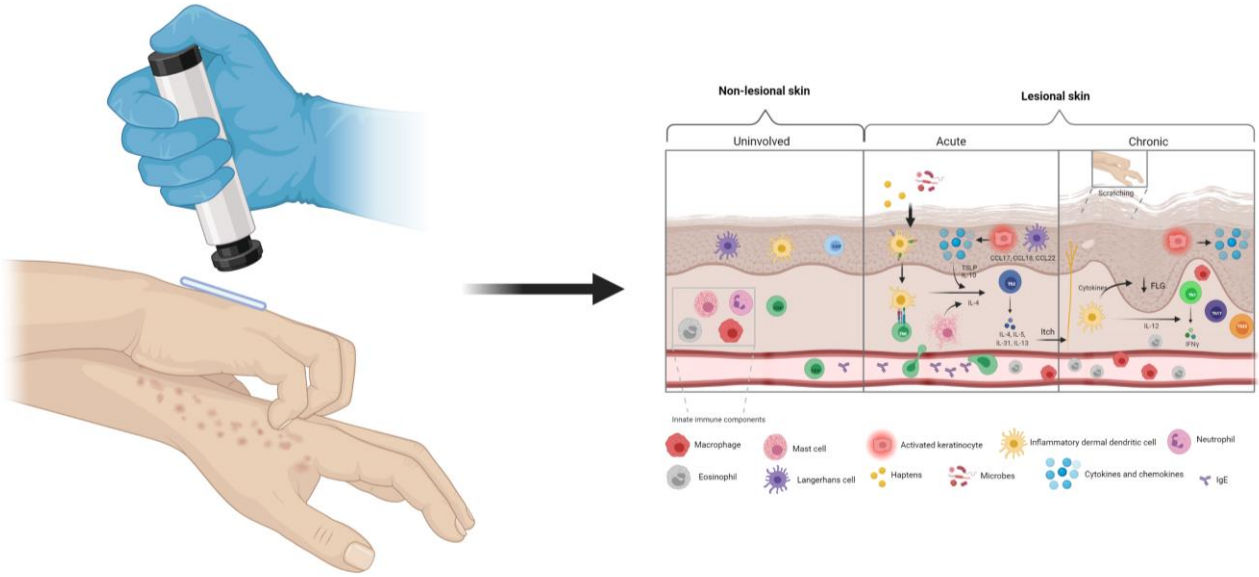
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

PhD Thesis

Julie Breinholt Kjær Sølberg

The skin transcriptome



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The skin transcriptome

PhD Thesis

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This PhD thesis is the product of a scientific collaboration between:

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Preface

This thesis is based on the cooperative work between the National Allergy Research Centre, the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte and the Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark between 2016 and 2021. In addition, the proteomic investigations of manuscript III are based on a collaboration with the Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. The project was funded by the Leo Foundation and received additional financial support by Helsefonden, which are gratefully acknowledged.

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Thanks to all my colleagues at both the National Allergy Research Centre as well as the Section of Forensic Genetics. The positive and inspirational atmosphere at both workplaces have made even the rainiest days a true joy.

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Julie Breinholt Kjær Sølberg
Copenhagen, August 2021

Included studies

This PhD thesis is based on the following three manuscripts:

Manuscript I

J. Sølberg, S.B. Jacobsen, J.D. Andersen, T. Litman, N.H. Ulrich, M.G. Ahlström, M.L. Kampmann, N. Morling, J.P. Thyssen, J.D. Johansen, The stratum corneum transcriptome in atopic dermatitis can be assessed by tape stripping, J. Dermatol. Sci. 101 (2021) 14–21. https://doi.org/10.1016/j.jdermsci.2020.10.011.

Manuscript II

J.B.K. Sølberg, A.S. Quaade, S.B. Jacobsen, J.D. Andersen, M-L. Kampmann, N. Morling, T. Litman, J.P. Thyssen, J.D., Johansen. The transcriptome of hand eczema assessed by tape stripping. Submitted for publication in Contact Dermatitis, July 2021.

