarding the underlying pathological mechanism involving the hair follicle. As part of a pilot study, conducted by Mohammad Shalbaf, with the main aim of detecting lupus related molecular signatures from plucked hair in order to identify CDLE related inflammation, hair follicles from patients with scalp psoriasis were also obtained and examined. While inflammation in CDLE results in skin atrophy, scarring and loss of hair follicles, psoriatic inflammation leads to “excessive tissue regeneration” with hyperproliferation, thickening of the epidermis, without affecting the hair follicle. As described in the methodology chapter, hair follicles were obtained from five healthy controls, six psoriasis (both lesional and non-lesional samples) and seven CDLE (both lesional and non-lesional) patients. The hair follicles of 4 to 5 anagen hairs were used for RNA isolation (RIN 7.5 – 10). mRNA expression of target genes was measured by RT-qPCR.

In CDLE there was a significant upregulation of a number of IFN-stimulated genes (including myxovirus protein 1 (Mx1), IFN inducible protein 6 (IFI6), CXCL9, CXCL10, bone marrow stromal antigen 2 (BST2), IFN-induced helicase C domain-containing protein 1 also known as melanoma differentiation-associated protein 5 (IFIH1/MDA5)), apoptosis-related genes (XIAP-associated factor 1 (XAF1)), MHC class I antigen presentation pathway (β2 microglobulin (β2M)) and complement factors (C3) compared to both non-involved and psoriatic lesional samples (see Table 4.1).

Fold difference				
Gene symbol	**Main features**		**Lesional CDLE vs healthy**	**Lesional psoriasis vs healthy**
Mx1		IFN stimulated genes	266*	2.5
IFI6			156*	1.3
CXCL9	CD8 infiltrate, autoimmune response		498*	1.7
CXCL10			40*	-
BST2			2742	5
IFIH1/MDA5			8.5*	0.6
BMP2	Atrophy, scarring		0.09	1.4
XAF1	Apoptosis / dying cells		101*	2.2
C3	Positive direct immunofluorescence		12.6*	2.4
β2M	Tissue repair		13*	1
KDR	Vascular endothelial growth factor receptor 2 (VEGFR2)		2.8	275

Table 4.1 Differential expression (fold change) of RT-qPCR validated genes between lesional CDLE or psoriasis versus healthy controls.

BMP2 - bone morphogenetic protein 2; β2M - β2 microglobulin; BST2 - bone marrow stromal antigen 2; IFIH1/MDA5 - IFN-induced helicase C domain-containing protein 1 / melanoma differentiation-associated protein 5; IFI6 - IFN inducible protein 6; KDR - kinase insert domain receptor; Mx1 - myxovirus protein 1; XAF1 - XIAP-associated factor 1 (XIAP - X-linked inhibitor of apoptosis). * indicates significance. Table adapted from [378].

No upregulation of IFN related signatures were found in psoriasis, which also supports the fact that psoriasis is mainly an interfollicular disease. However, bone morphogenetic protein 2 (BMP2), a tissue repair-related gene, and kinase insert domain receptor (KDR) which codes for vascular endothelial growth factor receptor 2 (VEGFR2) were both significantly upregulated in lesional psoriatic samples compared to CDLE, with the latter also being overexpressed compared to healthy (see Figure 4.15).

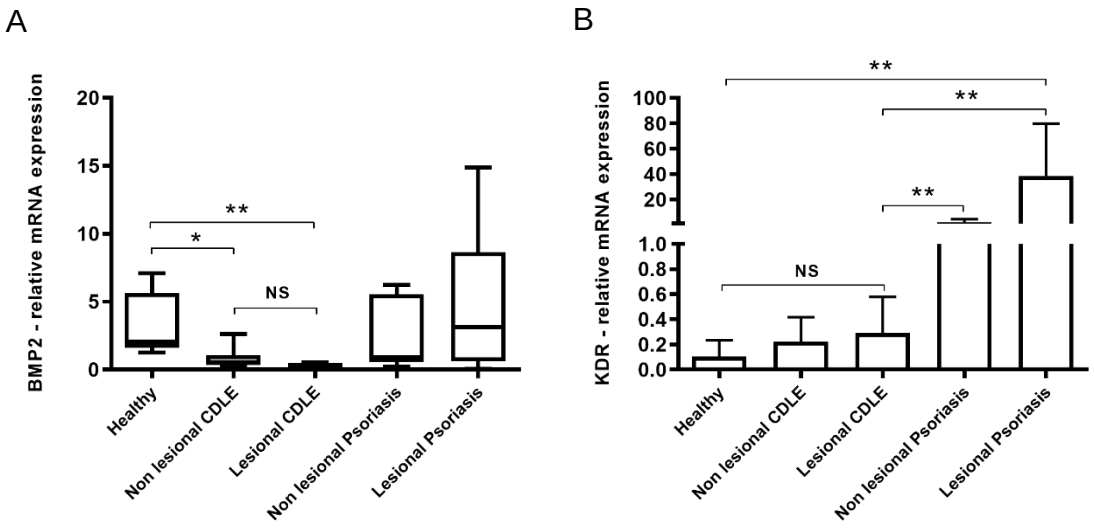

Figure 4.15 mRNA expression of BMP2 and KDR in hair follicle samples obtained by hair-plucking.

(A) BMP2 expression is significantly reduced in non-lesional and lesional CDLE compared to healthy, non-lesional and lesional psoriasis; (B) Significant upregulation of KDR mRNA expression in psoriatic non-lesional and lesional hair follicle samples compared to healthy controls and CDLE. Results are depicted as mean±SEM. Figure adapted from [378].

These preliminary results indicate that hair-plucking could be a suitable non-invasive diagnostic approach to identify scalp pathologies. Further investigations on the protein level, including measurements of markers identified by MS in tape stripping samples with a biomarker potential for psoriasis are necessary to see if hair follicles could be used as a simple diagnostic tool to differentiate scalp inflammation e.g. scalp psoriasis from seborrheic dermatitis.

4.8 The diagnostic value of tape stripping

The results presented in this chapter have been based on a study cohort constituted from patients diagnosed with typical plaque psoriasis and AD. However, the real diagnostic challenge in clinical practice is represented by cases where the otherwise characteristic disease features are vague due to minimal inflammation, overlap (often seen in distinct anatomical locations such as the palmoplantar region, flexural areas, scalp), have an atypical presentation, evolution or response to treatment. In order to assess the diagnostic value of IL36γ and elafin in a real clinical setting, tape stripping samples were collected from patients with atypical inflammatory phenotypes. The following cases demonstrate the remarkable potential of these two markers to distinguish psoriasis from AD in tape stripping material.

Tape samples have been taken from a 61 years old male patient who based on the clinical appearance and symptoms, had been diagnosed with chronic palmoplantar eczema (see Figure 4.16). There were no signs of eczema or psoriasis elsewhere on the body. The patient had no personal history of atopy, family history was positive for allergic asthma and negative for psoriasis. He had a positive patch test and a high total serum IgE level (767 ku/l), without detectable specific IgE. Mycology microscopy and culture were negative for fungal infection. The patient had no clear benefit from immersion psoralen and ultraviolet A (PUVA) therapy nor from oral alitretinoin 30 mg/day. During the disease course lesions gained a different appearance as coarser scales developed with the existence of a few pustules in association with characteristic nail changes suggestive of palmoplantar psoriasis. Results from an initial tape strip sample were analysed and clearly indicated the diagnosis of psoriasis with significantly enhanced levels of both IL36γ and elafin (IL36γ =959.32 pg/µg, elafin = 6495.11 pg/µg, CXCL1 = 15.979 pg/µg, IL8 = 29.22 pg/µg, IL18 = 6.44 pg/µg, S100A8/A9 = 443.830 ng/µg, HBD2 366.349 pg/µg; TARC and TSLP were not detected). Histopathological examination following clinical reassessment confirmed the diagnosis of palmoplantar psoriasis.

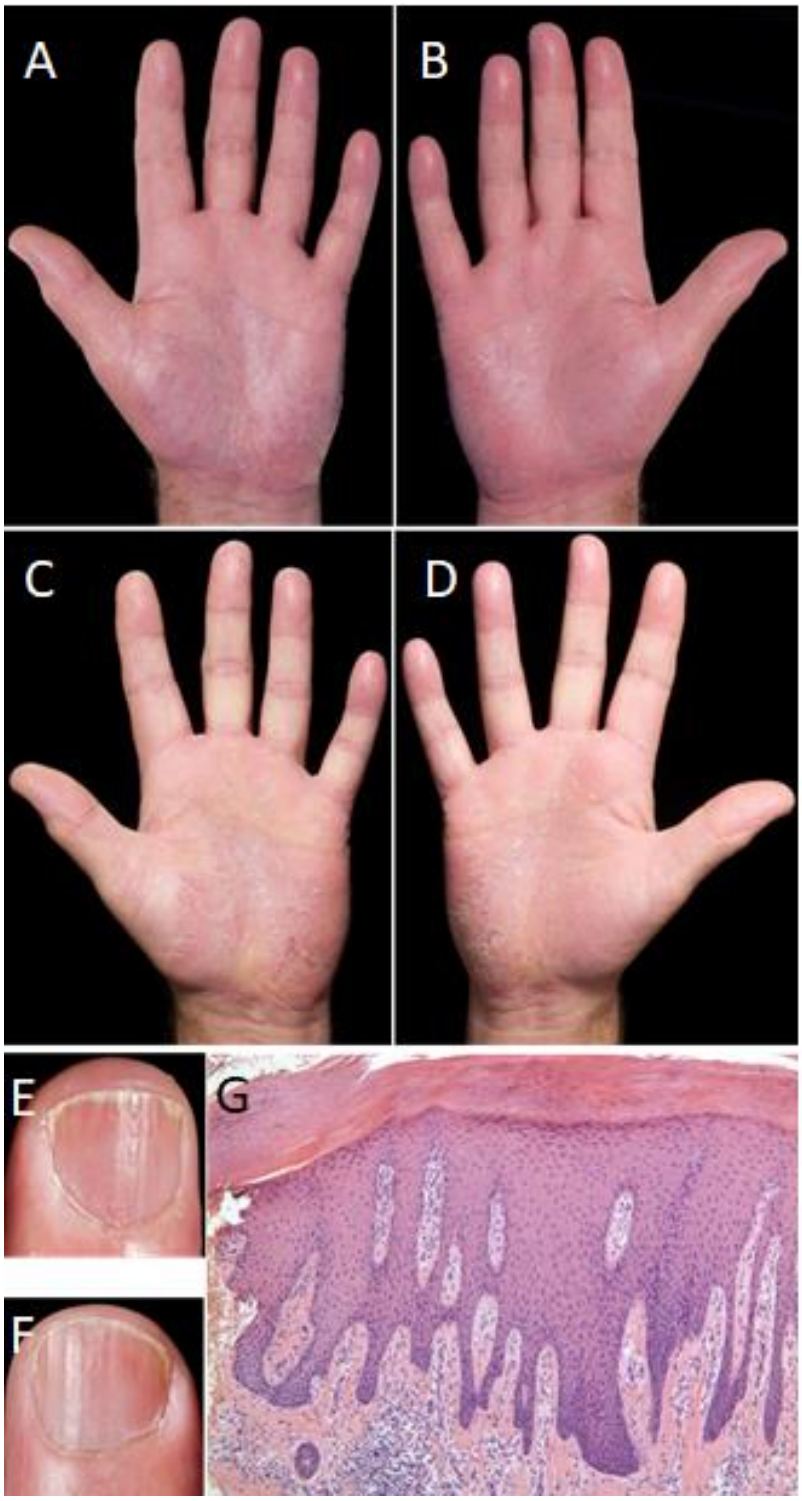

Figure 4.16 Shifting clinical phenotype representing a diagnostic challenge.

Figure 4.16 ***(continued)*** (A), (B) Clinical phenotype characteristic of predominantly vesicular type hand eczema: deeply seated palmar vesicles with multiple erosions and fine, superficial scales at an erythematous, infiltrated base accompanied by intense itch described by the patient. (C), (D) Shift of the clinical phenotype towards palmar psoriasis in the same patient following 3 months: presence of intact palmar pustules, coarse scaling, accentuation of demarcation lines. (E), (F) Nail changes resembling characteristic psoriatic pitting and ridging. (G) Histopathological examination shows regular epidermal hyperplasia, with clubbing and anastomosis of the elongated rete ridges associated with suprapapillary plate thinning. There is mild, patchy, perivascular dermal infiltrate composed of lymphocytes, without eosinophils and mild exocytosis. The granular layer is still present. There is, however, some parakeratosis in which collections of neutrophils are identified. (hematoxylin-eosin stain; high power magnification x400; histopathologic image provided by Dr Sara Edward). Figure adapted from [379].

The following two cases were referred by the rheumatology department, presented with unclassified polyarthralgia, a generic diagnosis of patients suffering from pain in multiple joints. As polyarthralgia has multiple etiologies, the exclusion of PsA was of high importance especially as the patients presented with skin lesions, in order to make a correct treatment decision. Hence, a diagnosis of psoriasis would have indicated PsA, and opened different treatment pathways compared to a diagnosis of eczema. In the first case, the presence of relatively well circumscribed margins and a thick, adherent scale made it impossible to completely rule out psoriasis (see Figure 4.17). However, low expression of IL36γ in tape stripping samples failed to suggest psoriasis (IL36γ = 25.20 pg/µg, GROα = 0.116 pg/µg, IL18 = 0.265 pg/µg, S100A8/A9 = 8.334 ng/µg, CTACK = 1.214 pg/µg, IL8 = 0.252 pg/µg, TARC and TSLP were below detection levels). Subsequent histopathological examination confirmed the diagnosis of chronic eczema. In the second case, establishing the correct clinical diagnosis might have been simple for a well-trained dermatologist as lesions were highly suggestive of a fungal infection (see Figure 4.17). However, for other specialists, the physical observation of erythema and scales might have suggested the possibility of psoriasis, while the presence of erosions and vesicles were suggestive of eczema. However, IL36γ levels were below the cut off level of 214 pg/µg, thus eliminating psoriasis from the diagnostic options based on tape stripping (IL36γ = 120.88 pg/µg, GROα = 0.041 pg/µg, IL18 = 1.451 pg/µg, S100A8/A9 = 55.248 ng/µg, HBD2 = 89.81 pg/µg, CTACK = 0.842 pg/µg, IL8 = 0.252 pg/µg, TARC and TSLP were below detection levels). Subsequent mycological testing confirmed infection with *Trichophyton rubrum*.

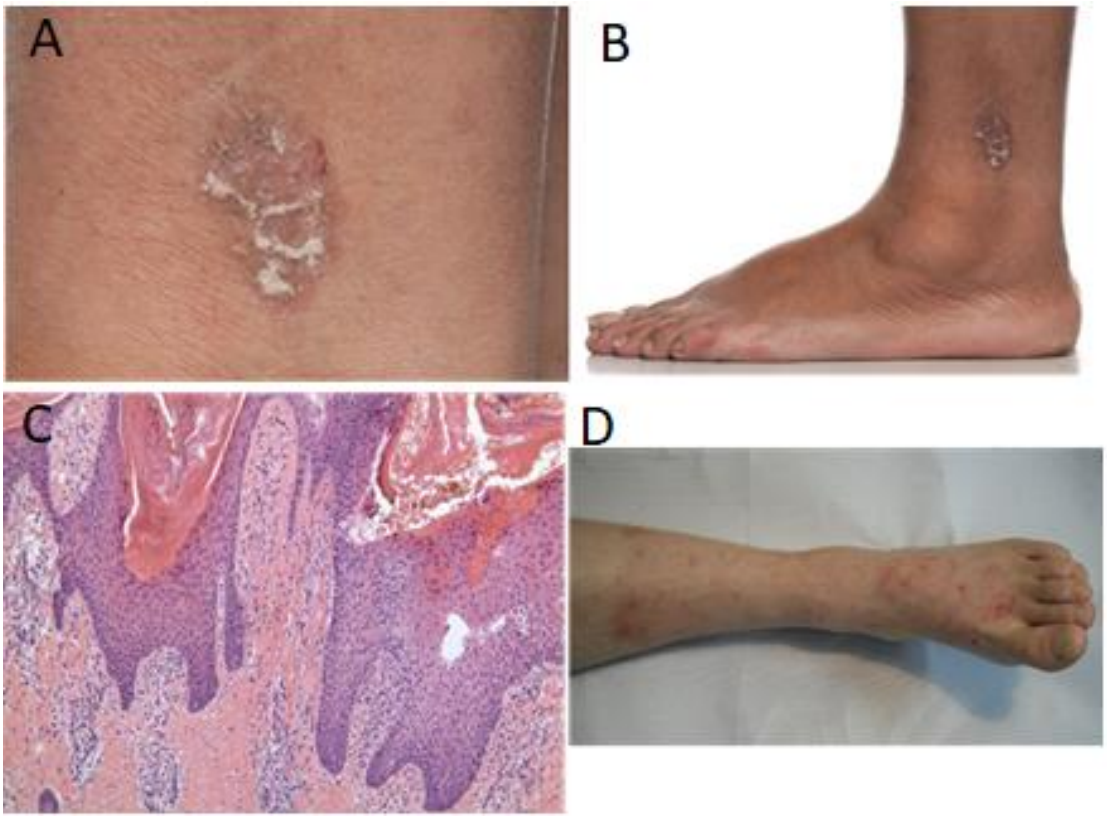

Figure 4.17 Overlapping clinical features of psoriasis and eczema.