Manuscript III

J.B.K. Sølberg, A.S. Quaade, L. Drici, K. Sulek, J.P. Thyssen, M. Mann, J.D. Johansen and B. Dyring-Andersen. The proteome of hand eczema assessed by tape stripping, Manuscript in preparation.

Additional publications

J. Sølberg, N.H. Ulrich, D. Krustrup, M.G. Ahlström, J.P. Thyssen, T. Menné, C.M. Bonefeld, A.Ø. Gadsbøll, E. Balslev, J.D. Johansen, Skin tape stripping: whichlayers of the epidermis areremoved?, Contact Dermatitis. (2018) cod.13199. https://doi.org/10.1111/cod.13199.

R.D. Bjerre, J.B. Holm, A. Palleja, **J. Sølberg**, L. Skov, J.D. Johansen, Skin Dysbiosis in atopic dermatitis is site-specific and involves the bacteriome, mycobiome and virome. Accepted for publication in BMC Microbiology, August 2021.

A.M. Andersson, **J. Sølberg**, A. Koch, L. Skov, I. Jakasa, S. Kezic, J. P. Thyssen. The association between tape strip and skin biopsy derived biomarkers and childhood atopic dermatitis severity and food allergy. Submitted for publication in Allergy, June 2021.

Abbreviations

AD	Atopicdermatitis
ACD	Allergic contact dermatitis
AMPs	Antimicrobial peptides
APCs	Antigen-presenting cells
DDA	Data-dependent acquisition
DEGs	Differentially expressed genes
DEPs	Differentially expressed proteins
DIA	Data-independent acquisition
EASI	Eczema Area and Severity Index
FLG	Filaggrin
HE	Hand eczema
HE^{+AD}	HE with AD
HE^{-AD}	HE without AD
HECSI	Hand Eczema Severity Index
ICD	Irritant contact dermatitis
LC-MS	Liquid chromatography mass spectrometry
LOR	Loricrin
LPS	Lipopolysaccharides
mRNA	Messenger RNA
NGS	Next generation sequencing
NMF	Natural moisturizing factor
PAMPs	Pathogen-associated molecular patterns motifs
PCA	Principal component analysis
PRRs	Pattern recognition receptors
PCD	Protein contact dermatitis
RIN	RNA integrity score
SC	Stratum corneum
TLRs	Toll-like receptors
WTS	Whole transcriptome sequencing

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Summary

Dermatitis is a very prevalent disease affecting approximately 40% of the Danish population from all age groups. Knowledge about the pathogenesis of dermatitis is very important especially with the emergence of new more targeted treatment options.

To perform a molecular investigation of e.g. the immunology of the skin a skin sample must be obtained. The gold standard to obtain a skin sample today is a skin biopsy. This procedure captures cells from all skin layers and is very suitable for histologic investigations, however, the procedure is associated with discomfort and risk of infections and scarring for the patients. Therefore, the technique is not very favorable when investigating sensitive skin areas such as the hands or the skin of children.

During the last decades the use of non-invasive skin sampling techniques has become increasingly popular in dermatological research. One such method is tape stripping that captures the corneocytes of stratum corneum. These cells do not have a nucleus and are therefore by definition dead skin cells, however, several studies have successfully retrieved RNA and proteins suitable for molecular investigations from tape strip skin samples.

The main aim of this thesis was to investigate if the tape stripping procedure could be used to assess the skin of atopic dermatitis and hand eczemaby full transcriptome and proteome investigations. Previous studies using tape strip samples has stored the samples at -20°C or colder, however, to make the skin sampling technique suitable for transport by mail e.g. in an outpatient sampling study, we stored the tape strip samples at room temperature for up to three days.

In *manuscript I* we investigated the global difference between RNA from tape strip samples stored at room temperature for up to three days and biopsy samples. Skin samples were obtained from both healthy and atopic dermatitis skin and the study showed that, despite a global difference between biopsy and tape strip samples, the tape strip samples could be used to assess the transcriptome of the skin of both healthy and atopic dermatitis skin.

In *manuscript II and III* we investigated if RNA and proteins from tape strip samples could be used to assess the transcriptome and proteome of skin from the hands of both healthy subjects and hand eczema patients respectively. For the RNA investigations tape strip samples were stored for up to

three days at room temperature before RNA extractions, however, for the protein investigations tape strip samples were stored at -80°C.

We found that the transcriptome and proteome of healthy skin from the hands as well as hand eczema skin could be assessed by tape strip samples. We also found that suitable tape strip samples could be obtained from both the dorsal- and palmar aspects of the hands. Furthermore, the tape strip samples show very promising results in the investigations of different subtypes of hand eczema. As an example, we found a higher mRNA expression of inflammatory markers, such as CXCL8 and IL-1B for non-lesional skin of hand eczema with atopic dermatitis as compared to hand eczema without atopic dermatitis. On protein level the difference between hand eczema with and without atopic dermatitis was largest at the lesional skin areas. Here, we found a higher expression of FLG2 and LOR and a lower expression of KRT16for hand eczema with atopic dermatitis as compared to hand eczema without atopic dermatitis. On mRNA level we identified six markers that could differentiate irritative contact dermatitis from allergic contact dermatitis including EPHA1which is important for epidermal differentiation.

This thesis showed that tape strip samples stored for up to three days at room temperature could be used to assess the transcriptome of both healthy, atopic dermatitis and hand eczema skin. Furthermore, the tape strip samples stored cold could be used to assess the proteome of healthy skin from the hands as well as hand eczema skin. Our studies did not only find already known molecular markers associated with the different skin areas, but also showed potential in the molecular investigations of the different subtypes of hand eczema.

Dansk Resumé

Eksem er en meget udbredt sygdom, der rammer ca. 40% af den danske befolkning fordelt på alle aldersgrupper. Viden om patogenesen af eksem er meget vigtigt, specielt med udviklingen af nye mere målrettede behandlingsmuligheder.

For at undersøge de molekylære mekanismer af f.eks. immunologen i huden skal man bruge en hudprøve. Standardproceduren til at tage en hudprøve er i dag en hudbiopsi. Med denne metode får man en hudprøve der indeholder celler fra alle hudlag. Derfor er disse hudprøver yderst velegnede til histologiske undersøgelser. En hudbiopsi er dog associeret med ubehag og risiko for infektioner og ardannelse for patienterne og derfor er teknikken ikke særlig favorabel når der skal tages hudprøver fra sensitive hudområder såsom hænderne eller hudprøver fra børn.

I løbet af de sidste årtier har brugen af ikke-invasive hudprøve-teknikker vundet frem i dermatologisk forskning. En af disse metoder er ”tape stripping” som fanger de døde hudceller fra stratum corneum. Selvom disse hudceller ikke har nogen cellekerne, og derfor per definition er døde celler, har flere studier vist at RNA og proteiner fra tape strips kan bruges til molekylære undersøgelser af huden.

I denne afhandling undersøgte vi om tape-strip-prøver kan bruges til at undersøge transkriptomet ogproteometi huden fra raske personer samt patienter med atopisk eksem og håndeksem. Tidligere studier har opbevaret tape-strip-prøver ved -20°C eller koldere, men i vores studier ønskede vi at undersøge om tape-strip-prøver kan sendes med intern post f.eks. i et klinisk studie hvor patienterne selv tager prøverne. Derfor opbevarede vi tape-strip-prøverne ved stuetemperatur op mod tre dage.

I *manuskript I* undersøgte vi den globale forskel mellem RNA fra tape-strip-prøver opbevaret ved stuetemperatur og RNA fra biopsier. Vi tog hudprøver fra både raske forsøgspersoner og patienter med atopisk eksem. Studiet viste at tape-strip-prøver kan bruges til undersøge det fulde transkriptom fra både rask hud og hud fra atopisk eksem, på trods af en stor forskel i forhold til hudbiopsierne.

I*manuskript II og III*undersøgte vi om RNA og proteiner fra tape-strip-prøver kan bruges til at undersøge transkriptomet og proteometfor hud fra hænderne fra både rask hud og eksemhud. Til RNA-undersøgelserne opbevarede vi tape-strip-prøverne ved stuetemperatur op mod tre dage før RNA-ekstrahering, hvorimod tape prøver til proteinundersøgelserne blev opbevaret ved -80°C.