(A), (B) Relatively well demarcated, slightly erythematous, intensely itchy lesion cowered by thick, adherent scales with left lateral supramalleolar localisation of a 51 years old female patient. (C) Histopathological examination shows irregular epidermal hyperplasia in association with hyperkeratosis and hypergranulosis. There is patchy epidermal spongiosis with focal lymphocytic exocytosis. There is no club-shaped elongation of the rete ridges and neutrophils are not identified. The features favour of those of a lichenified eczema (hematoxylin-eosin stain; high power magnification x400, histopathologic image provided by Dr Sara Edward). (D) Unilateral lesion measuring several centimetres on the left lower leg with erythematous, slightly raised borders and a clearer centre, a clinical picture characteristic of fungal infection. Slight scaling, vesicles and erosions are also present in association with itch. Figure adapted from [379].

Early recognition and treatment of PsA is crucial in order to prevent further joint damage and to improve disease course. The above cases were representative of the daily diagnostic challenge rheumatologists face in order to establish a correct diagnosis in the presence of minimal skin lesions and atypical presentations.

Tape stripping is a promising, simple method, with reliable disease specific markers for psoriasis, suitable to be used not only by dermatologist specialists but by other health professionals as well in order to facilitate diagnosis and guide the therapeutic pathway.

4.9 Discussion

Both psoriasis and eczema are chronic inflammatory skin diseases which often have considerable impact on the patients' quality of life. Their accurate early diagnosis is of high importance especially in cases where the apropriate diagnosis and treatment may prevent or alter the course of the disease and/or the development of co-morbidities with potentially irreversible alterations, such as seen in PsA.

Although histopathology is considered the gold standard in diagnosing clinically challenging dermatology cases, it is an invasive procedure with potential short- and long-term complications. Moreover, it is costly, requires well trained medical personnel and lengthy histopathological assessment which often delays start of appropriate treatment. Furthermore, histopathologic differentiation of psoriasis from eczema in some anatomical localisations, such as palmoplantar, is difficult [64, 66]. The often similar initial therapeutic response of eczema and psoriasis to topical corticosteroids or UV therapy also contributes to diagnostic difficulties in some cases. Although their treatment with 'conventional' therapies overlap (topical steroids, UV therapy, retinoids, MTX, cyclosporine A), the therapeutic decision regarding dosing regimens and choice of immunosuppressant in order to treat associated comorbidities (PsA) depends on the accurate diagnosis. Furthermore, now when newly developed targeted biologic therapies become more available and relatively affordable, diagnostic precision is more important than ever. Tape stripping proves to be an easy, robust, non-invasive technique to obtain skin samples, with potential application in clinical settings, which can complement or even replace invasive skin biopsies in the future.

Tape stripping has been used previously to identify epidermal mRNA or proteins [196, 201]. CCL17, TSLP and IL8 protein/mRNA have previously been described to be detectable in eczema lesions by means of tape stripping [195, 196, 380, 381]. We initially included RNA analysis as an outcome measure in order to compare our results with those from previous studies utilising RNA profiling. However, we experienced significant RNA degradation in our experimental approach and concluded that tape stripping is not a robust method to quantify epidermal mRNA expression due to potential false negative results. Ribonuclease (RNase) activity and RI (RNase inhibitor) are upregulated during terminal keratinocyte differentiation, but due to degradation RI is absent from the stratum corneum, contributing to the antimicrobial defence of the skin [382, 383]. Consequently, the stratum corneum is abundant in RNase activity. As OCT analysis showed, tape stripping (10 tapes) samples the very top layer of the epidermis and RNA degradation is likely due to high local RNase activity rather

than the result of the extraction process. It has also been shown that RNA isolated from tapes is most likely derived from cells associated with the stratum corneum (e.g. adnexal structures) rather than from corneocytes [384]. In addition, published studies show that correlation of mRNA expression and corresponding protein levels can be poor [385], indicating that protein expression more accurately reflects underlying disease processes. For these reasons an RNA profiling approach was not further pursued.

Both IL36γ and elafin are constitutively expressed by epithelial cells. IL36γ has recently emerged as an important psoriatic mediator, connecting tissue and leukocyte responses [144, 386-388]. Importantly, emerging evidence implicates IL36γ in the induction of IL23 by antigen presenting cells [53, 389, 390] and in angiogenesis in psoriasis [53]. The role of elafin in the pathogenesis of psoriasis is not yet extensively researched. As previously discussed, elafin is a neutrophil serine protease inhibitor, known to play a role in tissue protection against proteases derived from invading neutrophils in psoriasis [229, 239], but also with antimicrobial properties against a number of pathogens, including *P. aeruginosa* and *S. aureus* [391]. Both the expression of IL36γ and elafin has been shown to be greatly elevated upon stimulation with psoriasis-associated cytokines such as IL17 and TNFα [43, 240, 241, 392, 393]. Our results provide evidence that IL36γ and elafin expression is consistently high in psoriatic compared to eczematous inflammation, and they can be used as reliable, specific and sensitive biomarkers to identify psoriasis in epidermal samples. This finding is supported by previous work which demonstrated that the detection of IL36γ by immunohistochemistry allows differentiation of clinically unclear cases of psoriasis from eczema [144], while *in situ* hybridization previously showed that the expression of elafin is less abundant in chronic AD compared to psoriasis [229].

Importantly, our work indicates that the biomarker potential of IL36γ and elafin is so robust it is not affected by the location or the depth of sampling which, despite standardised tape stripping, will likely vary between anatomical sites, disease phenotypes and disease activity. Although OCT imaging demonstrated that tape stripping only samples the cornified layer of the epidermis in healthy skin, this might be different in lesional skin where epidermal differentiation and barrier function are dysregulated as is well described for psoriasis and eczema respectively. More in depth future investigation for different disease phenotypes, lesion severity and lesion chronicity is needed to appreciate resulting differences of sampling with the tape stripping approach.

A plethora of mediators, cellular and surface molecules have been described to be upregulated in psoriatic lesions [1]. In our approach we could confirm the neutrophil chemoattractants IL8 and CXCL1 [374] as well as the chemoattractant

for IL17 producing cells, CCL20, to be elevated in psoriatic as compared to eczema lesions. The same was true for S100A8/9 and the antimicrobial peptide hBD3, which have been reported to be overexpressed in psoriasis. Unlike in eczema, where superinfection of skin lesions is an important concern, the markedly increased production of antimicrobial peptides, such as S100 and hBD family members, by psoriatic keratinocytes protect lesions from external pathogens [182]. We could not, however, detect a significant difference in levels of hBD2 in psoriasis compared to eczema samples by tape stripping as previously indicated by our MS analysis. The discussion regarding how hBD2 expression differs between psoriasis and AD is still ongoing. There is data indicating high expression in acute AD in opposed to chronic lesions, although with higher levels in psoriasis [162], while others only show high expression in psoriasis [370]. Increase in the genomic copy number of beta defensins has been shown to be associated with psoriasis susceptibility [394], although a similar study on larger cohorts concluded that the association is not as strong as previously found [395].

As for AD, it has been reported that total IgE [396, 397], peripheral eosinophil count [123], CCL17 [125] and CCL27 levels [128, 372] correlate with eczema severity. Among these, CCL17 serum levels proved to be the best biomarker for AD regarding both severity and therapeutic response [398]. CCL27, identified to play a role in the pathogenesis of AD, worked well as a diagnostic marker in skin biopsy derived RNA [399, 400]. Contrary to work showing reduced expression of CCL27 and its receptor in psoriasis [173] and the possible role of CCL27 as a biomarker for AD [174, 401], we did not find significant differences in the amount of this chemokine when sampling the outermost skin layers. A similar finding has been reported in the serum of patients with AD and psoriasis [371]. Previously it has been shown that CCL27 plays a crucial role in the recruitment of T-cells in both inflammatory conditions [402]. Comparable presence of the chemokine suggests active recruitment of skin-homing T cells and thus points to inflammatory activity in both conditions. The differences between our findings and that of some others may be due to the method and site of sampling.

Although more research need to be conducted, it seems that hair-plucking could be a suitable alternative for tape stripping to examine the underlying inflammatory processes in pathologies involving the scalp.

The presented results demonstrate that IL36γ and elafin are reliable, specific, sensitive and robust biomarkers of psoriatic inflammation in tape stripping, allowing to differentiate psoriatic from atopic inflammation not only in typical, but also in atypical cases, where the otherwise characteristic clinical features overlap. Their combined, non-invasive detection in tape stripping samples could potentially replace invasive biopsies in the future. Both mediators are also highly

informative on the disease ethiopathogenesis. Other markers such as IL8 or CXCL1 could play a role in “molecular endotyping” and in the development of an objective disease classifier for psoriasis.

Chapter 5 Tape stripping biomarkers in psoriatic disease - follow up

5.1 Introduction

Following the identification of the excellent biomarker potential of IL36γ and elafin to distinguish psoriatic from eczematous inflammation, the next step was to study the dynamics of these markers in response to treatment. The question to answer was whether the most commonly used traditional psoriatic treatments would influence elafin and IL36γ levels, potentially limiting their diagnostic use in treated patients, and whether they could predict therapeutic response to these treatments. Therefore a pilot study was conducted to study the effect of common antipsoriatic drugs on the detection levels of IL36γ and elafin following the induction of an *in vitro* psoriatic phenotype with IL17 and TNFα. Experiments were conducted initially on human keratinocyte cell line HaCaT, then on primary human keratinocytes. Stimulation of cells was carried out before the administration of treatment in order to reflect clinical reality. The tested drugs included hydrocortisone and betamethasone (topical steroids), calcipotriol (vitamin D analogue), acitretin (retinoid), fumaric acid, cyclosporine A, MTX (immunosuppressive drugs), apremilast (phosphodiesterase type-4 (PDE4) inhibitor, oral biologic) and tofacitinib (janus kinase (JAK) inhibitor) at different concentrations.

5.2 The stability of different biomarkers related to treatment

5.2.1 Establishing the experimental set up

To induce a “psoriatic phenotype”, HaCaT cells were initially incubated with TNFα and IL17. TNFα and IL17 were selected not only for their known role in the pathogenesis of psoriasis but also as both have been shown to upregulate IL36γ [403] and elafin production [240, 241].

The first experiments indicated that cells should be stimulated for 24h in order to see substantial differences between the modulation of these inflammatory markers, therefore in all subsequent experiments there was a 24h stimulation period, followed by treatment and incubation with the specified drugs for a further 24h (see methodology chapter).

In agreement with the literature, elafin production was substantially induced by the combination of IL17 and TNFα. Furthermore, it became evident from the data

of the first experiments that topical steroids applied in low concentrations, hydrocortisone (0.15 µg/ml) and betamethasone (10^{-8} M), can enhance elafin production (see Figure 5.1). This is supported by the literature, which indicates that the anti-inflammatory/anti-proliferative effect of topical steroids is indeed dose dependent, in low doses topical steroids being able to further increase cell proliferation [404]. Therefore, in following experiments the doses of hydrocortisone for the treatment phase was increased. From the tested drugs, cyclosporine A and the combined use of cyclosporine A and MTX were found to have the strongest impact on the reduction of elafin levels produced by HaCaTs following stimulation (see Figure 5.2), while low dose acitretin, tofacitinib and fumaric acid further increased elafin production (see Figure 5.3).

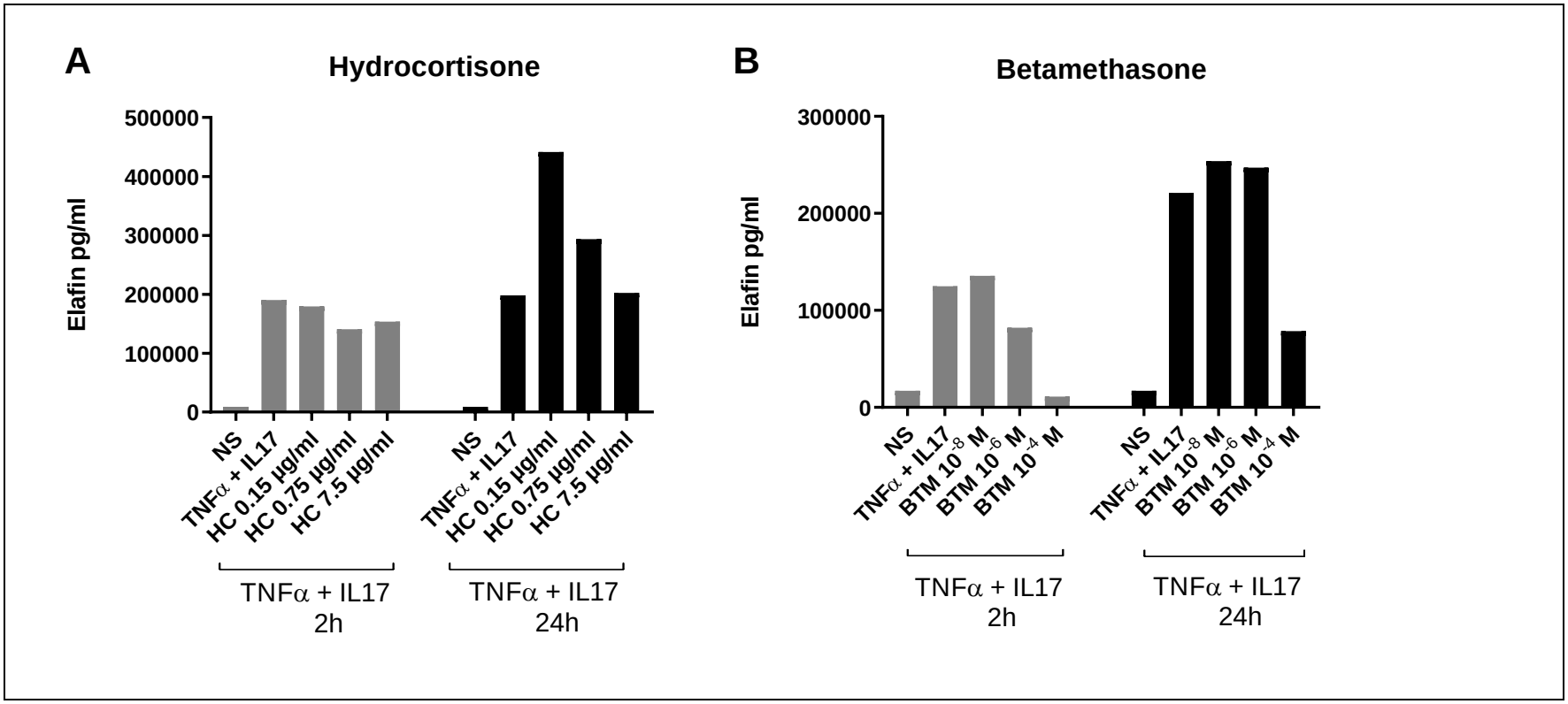

Figure 5.1 Dose response histogram of elafin secretion by HaCaT cells following 2h and 24h stimulation with pro-inflammatory cytokines (IL17, TNFα) and subsequent treatment with topical steroids (A) hydrocortisone and (B) betamethasone for 24h.

Elafin production is substantially increased following stimulation of HaCaT cells with a psoriatic cocktail of IL17 (20 ng/ml) and TNFα (50 ng/ml), both following a 2h and 24h incubation period. However, the modulation in elafin levels is more obvious following the longer incubation period. Both low dose hydrocortisone (0.15 µg/ml) and betamethasone (10^{-8} M) enhances the production of elafin. Elafin measurements are represented as absolute values of a single preliminary experiment. NS – nonstimulated; HC – hydrocortisone; BTM – betamethasone.