Vi fandt at transkriptomet og proteometi både rask og lesional hud fra hænderne kan undersøges ved brug af de ikke invasive tape-strip-prøver. Disse undersøgelser kunne laves uafhængigt af om tape prøverne var taget fra den dorsale- eller palmare side af hænderne. Derudover fandt vi at tape-strip-prøver har stort potentiale til at undersøge forskellige subtyper af håndeksem. Som eksempel fandt vi et højere mRNA udtryk af inflammatoriske markører såsom CXCL8 og IL-1B for den ikke-lesionelle hud hos håndeksem patienter med atopisk eksem sammenlignet med håndeksem patienter uden atopisk eksem. På protein niveau var forskellen mellem håndeksem med og uden atopisk eksem størst for den lesionelle hud. Her fandt vi et højere udtryk af FLG2 og LOR og et lavere udtryk af KRT16 for håndeksem med atopisk eksem sammenlignet med håndeksem uden atopisk eksem. På mRNA niveau fandt vi seks markører der kunne differentiere irritativt kontakteksem fra allergisk kontakteksem. En af disse markører var EPHA1 som er vigtig for den epidermale differentiation.

Denne afhandling viser at tape-stripprøver, opbevaret ved stuetemperatur i op mod tre dage, kunne bruges til at undersøge transkriptomet i både rask, atopisk eksem samt håndeksemhud. Derudover kunne tape-strip-prøver opbevaret på frost bruges til at undersøge proteomet af rask hud fra hænderne samt håndeksemhud. Udover at genfinde kendte markører for rask såvel som eksem hud viste vores studier også et stort potentiale for tape-strip-prøverne til at undersøge de molekylære mønstre for forskellige subtyper af håndeksem.

1. Introduction

It is estimated that the life-time prevalence of dermatitis among Danes is around 40%, affecting all age groups[1]. Dermatitis consists of several disease entities with different etiologies, clinical characteristics and anatomical localization. In this Thesis, the focus will be on atopic dermatitis (AD) and hand eczema (HE). The terms dermatitis and eczema will be used synonymously.AD is the most prevalent inflammatory skin disease affecting up to 20 percent of the European population, primarily affecting children[2,3]. AD often manifests before the age of two but can persist, relapse or even begin in adulthood[4,5]. AD patients generally have an impaired skin barrier and are therefore more prone to react to skin irritants.It is hypothesized that the impaired barrier may promote sensitization to proteins and type I allergy, while the relationship to contact sensitization to chemicals is not so straight forward[6–8].Persons with AD develop HE in 1/3 to 2/3 of cases, depending on genetic predisposition (filaggrin mutations)[9,10].

HE is a prevalent disease having a 1-year prevalence of 9% in the general population[10]. The etiologies of HE is many, including AD and occupational or domestic exposure to allergens or irritants[11,12].

The diagnosis and treatment of AD and HE areprimarily based on the clinical features of the eczema as well as the history of the patients, and for AD the diagnosis can be made using a set of criteria, where the first and most extensive was the Hanifin and Rajka criteria[3,13]. However, the increased availability of high-throughput techniques enables researchers and clinicians to makedetailed molecular characterizationsof the individual.

Currently, the gold standard to investigate the immunological print of the skin is a full-thickness skin biopsy. The procedure, however, includes removing a full-thickness piece of the skin, and therefore it is not a desired skin sampling technique when investigating sensitive skin areas such asthe face, hands, or fingers[14]. Due to the invasiveness of the skin biopsy, recruitment to studies including many skin samples to be taken can become difficult. Furthermore, skin biopsies need conservation to avoid sample degradation, such as freezing or formalin and eosin fixation.

Due to the drawbacks of skin biopsies, the use of non-invasive skin sampling methods in dermatological researchare increasing. One such method is tape stripping, which can be used to obtain skin cells from the outermost layer of the skin called stratum corneum (SC). This method

does not cause scarring or pain for the patients and is well suited to obtain skin samples from even the most sensitive skin areas including premature skin[15].