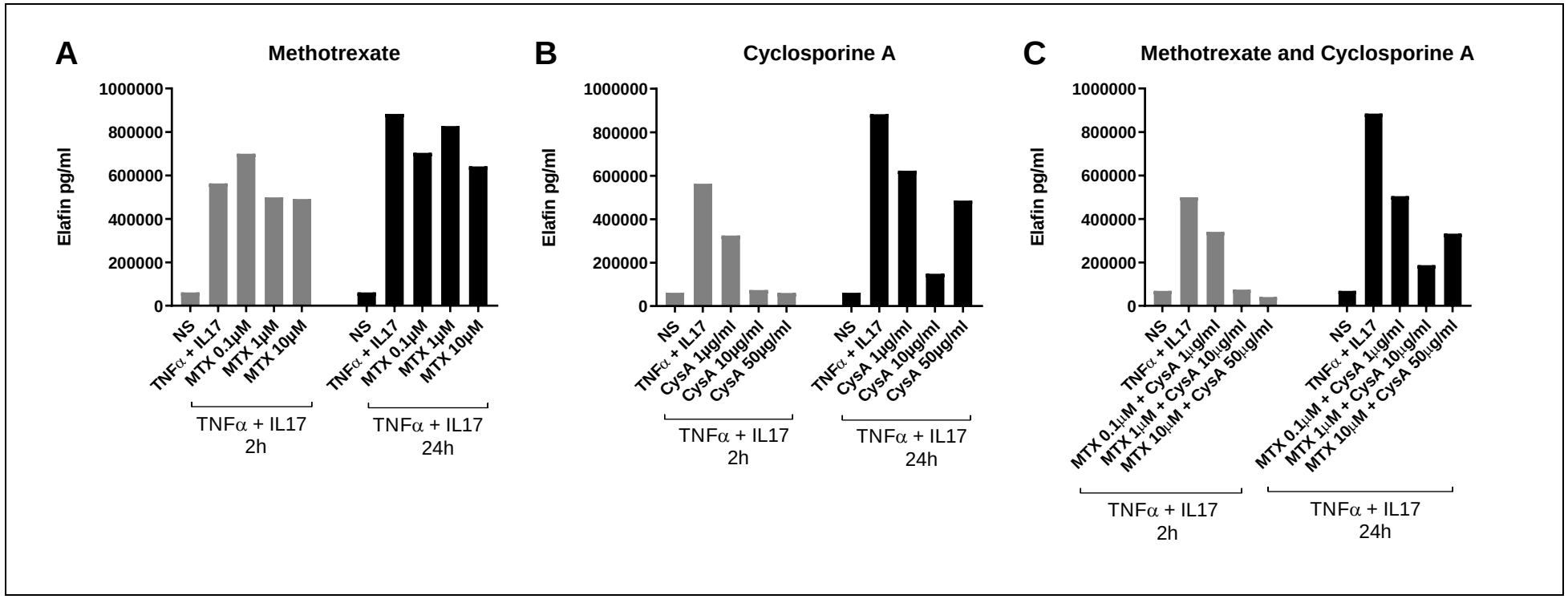

Figure 5.2 Dose response histogram of elafin secretion by HaCaT cells following 2h and 24h stimulation with pro-inflammatory cytokines (IL17, TNFα) and subsequent treatment for 24h with (A) methotrexate, (B) cyclosporine A, and (C) the combination of methotrexate and cyclosporine A.

Cyclosporine A (10 μg/ml) and the combination of MTX (1 μM) with cyclosporine A (10 μg/ml) have the strongest inhibitory effect on the production of elafin by HaCaTs stimulated with pro-psoriatic cytokines IL17 and TNFα. NS – nonstimulated; MTX – methotrexate; CysA – cyclosporine A.

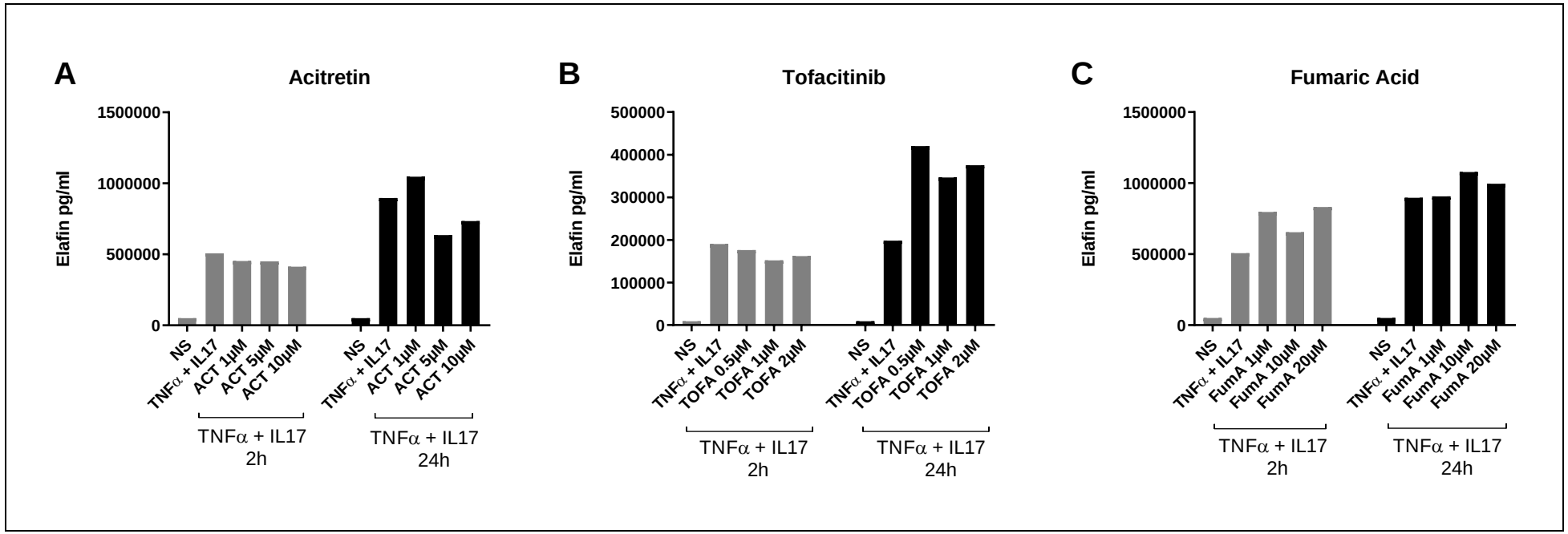

Figure 5.3 Dose response histogram of elafin secretion by HaCaT cells following 2h and 24h stimulation with pro-inflammatory cytokines (IL17, TNFα) and subsequent treatment for 24h with (A) acitretin, (B) tofacitinib, and (C) dimethyl fumarate (fumaric acid).

Low dose acitretin (1 μM) as well as tofacitinib and fumaric acid enhance elafin production in HaCaT cells, following the induction of the psoriatic phenotype with IL17 and TNFα. NS – nonstimulated; ACT – acitretin; TOFA – tofacitinib; FumA – fumaric acid.

5.2.2 The modulation of elafin and IL36γ production in HaCaT keratinocytes with psoriatic phenotype following different psoriatic treatments

The above mentioned first experiments, where HaCaT cells were stimulated for 24 hours with a psoriatic cocktail composed of TNFα (50 ng/ml) and IL17 (20 ng/ml), followed by a 24 hour incubation period with the most commonly used psoriatic treatments in dermatology at different concentrations, were repeated three times in total in order to exclude experimental bias (different growth rate of HaCaTs, pipetting error).

Initially only cell supernatants were collected in order to analyse the dynamics of elafin and IL36γ secretion by HaCaT cells following different treatments. However, IL36γ protein was only detectable at very low levels by ELISA in the cell supernatants. Therefore, cell lysates were also collected beginning with the third set of experiments. The lack of IL36γ in cell supernatants confirm data from the literature, that special signalling mechanisms are needed for its release from the cells, but what leads to its secretion and activation it is still poorly understood [405]. Recent results from our group indicate that one of the release mechanisms involve pathogen induced cellular damage [406].

As cell lysates were only collected for the third set of experiment for most drugs, a separate histogram was created for the third experiment, depicting the dynamics of the expression of elafin and IL36γ in both cell supernatants and lysates in relation to each other and to the positive control (stimulated with IL17 and TNFα, without treatment).

5.2.2.1 Topical steroids and vitamin D3 analogue

Topical treatment with steroids and vitamin D3 analogues, as well as the combination of these two treatments is one of the most frequently used in dermatology for the management of psoriasis, either alone in case of limited-to-mild disease or in association with a systemic drug in case of moderate-to-severe condition. The most frequently combined topical steroid and vitamin D3 analogue, available as one product, is betamethasone dipropionate (a potent topical steroid) and calcipotriol. In contrast to betamethasone dipropionate, hydrocortisone is the least potent topical steroid, usually prescribed for the treatment of facial lesions or for the paediatric population. Due to their frequent use in dermatology, these drugs (hydrocortisone, betamethasone, calcipotriol and the combination of betamethasone and calcipotriol) were selected at three different concentrations for the treatment of HaCaTs following the induction of a psoriatic phenotype. No significant effect was observed for either mediator following treatment with **hydrocortisone** (see Figure 5.4). By itself, neither **betamethasone** (see Figure 5.5) nor **calcipotriol** (see Figure 5.6) had significant effect on IL36γ or elafin levels compared to the positive control. However, when the highest concentrations of both drugs were used in combination, there was a significant decrease in the total amount of IL36γ expression in the cell supernatant and lysate (see Figure 5.7).

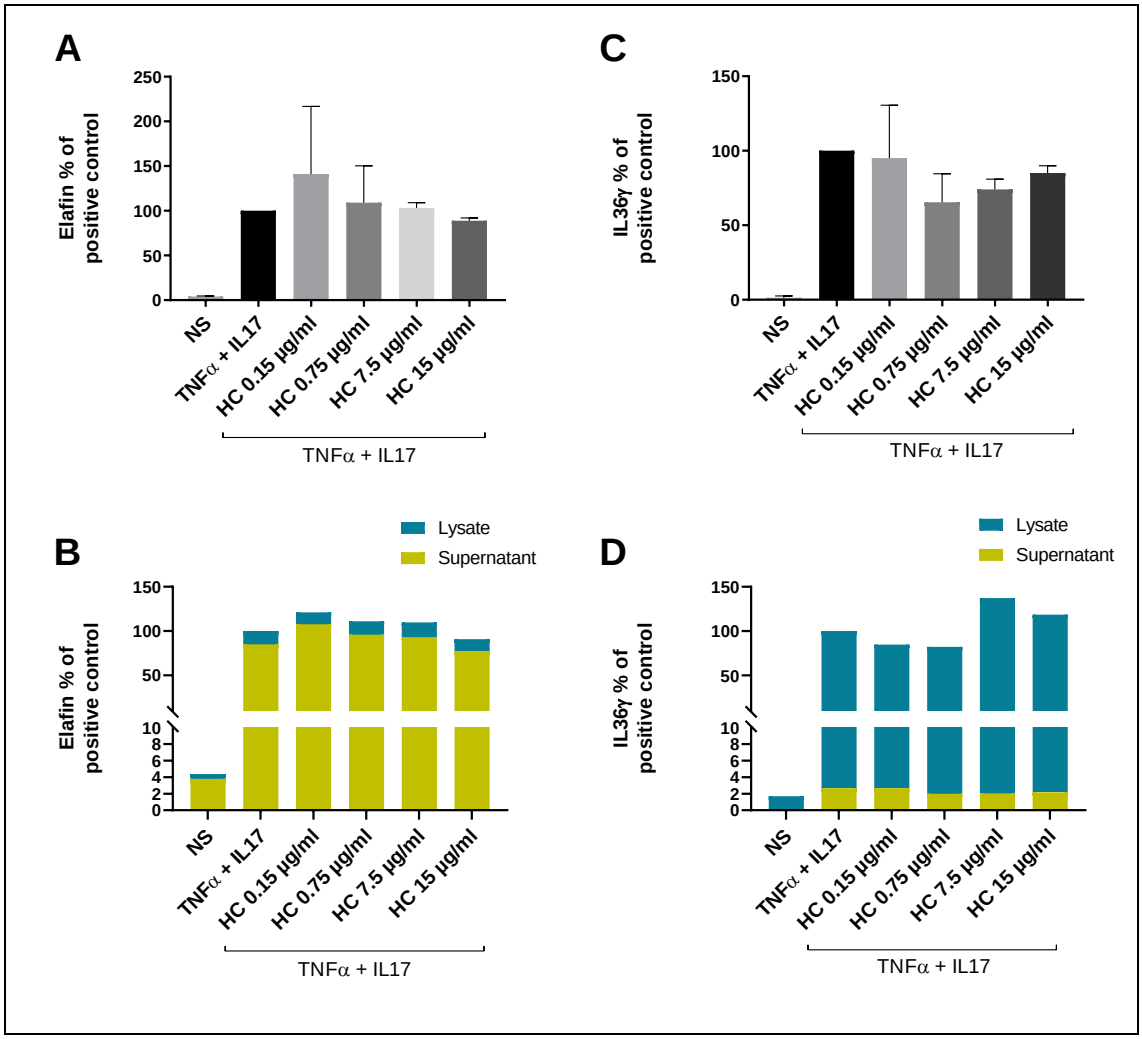

Figure 5.4 Dose response of HaCaT cells to hydrocortisone following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. *significance level $p<0.05$. HC - hydrocortisone; L - lysate; NS - nonstimulated; SN - supernatant.

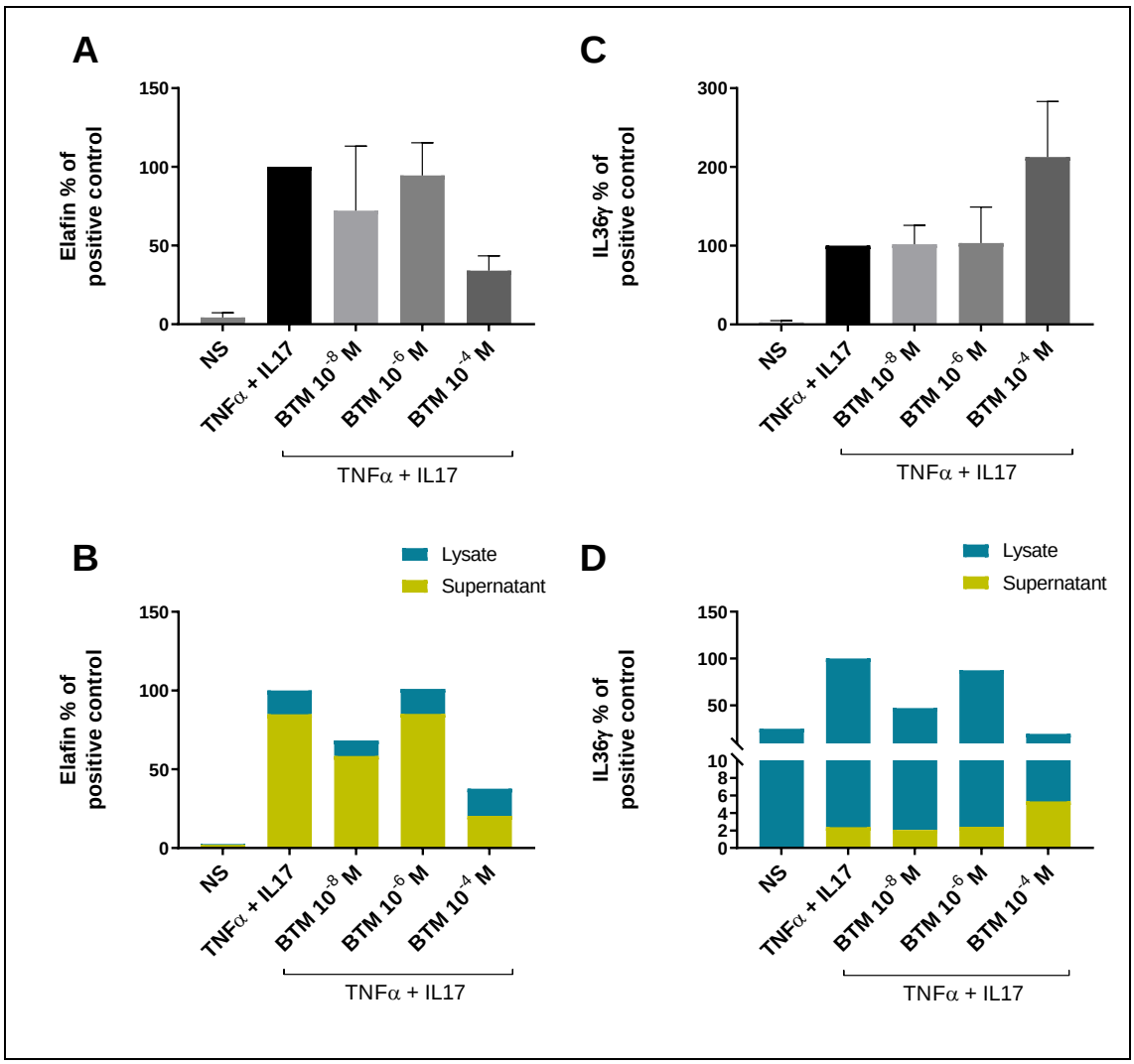

Figure 5.5 Dose response of HaCaT cells to betamethasone following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. BTM - betamethasone; L - lysate; NS - nonstimulated; SN - supernatant.