The aim of the studies included in this thesis was to investigate the transcriptomic differences and similarities of tape strip samples and skin biopsies of AD patients and healthy controls. Furthermore, we investigated if the tape strip method could be used to assess the transcriptome and proteome of HE.

2. Background

2.1 Atopic dermatitis

AD is the 15th most prevalent non-fatal disease worldwide and have the greatest disease burden of all skin diseases[16]. AD is a chronic inflammatory skin disease and though the causing factor of the disease is not yet fully understoodit is the result of an interplay between genetics,skin barrier defects, immunologic dysbiosis and environmental factors leading to a complex immunopathology (Figure 1). Environmental factors include exposure to exogeneous substances such as allergens, irritants and pathogens which can trigger or exacerbate AD. Skin barrier defect can be caused by genetic mutations in structural genes, tight junction defects or by alterations of the skin barrier upon inflammation or damage of the skin[17–19]. The dysregulated immune response includes alterations of important inflammatory markers such as an induced expression of IL-18[20].

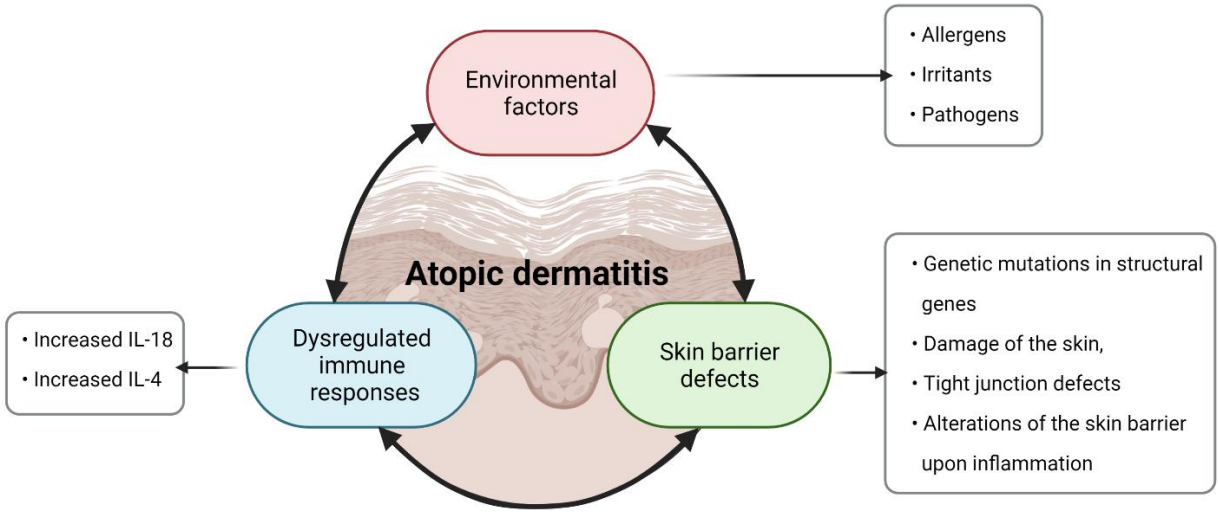

Figure 1. Atopic dermatitis is the result of a complex interplay between skin barrier defects, exposure to environmental factors and a dysregulated immune response. The schematic figure shows some examples of the three factors. Created with BioRender.com.

The prevalence of AD is increasing and today the life-time prevalence of AD is estimated to be up to 20% in Europe, primarily affecting children, with more women than men reporting to have eczema[2,3,21].

AD often starts in early childhood (45% before the age of 6 months and 80-90% before the age of 5 years) but in approximately 25% of the cases patients report to have adult-onset of AD, however, it should be noted that a recall bias most probably exist[22–25].In many children the clinical symptoms will disappear before reaching adulthood, however in approximately 10% the eczema persist as a chronic skin disease[3,5].