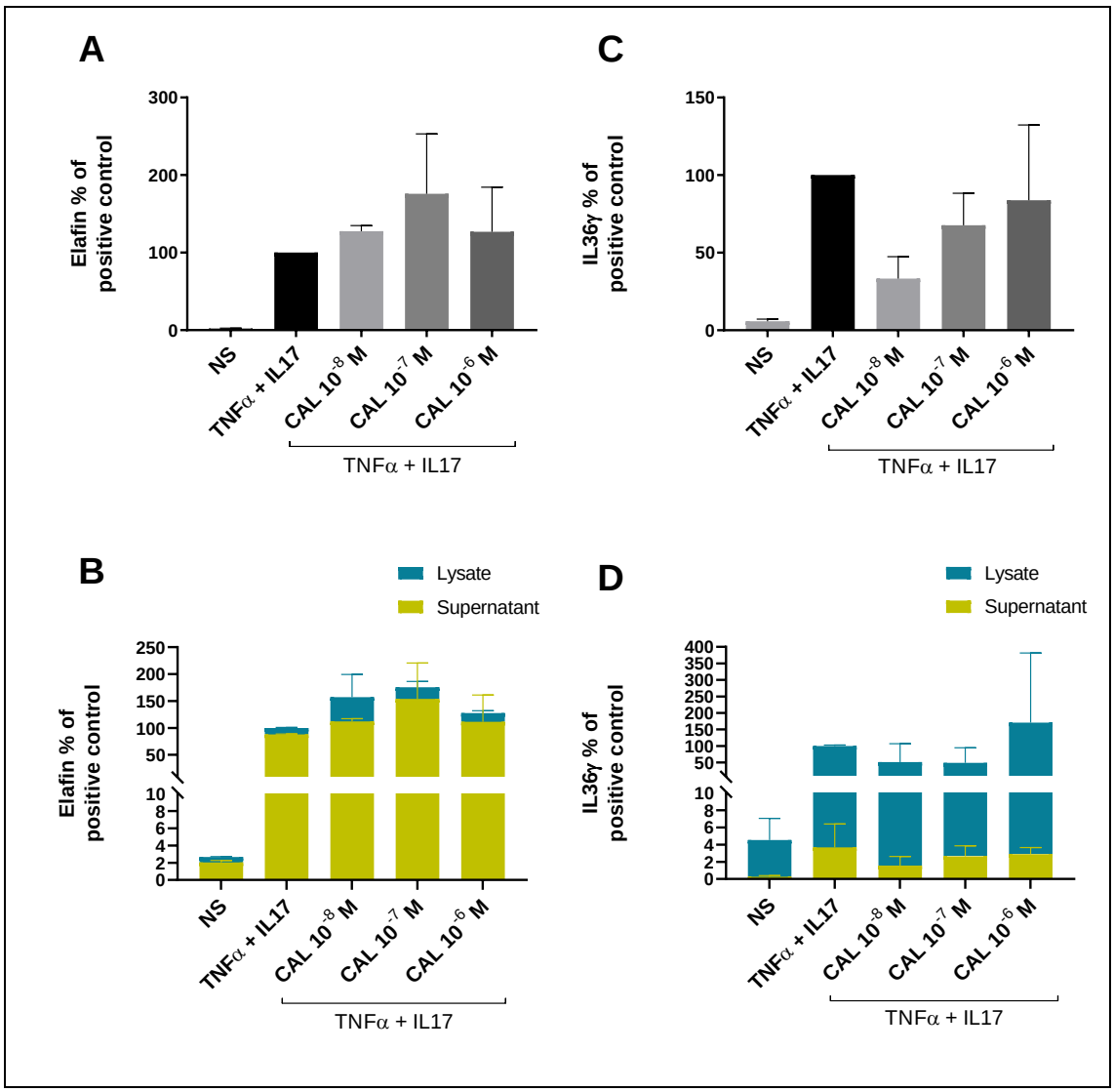

Figure 5.6 Dose response of HaCaT cells to calcipotriol following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of three independent experiments, in HaCaT cell supernatants *((sample SN/positive control SN)*100)* (A), (C) and in both supernatants and lysates *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)* (B), (D). Statistical analysis was performed using the absolute values. CAL – calcipotriol; L - lysate; NS - nonstimulated; SN - supernatant.

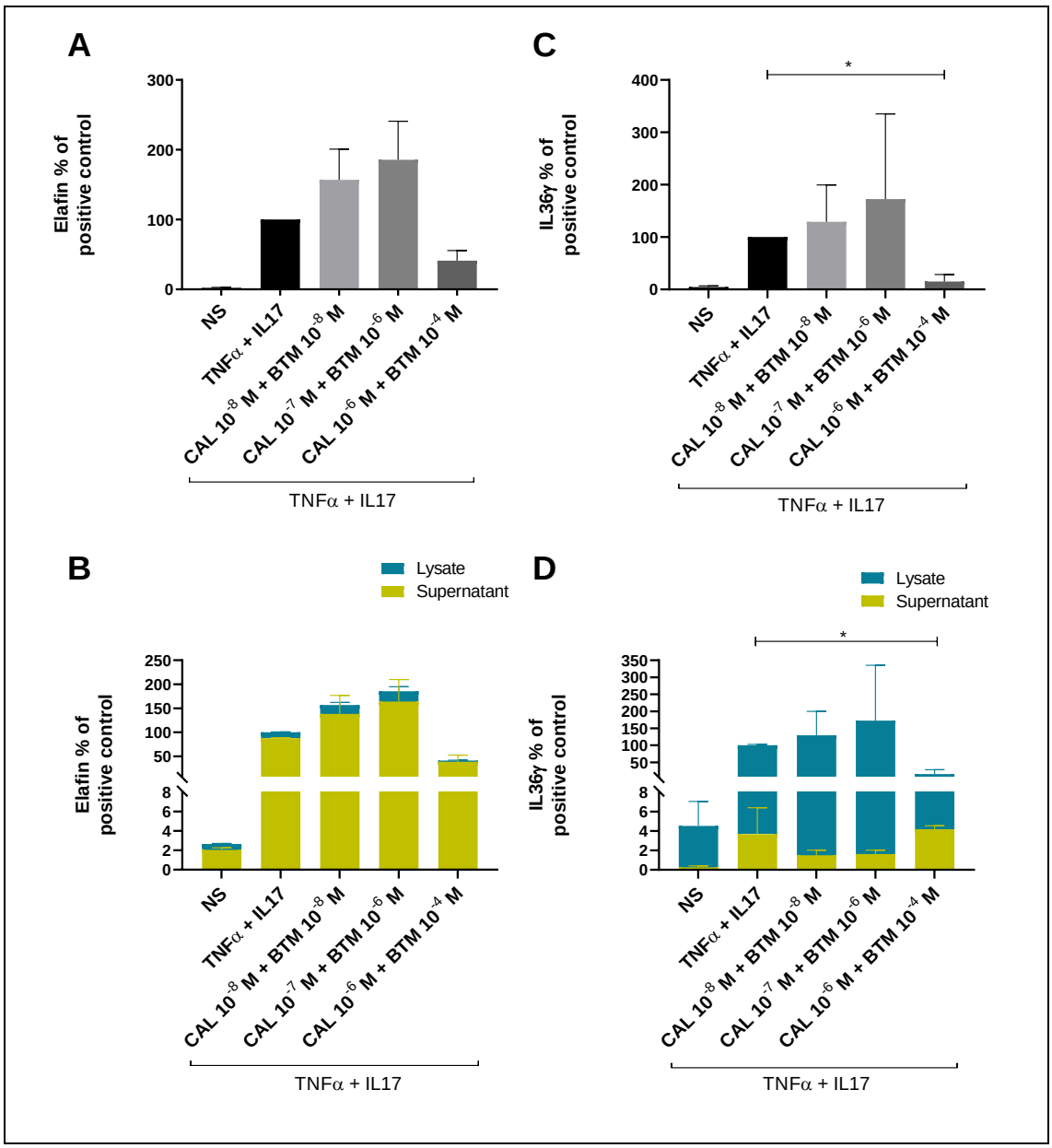

Figure 5.7 Dose response of HaCaT cells to the combination of calcipotriol with betamethasone following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) expression in HaCaT cell supernatants and lysates in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of three independent experiments, as the sum of mediator levels in HaCaT cell supernatants and lysates *((sample SN+L/positive control SN+L)*100)* (A), (C) and as a combined view of their expression in both supernatants and lysates *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)* (B), (D). Statistical analysis was performed using the absolute values. *significance level $p<0.05$. BTM - betamethasone dipropionate; CAL – calcipotriol; L - lysate; NS - nonstimulated; SN - supernatant.

5.2.2.2 Conventional systemic therapies (cyclosporine A, methotrexate, acitretin, fumaric acid)

According to current treatment guidelines, patients with moderate-to-severe psoriasis need to fail at least two conventional systemic treatment options, amongst them cyclosporine A and MTX, before becoming eligible for biologic therapy. Therefore most psoriatic patients with severe disease would receive these agents during the course of their disease. Other treatments for psoriasis are acitretin and fumaric acid esters. Phototherapy, another frequently used treatment, amongst other mechanisms induces apoptosis of both keratinocytes and lymphocytes and modulates cytokine expression (with an increase in IL10 and decrease in IL17/IL23) [407]. Acitretin is known to inhibit keratinocyte proliferation while also promoting differentiation. In keratinocytes it acts via the CRABP, the epidermal growth factor (EGF) receptor, and retinoic acid nuclear receptors [408]. Over 500 genes have been proposed to be modulated by acitretin [409], therefore it was plausible that even if indirectly, it could potentially regulate IL36γ expression. Fumaric acid esters are considered as first line treatment for moderate-to-severe psoriasis in Germany. It potentially interferes with the IL17A pathway, therefore it could affect IL36γ production of keratinocytes [410].

Cyclosporine A treatment, at concentrations of 10 µg/ml and 50 µg/ml, significantly reduced elafin levels compared to the positive control. On the other hand IL36γ secretion into the cell supernatant showed a tendency to increase with higher doses of cyclosporine A (see Figure 5.8). The three different doses of **MTX** (0.1 µM, 1 µM and 10 µM) had no significant effect on either mediators (see Figure 5.9).

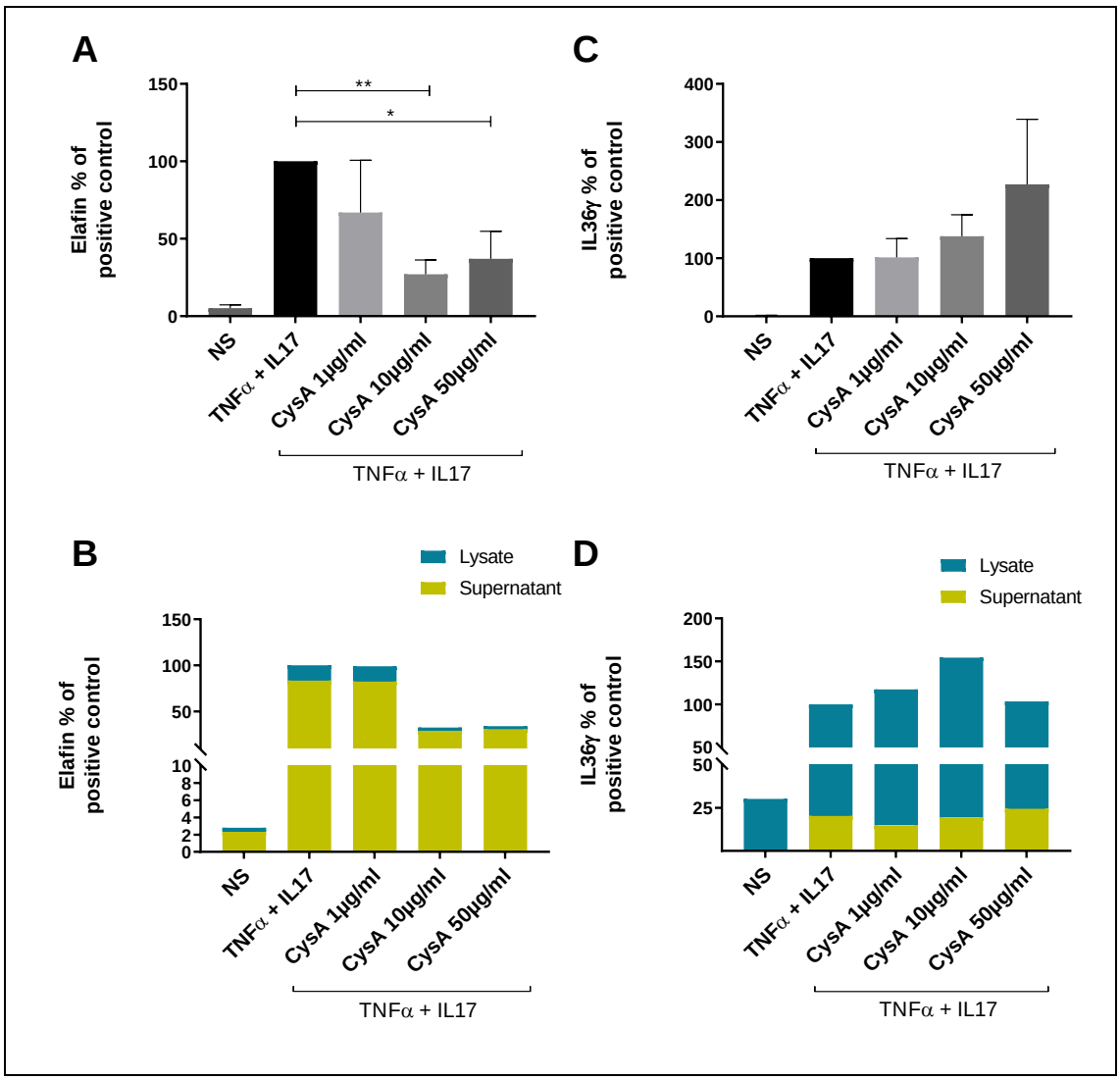

Figure 5.8 Dose response of HaCaT cells to cyclosporine A following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control, representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/ positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. *significance level $p<0.05$. CysA - cyclosporine A; L - lysate; NS - nonstimulated; SN - supernatant.

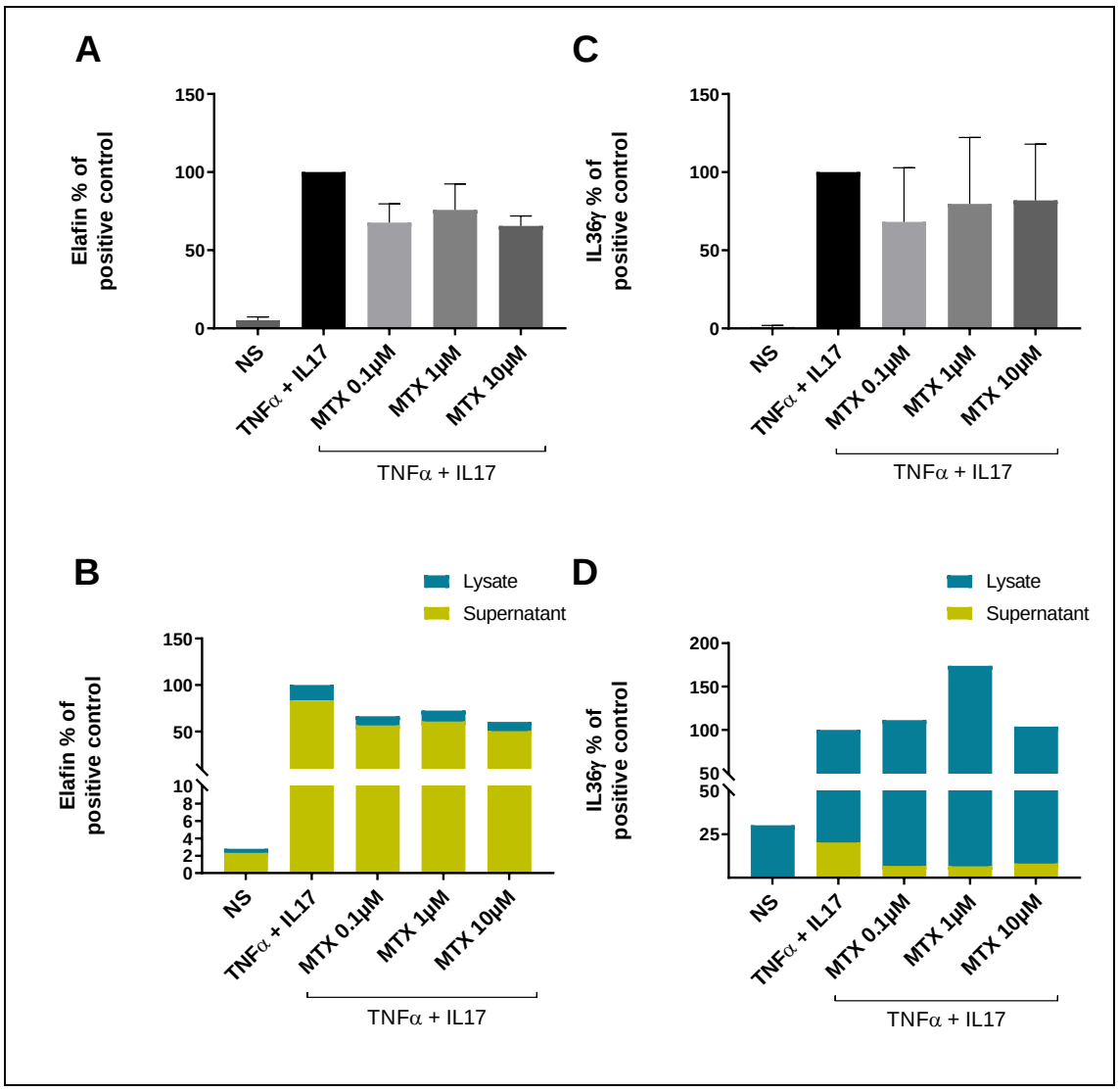

Figure 5.9 Dose response of HaCaT cells to methotrexate following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control, representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/ positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. *significance level $p<0.05$. L - lysate; NS - nonstimulated; MTX - methotrexate; SN - supernatant.

nUVB (310 to 311 nm) is often the first treatment option, especially in psoriatic patients with multiple comorbidities, due to its favourable side effect profile. In our experimental setup no significant difference was seen for IL36γ or elafin following 20mJ/cm^2 of nUVB treatment in TNFα and IL17 stimulated HaCaTs (see Figure 5.10).

Acitretin is often combined with nUVB therapy (also called retinoid PUVA - RePUVA) or administered as a monotherapy as a first line treatment option in plaque psoriasis, being the treatment of choice in pustular psoriasis. All three doses of acitretin showed a trend to reduce elafin detected in the cell supernatant after psoriatic stimulation. No effect was seen for IL36γ (see Figure 5.11).

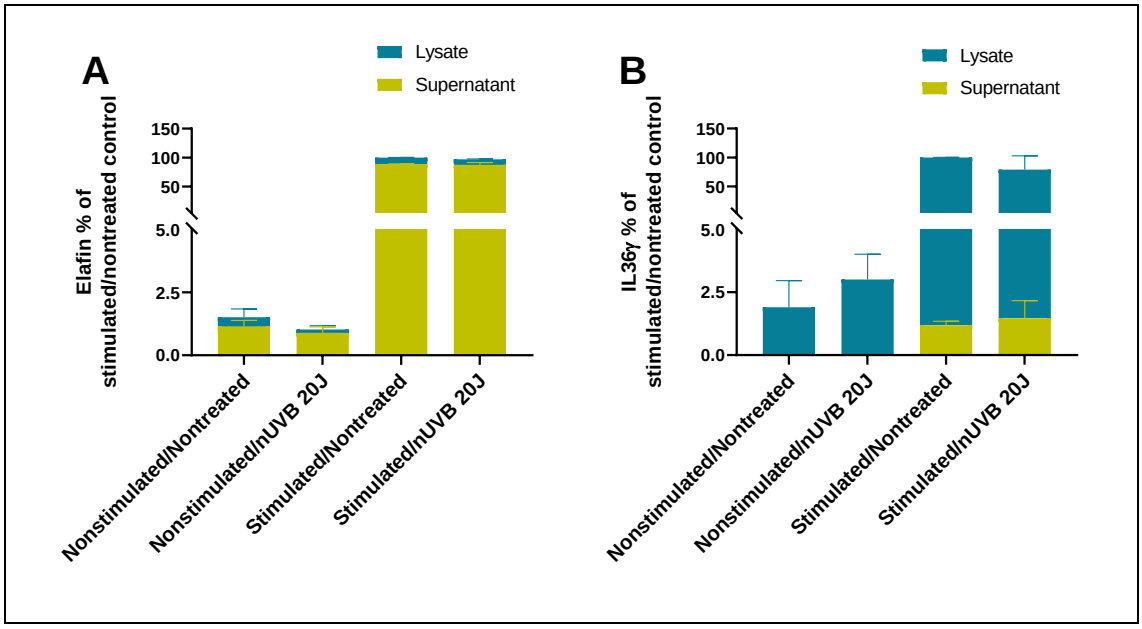

Figure 5.10 Dose response of HaCaT cells to nUVB following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A) and IL36γ (B) production in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of two independent assays, with readout of three wells for each assay, in HaCaT cell supernatants and lysates *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. L - lysate; NS - nonstimulated; nUVB – narrowband ultraviolet b; SN – supernatant.

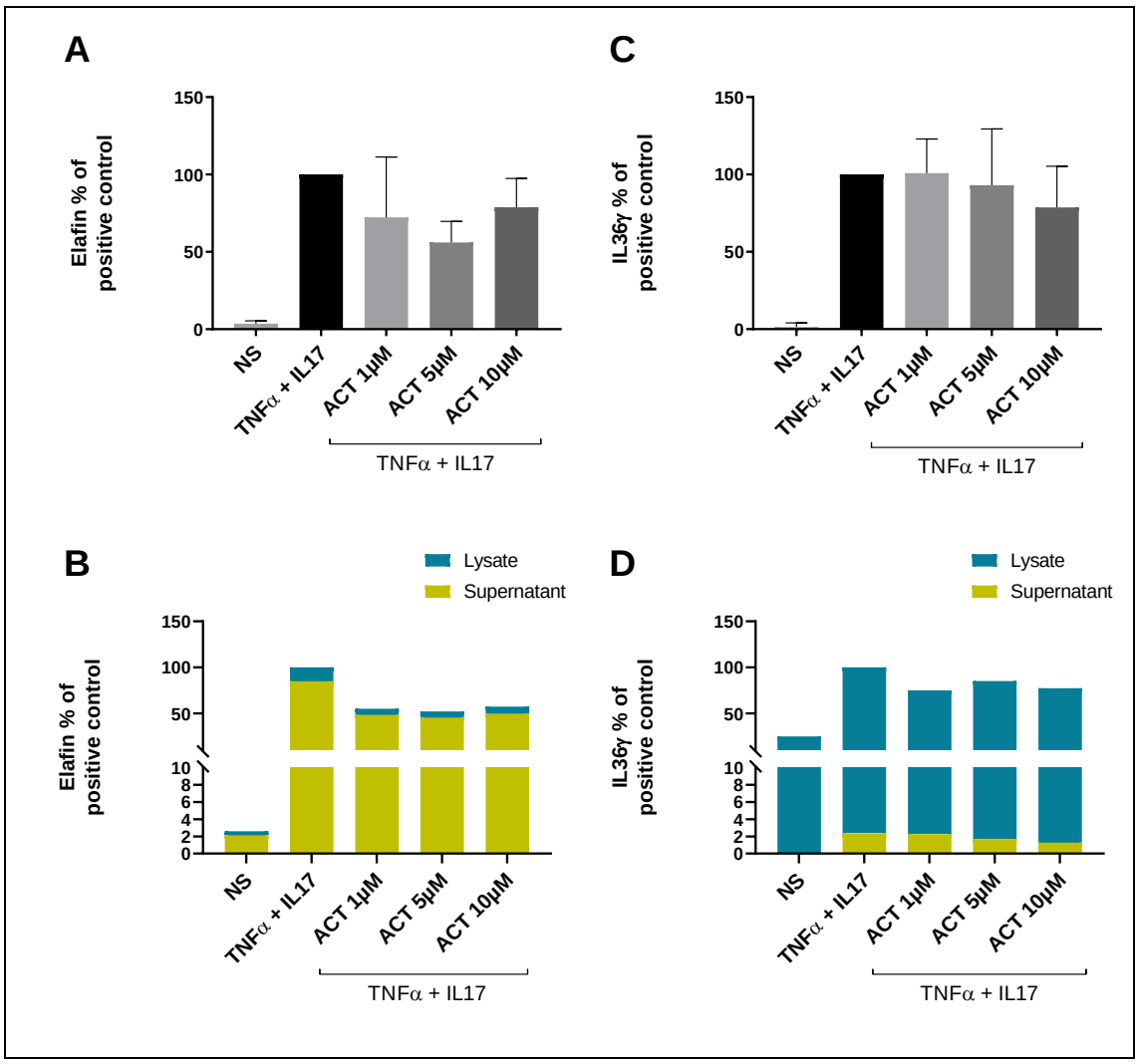

Figure 5.11 Dose response of HaCaT cells to acitretin following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control, representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/ positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. ACT – acitretin; L - lysate; NS - nonstimulated; SN – supernatant.

Fumaric acid, another nonbiologic treatment option for psoriasis, had no effect on IL36γ or elafin release in stimulated HaCaTs (see Figure 5.12).

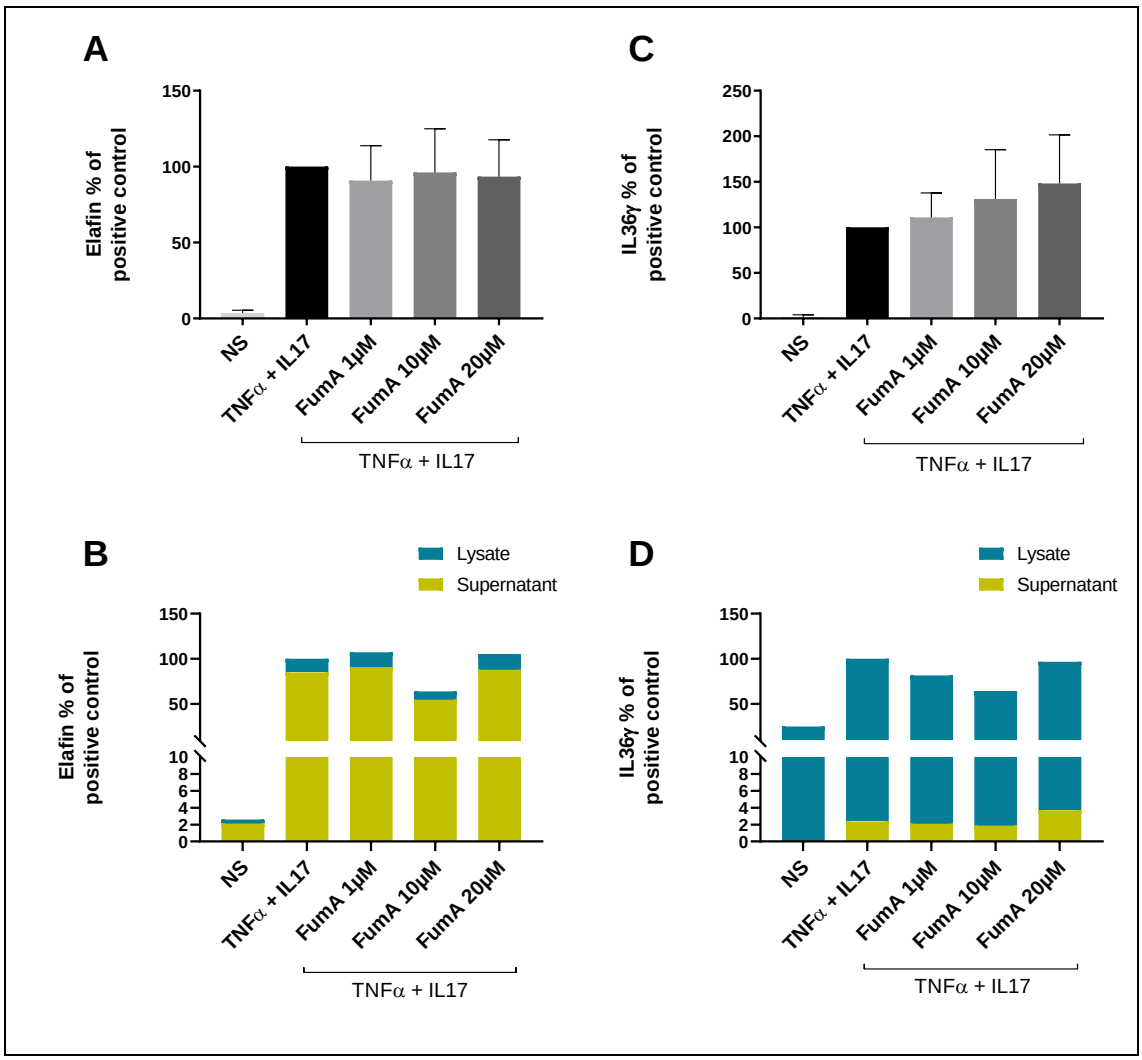

Figure 5.12 Dose response of HaCaT cells to fumaric acid following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) production in relation to the positive control, representing 100%. (A) Elafin and (C) IL36γ levels measured in the cell supernatants are presented as means ± SD of three independent experiments *((sample SN/ positive control SN)*100)*, while the dynamic change of (B) elafin and (D) IL36γ levels in the cell supernatants relative to the cell lysates are presented as percentage change in a single experiment *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. FumA - fumaric acid; L - lysate; NS - nonstimulated; SN – supernatant.

5.2.2.3 New oral treatments for psoriasis (tofacitinib, apremilast)

Both apremilast and tofacitinib are relatively new drugs. **Tofacitinib** is a JAK1/JAK3 inhibitor interfering with the JAK-STAT signalling pathway, hence with DNA transcription [411]. It is not approved for the treatment of psoriasis in the UK, although it has been approved for the treatment of PsA. **Apremilast** is a PDE4 inhibitor, suppressing the synthesis of pro-inflammatory mediators (TNFα, IL23) while facilitating those with anti-inflammatory effects (IL10) [412]. It has not yet been established if it has an effect on IL36 production.

Neither tofacitinib nor apremilast had an effect on elafin or IL36γ secretion by HaCaT cells (results not shown).

5.2.3 The modulation of elafin and IL36γ production in keratinocytes with psoriatic phenotype following selected psoriatic treatments

Based on the outcomes of the experiments on HaCaT cells, the following drugs which resulted in significant decrease in either elafin or IL36γ expression were selected to be tested on primary human keratinocytes: topical steroid betamethasone; vitamin D3 analogue calcipotriol; and the combination of these two treatments; MTX and cyclosporine A. The experimental set up was the same as in the case of HaCaT cells. Keratinocytes derived from healthy individuals were first stimulated with TNFα (50 ng/ml) and IL17 (20 ng/ml) for 24h in order to induce a psoriatic phenotype. At the end of the stimulation period, cells were treated with the selected drugs at three different concentrations for a further 24h. Following incubation, both the cell supernatant and lysate were collected, as previously described in the methodology chapter, for the detection of elafin and IL36γ cytokines with ELISA. Each experiment was repeated four times.

Just as in the case of HaCaT cells, elafin was mainly detectable in primary keratinocyte supernatants, while IL36γ was abundant in keratinocyte lysates with minimal detection levels in the cell supernatants.

Primary keratinocytes stimulated with TNFα and IL17 showed no significant response change following treatment with **betamethasone** or **calcipotriol** administered individually. But the combination of the two drugs applied at their lowest concentrations (10^{-8} M) significantly reduced total IL36γ levels, with further significant reduction seen with the highest drug concentrations of betamethasone (10^{-6} M) combined with calcipotriol (10^{-4} M) in both total IL36γ and elafin levels (see Figure 5.13).

These results are corresponding to those obtained with HaCaT keratinocytes.