Common manifestations of AD include dry and itchy skin (pruritus) and eczematous lesions with an age-related morphology and localization[3].The eczema lesions can affect any sites of the body, but typical localizations include the flexural folds for children and flexures, hands, wrists, ankles, and eyelids for the adults. For the head and neck form of the eczema lesions represent on the upper trunk, shoulders, and the scalp in adults (Figure 2)[3]. The skin inflammation of AD can manifest itself with periodic flare-ups followed by periods of remission, or it can be defined by continuous symptoms[3]. Some racial differences exist in the manifestation of AD e.g. for Asian and African American patients[26].

The diagnosis is mainly based on the observable disease manifestations in combination with the history of the patient. For AD patients diagnostic tools such as the Hanifin and Rajka criteria as well as the UK criteria are used[13,27]. Several tools exist to grade the severity of AD, including the Eczema Area and Severity Index (EASI), which grades the eczema according to four clinical symptoms and the size of the affected area giving an end score between 0 (no eczema) to 72 (very severe eczema)[28].Other scoring systems for AD include the SCORAD index which also take subjective measures such as itch and sleep disturbance into account[29,30].

Basic therapy of AD is the use of barrier stabilizing and hydrating agents and avoidance of inflammatory triggers.Topical treatment with corticosteroids and/or calcineurin inhibitorsis the mainstay of treatment for flare-ups and in some cases as preventive treatment[31,32]. For patients where topical corticosteroids does not give satisfactory disease control, systemic immunomodulators or photo-therapy can be applied[33]. New both systemic (biological) and topical treatments are being developed targeting key pathways in the immune response. Modulation of the skin microbiome is also a developing area[34].

A common complication of AD is a dysfunction of the microbial environment of the skin increasing the risk of recurrent infections[23]. A normal skin flora constitutes non-pathogenic microbes, such as *Staphylococcus epidermidis,* that uses the sebum of the skin as nutrients[35]. These non-pathogenic microbes serve to protect the skin from pathogenic microorganisms, however, in skin diseases this relationship can become dysfunctional and even non-pathogenic microorganisms can become pathogenic. The classic T_H2 polarization of AD decrease the number of antimicrobial peptides (AMPs), allowing pathogenic microbes, such as *Staphylococcus aureus,* to colonize and penetratethe skin[36,37]. These infections further increasethe skin barrier impairment and worsen the eczema as well as increase the risk of systemic infections[3,38,39].