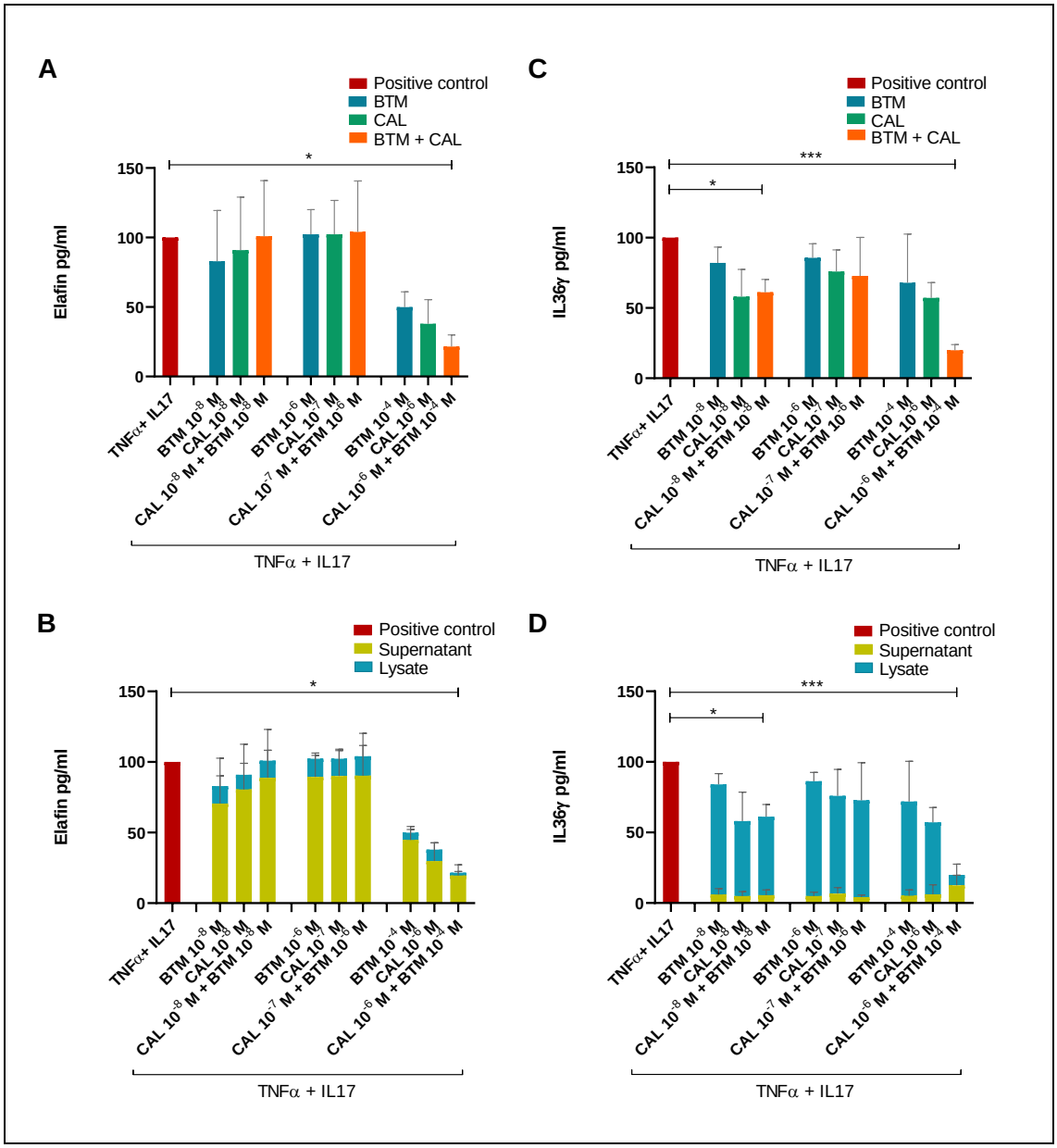

Figure 5.13 Combined view of dose response of primary keratinocytes to betamethasone, calcipotriol and the combination of betamethasone with calcipotriol following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) expression in keratinocyte supernatants and lysates in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of four independent experiments, as the sum of mediator levels in keratinocyte supernatants and lysates *((sample SN+L/positive control SN+L)*100)* (A), (C) and as a combined view of their expression in both supernatants and lysates *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)* (B), (D). Statistical analysis was performed using the absolute values. *significance level $p<0.05$; ***significance level $p<0.001$. BTM - betamethasone dipropionate; CAL – calcipotriol; L - lysate; NS - nonstimulated; SN - supernatant.

Cyclosporine A treatment at its highest concentration (50 µg/ml) significantly decreased IL36γ levels in primary keratinocytes previously stimulated with TNFα and IL17, but showed no significant effect on elafin expression (see Figure 5.14).

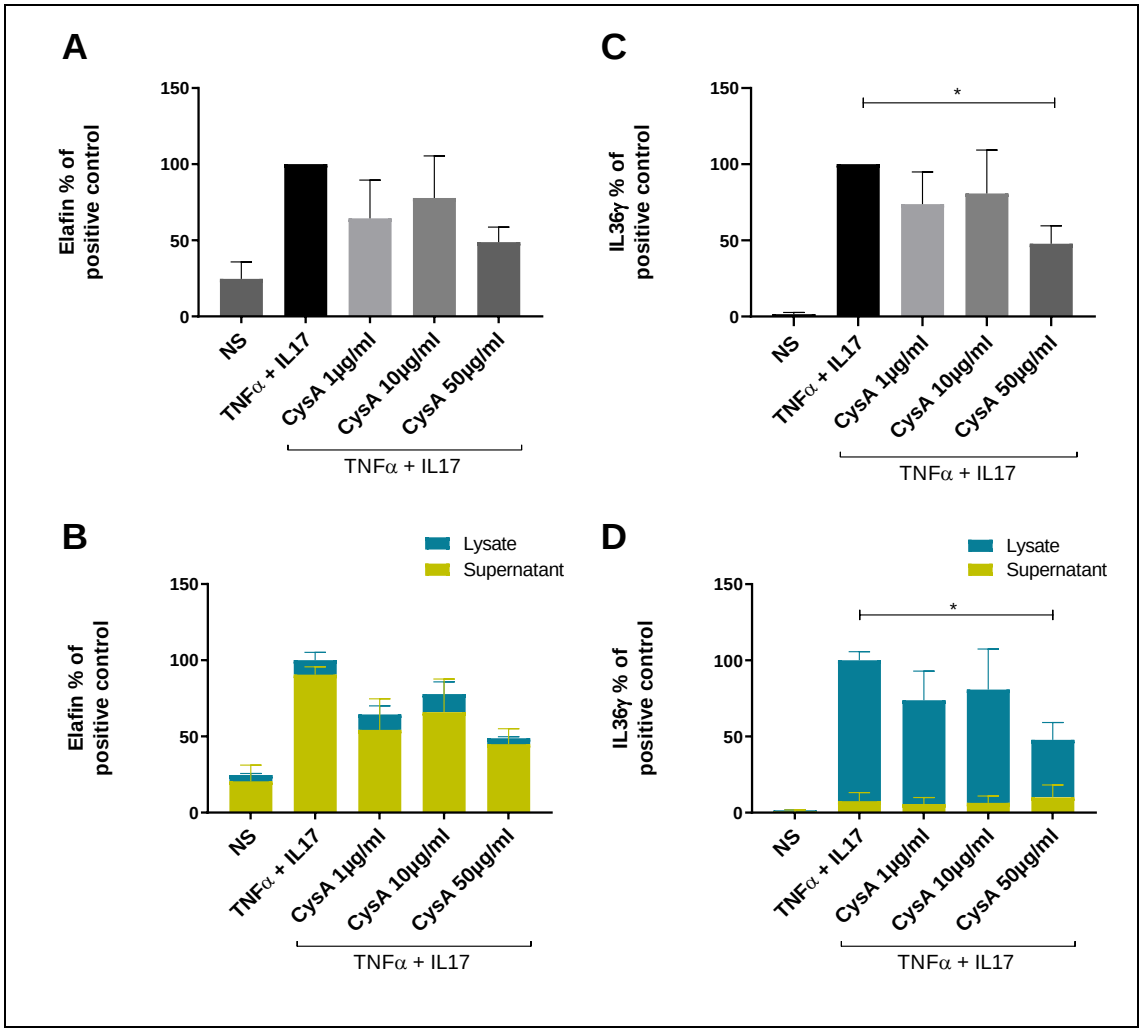

Figure 5.14 Dose response of primary keratinocytes to cyclosporin A following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) expression in keratinocyte supernatants and lysates in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of four independent experiments, as the sum of cytokine levels in keratinocyte supernatants and lysates *((sample SN+L/positive control SN+L)*100)* (A), (C) and as a combined view of their expression in both supernatants and lysates (B), (D) *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)*. Statistical analysis was performed using the absolute values. *significance level $p<0.05$. CysA - cyclosporine A; L - lysate; NS - nonstimulated; SN - supernatant.

No significant changes were detected for elafin or IL36γ in primary human keratinocytes following treatment with **MTX** (see Figure 5.15).

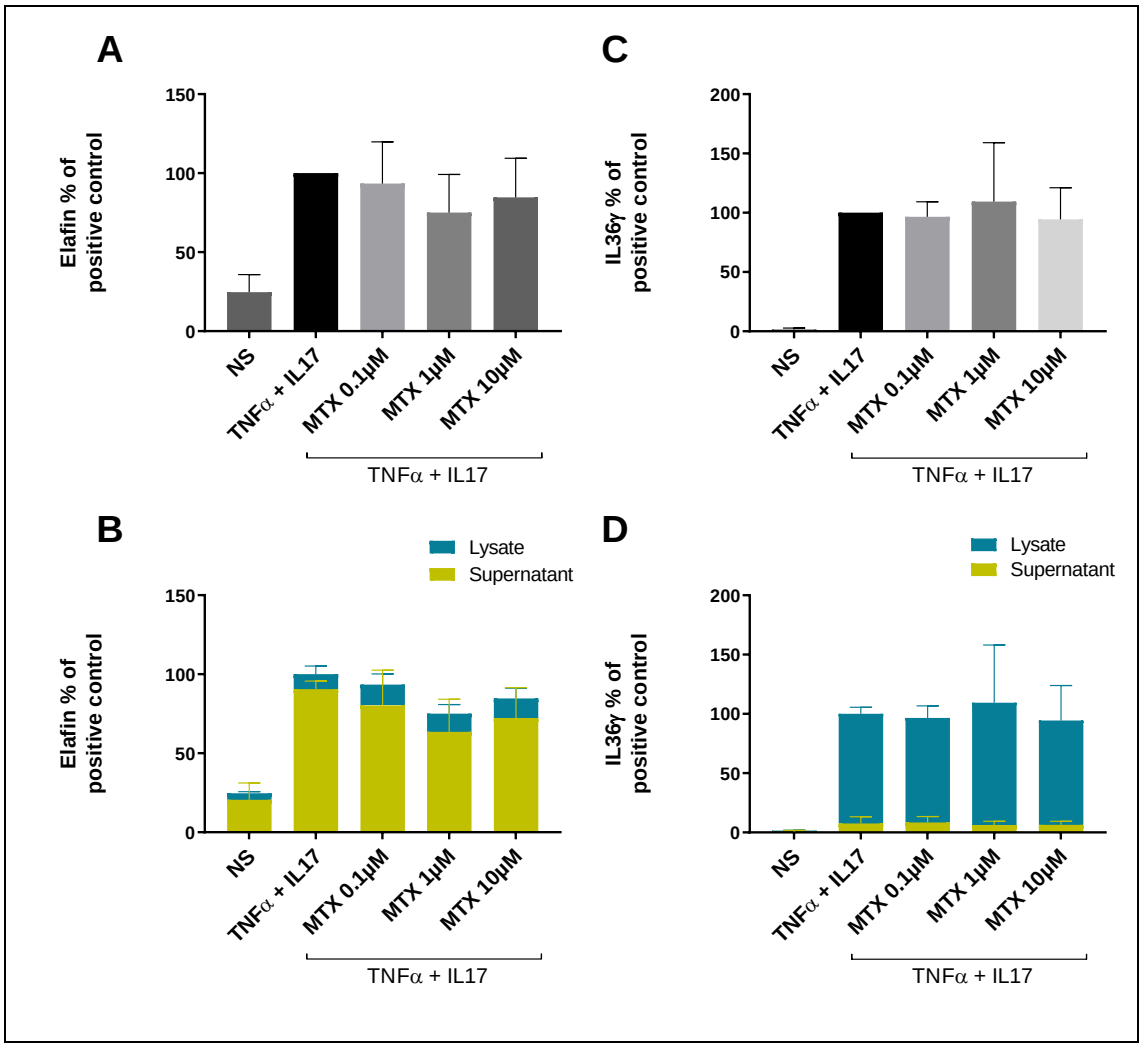

Figure 5.15 Dose response of primary keratinocytes to methotrexate following stimulation with TNFα and IL17.

The data are represented as the percentage change in elafin (A), (B) and IL36γ (C), (D) expression in keratinocyte supernatants and lysates in relation to the positive control, representing 100%. Elafin and IL36γ expression are presented as means ± SD of four independent experiments, as the sum of mediator levels in keratinocyte supernatants and lysates *((sample SN+L/positive control SN+L)*100)* (A), (C) and as a combined view of their expression in both supernatants and lysates *((sample SN/positive control SN+L)*100); ((sample L/positive control SN+L)*100)* (B), (D). Statistical analysis was performed using the absolute values. L - lysate; MTX - methotrexate; NS - nonstimulated; SN - supernatant.

5.3 Cell viability

LDH (Thermo Fisher Scientific) and ToxiLight (Lonza, Nottingham, UK) cytotoxicity assays were used in order to define drug induced toxicity of HaCaTs and primary keratinocytes, as described in the methodology chapter.

As the LDH activity in both supernatants and lysates was low, further measurements aiming to identify factors which could contribute to the low LDH activity were carried out.

As for cell lyses RIPA buffer was used and not the LDH lysis buffer provided in the kit, an additional experiment was performed in order to see if RIPA buffer could interfere with the assay in detecting LDH. Equal amounts of cells were lysed by RIPA buffer as well as by the LDH lysis buffer (250.000cells). No difference has been detected in the efficacy of the assay.

The effect of multiple thawing and freezing cycles on LDH activity was also tested by repeatedly exposing fresh samples to 3 consecutive freeze/thaw cycle. However, this did not interfere with LDH activity.

Finally, testing if long term storage at -80°C could decrease LDH activity was carried out, as both supernatants and cell lysates were stored at -80°C. Freshly produced HaCaT cell lysates were stored for 1 week at -80°C. Significant reduction in the LDH activity was visually noticeable compared to the same samples' LDH activity a week previously. Therefore, the LDH cytotoxicity assay was not suitable to evaluate cell toxicity in our samples following long term storage at -80°C, as also suggested by the literature, due to decrease in LDH activity [413].

Therefore, the ToxiLight bioassay was used to quantify the cytotoxic effect of selected drugs on primary keratinocytes, where samples were already stored at -80°C. The percentage of the AK content released into the cell supernatant was calculated relative to the total AK content of each individual sample (measured in the cell lysate and supernatant) and not to the negative (nontreated/nonstimulated) control, in order to avoid any bias deriving from difference in cell proliferation. One way ANOVA with multiple comparison test was used to identify significant differences between the negative control and nonstimulated but treated groups. As dimethyl sulfoxide (DMSO) was used as a diluent, the toxicity of the highest concentration used in the experiments was also tested, but showed no significant effect.

Significant release of AK compared to the DMSO control resulted from the combination of betamethasone (10^{-4} M) with calcipotriol (10^{-6} M) (by 35.53 ± 3.27%) (see Figure 5.16).

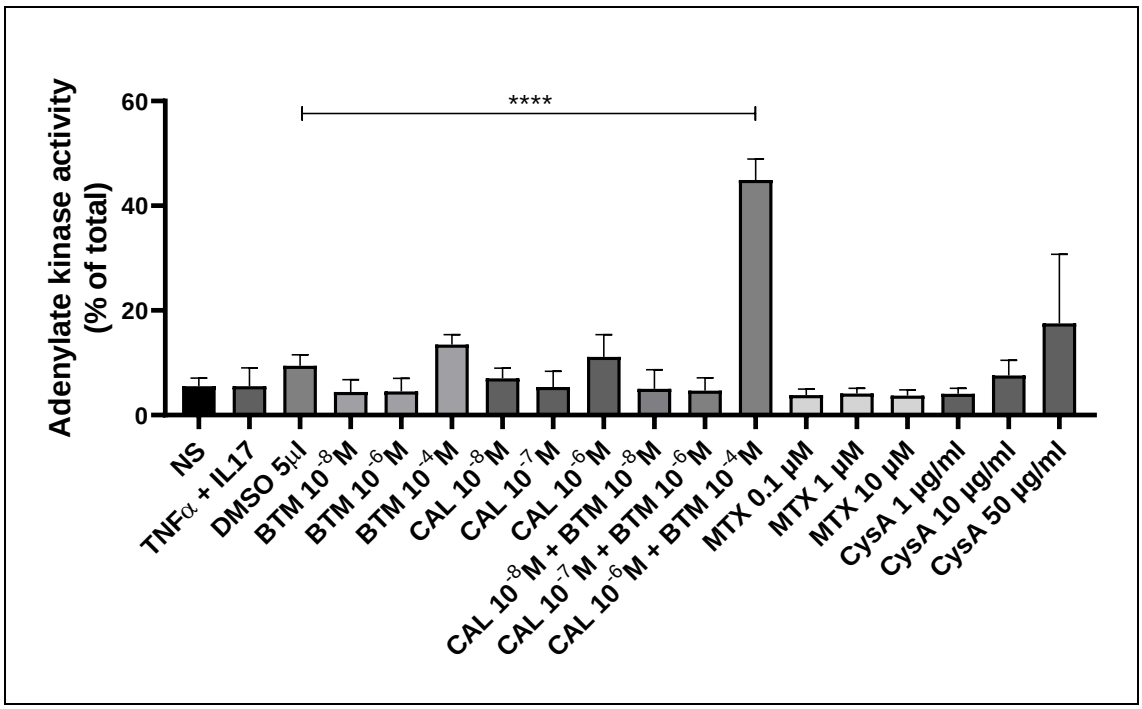

Figure 5.16 Release of cytosolic adenylate kinase from primary keratinocytes into the culture supernatant after 24h incubation with various psoriatic treatments.

AK activity in the keratinocyte supernatant is represented as the percentage (means ± SD) relative to the total amount of AK measured in the cell supernatant and lysate of each individual sample *((SN/SN+L)*100))*, of three independent experiments. NS was used as negative control, except for wells containing the highest concentration of DMSO: betamethasone (10^{-4} M), betamethasone (10^{-4} M) combined with calcipotriol (10^{-6} M) and cyclosporine (50 µg/ml). ****significance level p<0.0001. AK - adenylate kinase; BTM - betamethasone dipropionate; CAL - calcipotriol; CysA - cyclosporine A; DMSO - dimethyl sulfoxide; L - lysate; MTX - methotrexate; NS - nonstimulated; SN - supernatant.

In order to confirm the cytotoxicity of drugs also with the LDH assay, the experiment was repeated with primary keratinocytes, using the two highest concentrations of each treatments. As the only interest was to see if the applied drug is toxic or not, therefore the stimulation step with TNFα and IL17 was not applied. Following a 24h incubation period with the drugs of interest, both supernatants and lysates were collected with direct measurement of their LDH content. As this was a single experiment, no statistical analysis could been carried out. Compared to the negative control LDH activity in keratinocyte supernatants was increased by 4% following treatment with betamethasone at 10^{-4} M, by 11% following treatment with calcipotriol at 10^{-6} M, and by 32% following the combined treatment with calcipotriol at 10^{-6} M and betamethasone at 10^{-4} M. None of the other treatments increased the activity of LDH compared to the control (see Figure 5.17).

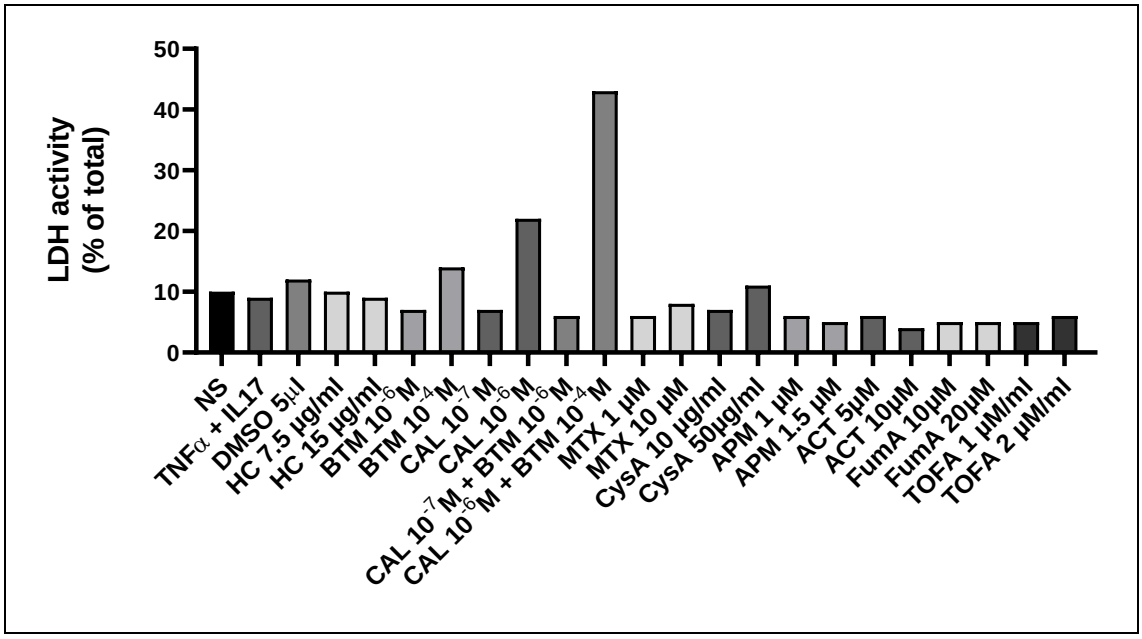

Figure 5.17 Release of cytosolic LDH from primary keratinocytes into the culture supernatant after 24h incubation with various psoriatic treatments.

LDH activity in the keratinocyte supernatant is represented as the percentage relative to the total amount of LDH measured in the cell supernatant and lysate of each individual sample *((SN/SN+L)*100))*. 10% of the total LDH were released into the cell supernatant of the NS control, while this was 12% with DMSO (5 µl),14% with betamethasone at 10^{-4} M, 22% with calcipotriol at 10^{-6} M, and 43% with calcipotriol at 10^{-6} M combined with betamethasone at 10^{-4} M. None of the other treatments had higher LDH release than the NS control. ACT - acitretin; APM - apremilast; BTM - betamethasone dipropionate; CAL - calcipotriol; CysA - cyclosporine A; DMSO - dimethyl sulfoxide; FumA - fumaric acid; HC - hydrocortisone; L - lysate; LDH - lactate dehydrogenase; MTX - methotrexate; NS - nonstimulated; SN – supernatant; TOFA - tofacitinib.

5.4 Discussion

In order to assess the full potential of elafin and IL36γ as psoriasis disease biomarkers, their expression and dynamic changes in response to the most frequently used psoriatic treatments was tested in HaCaT and primary human keratinocyte experiments. The question to answer was whether they could still act as biomarkers in patients who are already on active treatment and if they could potentially predict therapeutic response to certain treatments.

Epithelial IL36 gene expression requires p38 - mitogen-activated protein kinase (MAPK)/c-Fos, nuclear factor-kappa B (NF-κB) and phosphoinositide 3-kinases (PI3K) signaling, and is regulated by MAPK phosphatase (MKP1) [414]. The complete mechanistic understanding of the regulation of elafin expression in skin epithelia is still lacking. In fact it has been shown that it must involve regulation at multiple levels as it cannot be derived simply from keratinocyte transfection studies [415]. It is clear however, that similarly to IL36 gene expression, the p38-MAPK pathway is involved [416], while the role of the NF-kB pathway has been demonstrated in lung epithelia [417]. Drugs which interact with any of these

signalling pathways can potentially modulate IL36γ and elafin production in keratinocytes. However, the effect of “traditional” immunosuppressant drugs, such as MTX and cyclosporine A on keratinocytes is not well researched and the available data is often conflicting.

Although individually neither betamethasone nor calcipotriol had modulatory effects, their combined use at the highest concentrations significantly reduced both elafin and IL36γ levels in keratinocyte experiments. This result is especially important as the commercially available combination of these two drugs is one of the most frequently prescribed topical treatment for moderate-to-severe disease. However, the cytotoxicity assays performed indicated that this reduction is probably due to drug induced cellular toxicity. However, one could argue that the cytotoxic effect seen equals the therapeutic effect of these drugs, and it is the result of the combination of induced cell apoptosis and terminal differentiation of keratinocytes. Topical steroids exert their effect on inflammatory skin diseases, such as psoriasis, via their vasoconstrictor, anti-inflammatory and anti-proliferative properties. It is known that the anti-proliferative effect of topical steroids is dose dependent. At low concentrations, topical steroids tend to increase proliferation, and this may explain the trend for increased elafin and IL36γ levels following the application of low dose hydrocortisone. Betamethasone dipropionate has been shown to have the best antiproliferative effect compared to other topical steroids used in dermatology, arresting the cell cycle at the G2-phase and causing cell death via apoptosis [404]. Topical steroids also act via the glucocorticoid receptor (GR) which following translocation into the nucleus increases expression of anti-inflammatory cytokines (e.g. IL10, IL1RA) while inhibiting the expression of pro-inflammatory genes. Evidence suggests that the corticoid-GR complex also transrepresses the NF-kB pathway. Glucocorticoids also interfere with the MAPK signalling pathway by inhibiting c-Jun N-terminal kinase (JNK) via the MKP1 regulatory protein [418]. As both IL36γ and elafin expression and regulation occurs through these signalling pathways, we expected a reduction in their levels following betamethasone treatment. Higher doses might be necessary to interfere with their expression in keratinocytes. Calcipotriol regulates cell proliferation and differentiation via an intranuclear vitamin D receptor which acts as a transcription factor [419]. Calcipotriol also seems to have an inhibitory effect on the NF-kB pathway [420], which might be the pathomechanism through which it downregulates S1007/S10015 production by keratinocytes. It has been also suggested that calcipotriol inhibits the expression of IL17A receptor via the regulation of NF-kB [421]. As calcipotriol in higher concentrations can exert an irritative effect on the skin, which is seen in approximately 20% of patients and often results in treatment discontinuation

[422], calcipotriol and betamethasone are preferentially used together not only due to their potent combined therapeutic effect in reducing inflammation but also in order to decrease the chance of the development of an irritative dermatitis [423].

As nUVB, acitretin, fumaric acid, apremilast and tofacitinib did not show significant modulation in elafin and IL36γ levels in HaCaT cell experiments, no further experiments with these were carried out on primary human keratinocytes.

In primary keratinocytes, MTX did not influence elafin or IL36γ expression levels. MTX is an anti-folate drug used as the first line treatment for moderate-to-severe psoriasis. It has been first introduced as a cytotoxic chemotherapeutic agent. Studies investigating the effects of MTX on keratinocytes are scarce and conflicting, some showing that it has an antiproliferative effect [424], while only one study examined and demonstrated its positive effect on keratinocyte differentiation [425]. It has been demonstrated that T lymphocytes are more vulnerable to MTX cytotoxicity than keratinocytes. In addition MTX can significantly decrease TNFα production in lymphocytes [424]. It is generally accepted, that these effects on T lymphocytes are the main mechanisms how MTX exerts its anti-inflammatory properties in skin pathologies. However, keratinocytes are also an important source of TNFα [426]. It has been shown that TNFα expression is increased in psoriatic keratinocytes via the activation of p38-MAPK signalling pathways [427], while TNFα expression is significantly downregulated in the skin of psoriatic patients following MTX treatment [428]. Although there is no available research showing the mechanisms on how MTX treatment interferes with the cytokine production of keratinocytes, it is quite plausible that the clinical response seen in psoriasis following MTX treatment could be via its effect on keratinocytes, a question which is worth further investigation. However, our results suggest that MTX treatment does not influence IL36γ and elafin levels in cell culture experiments.

Cyclosporine A was the only systemic treatment which induced a significant effect on IL36γ levels in primary keratinocytes. Its effect on elafin reduction was only demonstrated in HaCaT cells. Cyclosporine A is one of the most effective systemic treatments not only in psoriasis, but also in AD, as well as in other dermatological conditions. Its long-term therapeutic use is however limited due to its side effect profile (especially due to nephrotoxicity and high blood pressure). The therapeutic/immunosuppressive effects of cyclosporine A are attributed to its inhibition of T lymphocytes via the calcineurin/nuclear factor of activated T-cells (NFAT) pathway. Cyclosporine also induces the expression of transforming growth factor β 1 (TGF-β1), leading to decreased IL2 dependent T cell proliferation [429]. The exact mechanisms on how cyclosporine A exerts its

therapeutic effects in the skin is not fully understood, but it has been shown that it has an antiproliferative effect on keratinocytes [430] and it can reduce inflammation in immunocompromised mice which lack functioning T cells [431]. It has been shown that keratinocytes also express calcineurin and NFAT1 [431]. As cyclosporine A also has some inhibitory effect on activated NF-kB and p38-MAPK, pathways which are also implicated in IL36 expression, it is likely that the reduction we have seen in IL36γ following cyclosporine A treatment is related to the modulation of these pathways. Our MS data also demonstrated high expression levels of cyclophilin A, the cytosolic receptor for cyclosporine A, in the upper epidermal layers of AD with even greater increase in psoriasis. The majority of patients are responsive to cyclosporine A with rapid disease amelioration compared to other systemic drugs, such as MTX. Based on our results, whether patients on cyclosporine A treatment, but with active skin lesions, would still present with elafin and IL36γ levels which could identify psoriatic inflammation in tape stripping needs to be investigated on a clinical level. Based on the *in vitro* experiment, although the level of reduction of these two markers is significant compared to the positive control, it is still relevantly higher compared to the negative control group.

Some of our results are supported by a previous study, which showed moderate downregulation of elafin expression on RNA level in skin biopsies taken from psoriatic patients following treatment with cyclosporine A, calcipotriol and dexamethasone. No alteration in elafin RNA levels with MTX and dimethylfumarate have been seen. However, they did see reduction in elafin following treatment with all-trans-retinoic acid [432]. We could not confirm this effect of acitretin on elafin expression at the protein level in our cell culture experiments. The presence of other immune players may be necessary to see the reduction of elafin expression, and it may also indicate that although studies based on RNA expression are highly valuable, RNA does not always translate to protein.

Furthermore, these *in vitro* experiments demonstrate why it is more beneficial to use a combination of biomarkers, rather than a single marker for the diagnosis of psoriasis in tape stripping. A single marker with such a high sensitivity and specificity such as elafin or IL36γ could potentially lose its biomarker quality if the patient is already on a certain treatment. However, although the potent combination of betamethasone dipropionate with calcipotriol (used as topical treatment) has a considerable effect on IL36γ and elafin expression as demonstrated, most systemic treatments do not seem to significantly alter IL36γ and elafin levels, except for cyclosporine A.

In patients the exact amount of topical treatments applied to a skin area is not predictable as it depends on multiple factors, such as the amount of product applied by the patient, topical blood flow, skin hydration, nature of the lesion (thickness of the plaque, presence of erosions/fissures), etc. At the same time it is also extremely difficult to establish the concentration a systemic treatment is actually delivering to the skin, as this is also influenced by a multitude of individual factors, such as speed and degree of metabolism (enzyme deficiencies or comorbidities such as kidney or hepatic dysfunction may slow down or speed up the metabolic process). However, as *in vivo* the mechanisms how these drugs exert their therapeutic effect is not dependent solely on keratinocytes, but are affected by other immune mechanisms as well as the cellular environment, clinical validation of the above findings is necessary.

Brief summary

In contrast to IL36γ, which showed very low detection levels in cell culture supernatants and was more abundant in the cytosol, elafin was mainly detectable in the supernatants, reflecting its active secretion.

Our results indicate that treatment with nUVB, acitretin, fumaric acid, apremilast, tofacitinib and MTX does not have a significant influence on the expression levels of elafin and IL36γ of psoriasis induced HaCaT cells and primary keratinocytes. From the investigated systemic treatments, only cyclosporine A had a significant effect on the expression of these markers.

The combination of topical steroid betamethasone with vitamin D3 agonist calcipotriol can induce a marked reduction in elafin and IL36γ levels. The combination of calcipotriol with betamethasone at their highest concentrations was the sole treatment which resulted in cytokine levels similar to that measured in the negative control. However, whether this was due to the synergistic cytotoxic effect or the result of the therapeutic effect of the combination therapy inducing terminal differentiation of keratinocytes, remains to be demonstrated.

Clinical validation of this pilot data in order to assess if active treatment with the above mentioned treatments could influence the biomarker potential of elafin and IL36γ in tape stripping is necessary.

Chapter 6 Discussion and Conclusions

Despite the advances in the molecular field, invasive skin biopsies combined with histopathological examination still represent the diagnostic gold standard in dermatology when clinical assessment fails to identify the underlying disease. The easy accessibility of skin for sampling combined with the availability of high sensitivity ELISAs offers the opportunity for non-invasive diagnostic tests based on molecular markers. This could guide the diagnostic and treatment pathway and avoid the need for invasive biopsies. Psoriasis and eczema are among the most common dermatological conditions for which patients seek medical advice, yet differentiating these two disease entities often leads to diagnostic challenge even for trained dermatologists. Furthermore, despite significant recent progress in understanding the pathogenesis of these inflammatory skin diseases, leading to the development of new biologic drugs and better patient care, a multitude of questions are still yet to be answered.

The work conducted in this thesis endeavoured to further develop tape stripping as a non-invasive diagnostic tool in order to differentiate psoriasis from eczema with the use of highly specific and sensitive epidermal biomarkers. It also aimed to show if this simple technique combined with cutting edge research technology could provide valuable information on molecular signatures detected in the upper layers of the epidermis in order to add to the existing knowledge on disease pathogenesis in psoriasis and eczema.

6.1 The importance of early and accurate diagnosis

In the majority of cases, the diagnosis of psoriasis and AD is usually straight forward in the presence of their distinct clinical features. The diagnostic difficulty arises when these characteristic features are not yet present (minimal inflammation at the early stages of disease) or they are blurred (in chronic inflammation). As an example, a slightly scaling erythematous plaque on the external surface of the ankle could represent either psoriasis or eczema. Furthermore, as depicted in chapter 1, due to the unique properties of certain anatomical locations such as the scalp, ear canal, face, intertriginous/flexural areas, genital area, palms and soles, the otherwise typical disease features often overlap not only on a clinical but also on histopathological level [66, 120]. Histopathologic differentiation in those anatomical areas can be similarly challenging, especially in case of atypical presentations or in very chronic lesions, as chronic eczema exhibits a psoriasiform histopathologic pattern [111, 433].

Munro microabcesses and Kogoj micropustules are characteristic histopathologic features of psoriasis, but often absent [71].

Yet, one could argue that distinguishing these two disease entities is not as important, as their initial treatment pathway is almost identical regarding the array of topical and “traditional” systemic immunosuppressants.

However, it is now recognised that psoriasis is associated with a number of important comorbidities. Therefore, early recognition and adequate screening for these relevant co-morbidities is essential in order to prevent disease progression, improve patients’ quality of life and to prolong life expectancy.

Cardiovascular disease (CVD) is one of the most significant psoriasis co-morbidity, and is estimated to decrease the life expectancy of patients with moderate to severe psoriasis by approximately 5 years. Therefore, screening for CVD in these patients is essential. The prevalence of cardiovascular risk factors, such as hypertension, obesity, cigarette smoking, diabetes mellitus, is also increased among psoriasis patients [434]. As an independent risk factor, psoriatic inflammation can contribute to the formation of atheromatous plaques, increasing the frequency of myocardial infarction and mortality [435, 436]. The other common comorbidity associated with psoriasis is PsA. In 85% of cases it develops with approximately 10 years delay from the onset of skin disease. However, in 15% of cases joint and skin involvement manifest at the same time, or the arthritis precedes skin involvement [114, 115]. As this is a seronegative arthritis, its differential diagnosis represents a real diagnostic challenge in the complete absence or in the presence of only minimal skin lesions [67]. As PsA is a debilitating disease affecting young individuals (age of onset between 35 and 45 years) [70], early recognition assuring adequate treatment at disease onset could potentially prevent irreversible joint damage, substantially improve patients quality of life and reduce healthcare costs.

6.2 The value of epidermal sampling (tape stripping) in clinical and research settings

As demonstrated in chapter 3 and 4, tape stripping can be successfully used to study molecular patterns in inflammatory skin diseases with epidermal involvement as well as for diagnostic purposes. It is due to increased sensitivity of detection methods that by the use of this simple, non-invasive, epidermal sampling technique, one can identify not only structural proteins such as keratin, but also highly relevant inflammatory mediators, such as IL36γ.

6.2.1 Tape stripping as a non-invasive diagnostic tool

One of the most relevant finding of the here presented work is the identification of IL36γ and elafin as robust biomarkers with overwhelmingly high sensitivity and specificity to distinguish psoriatic from AD inflammation in tape stripping material.

As presented in chapter 1, IL36γ has been recognised as a key pro-inflammatory cytokine in psoriasis and has previously been proposed as a serologic and histopathologic marker [144]. Elafin has also previously been shown to be increased in psoriatic skin [229, 239] and serum and to correlate with psoriasis disease severity [437]. As demonstrated in chapter 3 and indicated by previous research based on biopsy material [144, 164, 229], although both markers are increased in psoriatic as well as in atopic inflammation compared to healthy, their expression levels are significantly higher in psoriasis when the two inflammatory conditions are directly compared. This work is the first to highlight the combined diagnostic potential of IL36γ and elafin for psoriatic inflammation by using the non-invasive tape stripping method instead of an invasive biopsy technique.

Biopsies can lead to patient anxiety and potential surgical complications such as anaphylactic shock, pain, bleeding, infection, and scar formation. In addition it takes specialised medical training, nurse support, expensive equipment and a minimum twenty minutes time slot to perform. Histopathologic examination of the obtained specimen is necessary, with additional costs and on average for UK reality a four weeks diagnostic delay, albeit it is not uncommon to end up with an inconclusive histopathologic result. All these aspects limit the application of skin biopsies mainly to secondary dermatology care, and even in this setting extra caution is taken when multiple or repeated biopsies from the same patient are necessary. Therefore although it is considered the diagnostic gold standard, this technique is only performed if there is an absolute clinical diagnostic question and it is not suitable to be used routinely in a clinical setting for follow up, to evaluate disease progression or response to treatment.

The identification of serologic markers is intended to fill this void in clinical care, however as highlighted in chapter 1, serologic markers are unreliable indicators of skin inflammation as multiple systemic factors can influence their levels. In addition, although significantly less invasive than biopsies, phlebotomy is still an invasive procedure requiring specialised training. The non-invasiveness, easy to perform nature of tape stripping makes it a highly attractive clinical tool as it allows for multiple, repeated sampling from the same patient and even of the same site at different time points, causing minimal if any discomfort. As tape stripping directly samples the skin it is able to reflect the underlying tissue response more accurately than serological markers. Moreover, tape stripping is suitable to be

used in the paediatric population, where biopsies are avoided due to their invasiveness not only limiting diagnostics but also research. Tape stripping in itself does not require any specialised training or equipment other than the actual tapes.

As demonstrated in chapter 4, IL36γ and elafin are excellent epidermal biomarkers to distinguish psoriatic from AD inflammation, particularly as both are constitutively produced by keratinocytes, reflecting the skin's inflammatory status [144, 241]. These markers' high sensitivity and specificity combined with the simple, non-invasive sample collection, allow tape stripping to be developed into an accurate point of care test for the diagnosis of psoriasis which could be used not only in secondary but also in primary care settings. Future development of a lateral flow test, which would permit the immediate interpretation of results, would further increase its accessibility and use, in particular in primary care.

The question remains however, if the level of these two markers would be similarly low compared to psoriasis in other dermatology conditions, such as cutaneous lymphoma, mycosis fungoides (MF) and fungal infection. This is especially relevant in the case of IL36γ, as it has been proposed that it acts as an alarmin, contributing to the primary defence mechanisms of barrier tissues [438]. This is supported by its high levels not only in the skin but also in the bronchial [439] and intestinal epithelia [440, 441], as well as by its increased secretion upon microbial stimuli, such as bacterial lipopolysaccharides (LPS) [442], certain viral [443] and fungal infections [406, 414, 444, 445]. In fact, based on histopathological and clinical similarities, it has been proposed that psoriasis might well be the result of an uncontrolled IL36γ response to an initial fungal infection [445]. From a clinical point of view, fungal infections do have disease specific features and would only cause diagnostic difficulty with psoriasis in case of minimal skin inflammation. MF in its initial stages can mimic the appearance of plaque psoriasis, therefore can be misdiagnosed prior to its progression into tumour stage. Furthermore, acknowledging that misdiagnoses is a real possibility in early stages, studies also suggest that plaque psoriasis represent a true risk factor for the development of cutaneous lymphoma [446]. Available research data based on biopsy material indicate that although IL36γ is highly expressed in MF compared to healthy controls, it presents similar expression levels to those seen in AD [447]. Although this was not the focus of this work, a future project examining the expression levels of IL36γ and elafin in fungal and MF tape stripping samples compared to plaque psoriasis would be valuable.

High elafin levels have been measured in other skin pathologies characterised by neutrophil infiltration, such as in Sweet's syndrome, Behçet's syndrome, pyoderma gangrenosum, allergic vasculitis and acute infectious diseases [241]

and it has been proposed as a diagnostic biomarker for host versus graft disease [448], however, none of these pathologies represent a diagnostic challenge with psoriasis.

As a summary, although tape stripping needs further validation in a clinical setting, with the identification of highly specific and sensitive tape stripping disease biomarkers, such as IL36γ and elafin, this tool has an excellent diagnostic and disease follow up potential, suitable to be used not only in secondary but also in primary care settings.

6.2.2 The role of non-invasive, epidermal sampling in precision medicine

Precision medicine represents the future of healthcare, allowing to provide “the right treatment to the right patient at the right time”. This is believed to allow improved therapeutic benefit with less side effects. Such an approach may also allow for the prevention of disease progression and the development of potential co-morbidities. However, in order to achieve this, in addition to the development of a non-invasive point of care test already mentioned, there is a need for a new disease classifier in both psoriasis and eczema, based on molecular endotyping in addition to clinical signs and symptoms.

In chapter 3 and 4 we demonstrated that tape stripping in combination with MS and ELISA based techniques can be successfully used for the detection and study of different protein expression patterns, allowing for molecular disease endotyping. As IL36γ and elafin are highly specific diagnostic biomarkers, other markers described in these chapters such as IL8, CXCL1, CXCL20 as well as CCL17 and CCL27, and many other markers detected my MS could well be used to establish molecular subtypes and predict therapeutic response. Our future project is to combine our existing molecular data with the available patient related clinical data (including demographics, psoriasis risk factors, co-morbidities, treatment history) and use data mining techniques, such as machine learning, in order to not only identify the different molecular patterns, but also create a new disease classifier.

The description of such molecular subtypes would allow for a more objective approach to guide therapeutic pathways in both psoriasis and AD, further improving drug safety and effectiveness as well as healthcare outcomes. This is especially important now when currently available biologics (anti-TNFα, IL12, IL23, IL17 inhibitors) are highly effective for the treatment of psoriasis and PsA [449], with the potential to also prevent other systemic co-morbidities. Early treatment with targeted biologic therapy would also decrease side effects from

immunosuppressant treatments, such as cyclosporine A and MTX. The increased prevalence of skin cancer and lymphoma related to psoriasis might well be a direct effect of the combination of long lasting, high dose phototherapy and immunosuppressant treatments [450, 451], while liver cirrhosis is a well-recognised side effect of MTX [452]. In addition, a marker which could predict the development of treatment related severe side effects (e.g. the development of systemic lupus erythematosus (SLE) with anti-TNFα therapy) is also lacking. The range of available biologics for AD is currently restricted to Dupilumab (IL4/IL13 inhibitor). This has proved to be effective not only for the treatment of moderate-to-severe AD [453], but also for its most frequent co-morbidity, asthma [454]. Although, a number of new biologics are currently under investigation for the treatment of AD [455], the combined use of tape stripping with MS provides great potential to identify new therapeutic targets for both AD and psoriasis.

Furthermore, despite the numerous new biologics available for the treatment of psoriasis, there are 20-30% of patients with recalcitrant disease, who fail to respond [182]. In addition, most new biologics are either directly targeting IL17A or its receptor subunit IL17RA, shared with other IL17 isoforms. In the case of IL17A blockade, other IL17 isoforms (also produced by keratinocytes) with known pro-inflammatory role in psoriasis (IL17C, E and F) still remain active. On the other hand, although the inhibition of the common receptor could result in a greater therapeutic effect, the systemic inhibition of all IL17 isoforms could result in potentially severe side effects, as they exhibit different roles in other epithelial tissues. As an example, L17A [456] but also L17C and F [57] are essential for the maintenance of the integrity of the gut epithelium as well as for antimicrobial defence.

Therefore more skin specific biologic treatments would be of high interest. IL36γ has undoubtedly a central role in psoriasis pathogenesis. Its expression is highly increased by IL17A, TNFα, IL22 [403] and IL23 [53], key pro-inflammatory cytokines in psoriasis, but IL36γ can also stimulate its own production in an autocrine fashion [403]. Similarly, there is a positive feedback loop between IL36γ and LL37, an alarmin molecule well known for its role in the pathogenesis of psoriasis [457]. The question remains if a biologic therapy specifically targeting IL36γ or the IL36/IL36R pathway would prove as effective as anti IL17A biologics but with the avoidance of IL17 blockade related side effects, especially as recent research indicate that IL36γ/IL36R signalling has a crucial role in intestinal repair via the induction of IL22, IL23 and AMPs [458, 459]. Recently, a monoclonal antibody against the IL36 receptor used in a phase one study for the treatment of pustular psoriasis showed promising results regardless of the presence of the

IL36RN mutation, with mild to moderate side effects, including upper respiratory and urinary tract infection [460].

Less is known regarding the involvement of elafin in the pathogenesis of psoriasis. Similarly to IL36γ, elafin is increased by IL17 and TNFα [240, 241]. Beside the protective role it plays against tissue damage as a neutrophil serine protease inhibitor [229, 239] and its antimicrobial properties [391], elafin also exerts an anti-inflammatory role by inhibiting LPS related activation of NF-κB and activator protein-1 (AP-1), a transcription factor linked to cell proliferation, differentiation, and apoptosis [461, 462]. Furthermore, elafin reduces macrophage activation and TNFα production by binding LPS in the presence of serum, however, in its absence it can enhance TNFα production in macrophages [463]. The therapeutic, anti-inflammatory effect of elafin overexpression has been demonstrated in IBD, showing superiority to IL10 and TGFβ1 [464, 465]. In addition, elafin has been proposed as a potential treatment for atheromatous plaques, by limiting neutrophil elastase induced tissue damage, inhibiting TNFα and LPS induced IL8 production of endothelial cells and macrophage activation [461]. Elafin treatment has been also successfully studied for the limitation of neutrophil induced tissue injury in hypoxemic environment [466] and its functional variants have shown therapeutic benefit in pulmonary inflammation [467]. One study found lower elafin levels in scale and biopsy material of pustular psoriasis compared to plaque psoriasis. The authors hypothesized that insufficient elafin levels may be the culprit of excessive neutrophil damage promoting the formation of multiple epidermal pustules seen in pustular psoriasis and propose neutrophil elastase inhibitors as new treatment modalities for pustular psoriasis [468]. However, the potential therapeutic role of elafin in psoriasis has not been further investigated.

At the moment we lack objective tools to assess disease severity in dermatology. The available disease assessment tools, such as PASI in psoriasis and EASI in AD, are used to guide treatment decision pathways and to assess therapeutic response. However, there are significant interindividual variations in their application. Furthermore, the clinical signs assessed by these tools can be influenced by factors such as temperature or humidity in the case of erythema, or by the application of topical treatments in the case of scaling. In addition they are not suitable for the assessment of limited, although potentially severe disease, such as forms localised to the hands, face or genitalia. In addition to accurate early diagnosis and the prediction of therapeutic response, tape stripping markers could be also used for objective disease severity assessment and monitoring of treatment efficacy, potentially even predicting an imminent disease relapse prompting treatment change. More in depth laboratory as well as

clinical studies on how these markers react to different treatments is necessary, and should be extended not only on ‘traditional’ immunosuppressants but also to biologics.

As a summary, molecular endotyping and the use of non-invasive biomarkers would be highly valuable as they could allow for personalised medicinal approaches as well as save healthcare costs.

6.2.3 Tape stripping in understanding disease pathomechanisms

This work demonstrates that tape stripping, a simple, easy-to-apply, non-invasive technique, combined with a high-tech research equipment (LC-MS-MS) can identify valuable proteins with relevance in maintaining healthy skin balance or with an important role in disease specific inflammation.

Although not included in results, a number of proteins identified in tape stripping samples by MS have been selected for further investigation based on their differential expression between lesional and healthy skin and potential importance in skin homeostasis and disease etiopathogenesis, including: FABP5, galectin 7, CFL1, elafin, SFN 14-3-3, and cyclophilin A. Stable HaCaTs containing the expression plasmid for these genes have been successfully cloned. These clones can be used for future research to study the effects (including proliferation, differentiation, inflammatory mediator generation) of increased (gain of function) or decreased (loss of function) expression of these genes in healthy, psoriatic and AD conditions.

6.3 Limitations

This work mainly focused on plaque psoriasis, with limited number of samples available from other phenotypes. Although IL36γ and elafin expression levels are certainly higher in all phenotypes of psoriasis compared to AD, increasing the sample cohort would allow for the identification of more clear molecular patterns between disease phenotypes.

The main limitation of the tape stripping technique is to control the depth of sampling in the case of pathologically involved skin. As discussed in chapter 4, it has been demonstrated that in healthy skin, tape stripping is limited to the stratum corneum [342, 343]. However, due to the structural changes caused by inflammation, the question if tape sampling is also limited to the stratum corneum in pathologically involved skin remains open. Furthermore, the intra- and inter-individual variation in skin thickness across different body sites, and the different inflammatory status of involved skin, also contributes to the difficulty of clinical standardisation. Nevertheless, our results suggests that in case of a robust

biomarker, such as IL36γ and elafin, these potential variations did not alter the diagnostic potential of tape stripping. Therefore although clinical validation of tape stripping is still necessary, it undoubtedly offers an unmatched diagnostic potential to non-invasively identify psoriatic inflammation. When used to follow disease progression or treatment response, the first sample in itself can be used as a reference point for subsequent samples in order to track disease dynamics.

6.4 Conclusions

To conclude, the work presented in this thesis demonstrates the potential use of tape stripping as a valuable non-invasive clinical and research tool. Most importantly, it highlights the diagnostic potential of this technique to distinguish psoriatic from eczematous inflammation in clinically challenging cases, by identifying IL36γ and elafin as highly reliable, robust biomarkers. Furthermore, with the combined use of tape stripping and MS it describes a number of proteins with potential interest in the pathogenesis of psoriasis, offering the potential for the discovery of new therapeutic targets.

Future work should focus on the validation of tape stripping in clinical settings, with the aim to develop a reliable, non-invasive point of care diagnostic test as well as to demonstrate its use in the prediction of therapeutic outcome and disease follow up. Additional work investigating the effect of psoriasis therapeutics on these biologic markers is essential. Finally, the use of data mining techniques combined with proteomics data obtained via tape stripping can be used for the identification of distinct molecular patterns and represent the future for the development of a new, scientific based psoriasis classification.

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Appendix

All relevant data file is attached in an electronic format, including:

- MS LFQ data;
- Volcano plot analysis outcomes.