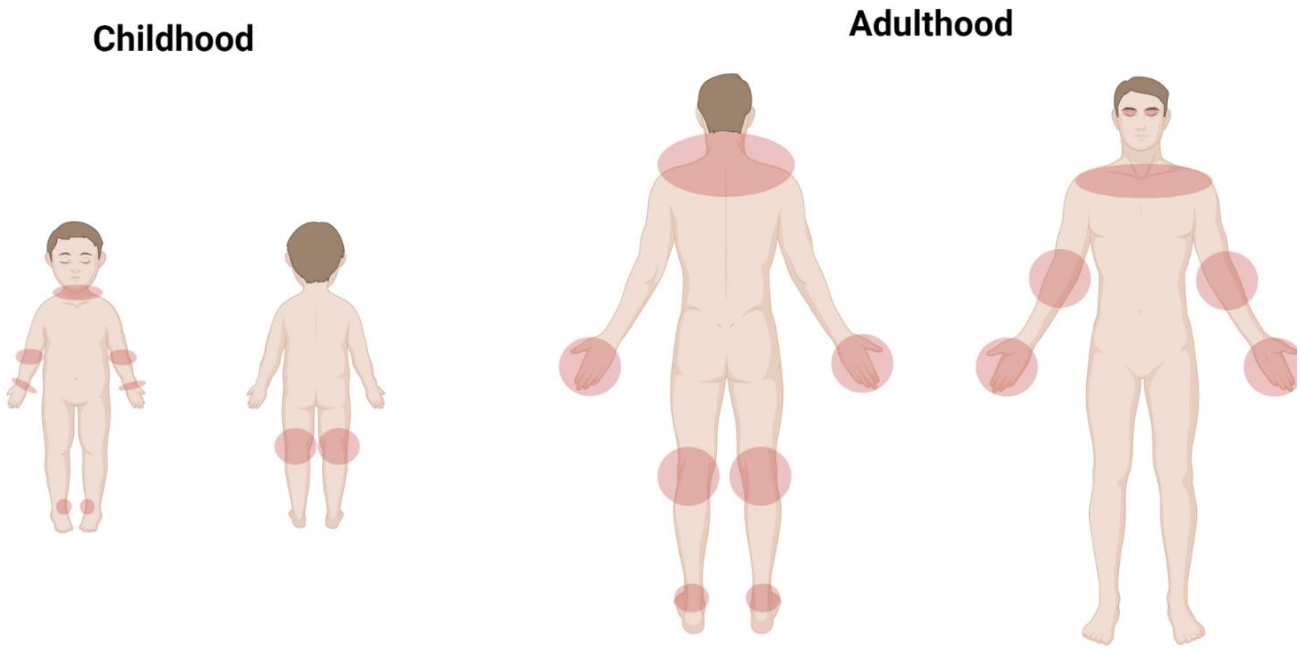

Figure 2. Atopic dermatitis lesions can present all over the body but often have an age-related localization pattern. Childhood eczema often represent in the flexures. In adulthood are the lesions often found to be located at the flexures, ankles, hands and eyelids. Furthermore, a special form of the eczema called head- and trunk eczema represent with lesions on the trunk, neck and scalp. Created with BioRender.com

2.2 Hand eczema

HE is a very prevalent inflammatory skin disease affecting up to 10% of the general population[10,40]. The acute state of HE is characterized by erythema, edema, and possibly vesiculation, whereas dry and scaling skin, hyperkeratosis and fissures are prominent in the chronic phase[41].

HE is the most common recognized occupational disease in many countries, including Denmark, and can affect the ability to work,quality of life and be a significant socio-economic burden[42,43].

Exposures to irritantsand/or allergens in the work environment or domestically are the most common causes of HE in patients investigated by dermatologistsreferred to as irritant contact dermatitis (ICD) or allergic contact dermatitis (ACD)[12]. Current AD or a history of ADis a prominent risk factor with a two- to four-fold increased chance of developing HE[6,44,45].

Irritants are most often detergents or solvents in the work environment. More than 2 hours of wet work daily or 20 hand washes are known risk factors for HE (ICD)and as mentioned already persons with AD are more susceptible to develop this type of HE due to their impaired skin barrier[46,47]. Common allergens causing HE includefragrances and biocides. Exposure to industrial chemicals such as epoxy and acrylates in the work environment may also cause ACD[48–50].

It is not possible to distinguish between ICD and ACD by its clinical presentation. The gold standard for detecting ACD is patch testing combined with exposure assessment[51]. Patch testing is a biological test, where the suspected allergens are applied in plastic or aluminum chambers of approximately 1 cm^2 to the upper back and affixed with special tape. The chambers are left in place for 2 days and the morphology of the reactions are optimally read at the day of removal, day 3 or 4 and day 7[51]. A reading scale is used to judge and grade reactions[49,51]. In case of a positive patch test, current or previous exposure to the allergen in question on the hands needs to be demonstrated or qualified to make the diagnosis of ACD.

The diagnosis of ICD relies on a quantitative exposure analysis demonstrating a sufficient exposure to known irritants which correlates with the debut or exacerbation of HE, in addition current ACD needs to be excluded. A rare form of contact dermatitis is protein contact dermatitis (PCD), which is caused byrepeated exposure to usually food allergens causing a type I reaction (urticaria), which may develop into dermatitis. PCD is mostly seen in workers inthe food industry, and the diagnosis is made byskin prick testing/prick-prick testing, clinical presentation and exposure analysis[51,52].

HE is a heterogeneous disease which can be classified into subtypes by many factors including; morphology (e.g. nummular, palmar hyperkeratoic or vesicular HE), etiology (irritant-, allergic-, protein contact dermatitis and/or AD), anatomical involvement (e.g. dorsal- or palmar pattern, pulpitis, and interdigital), and/or dynamics (acute, recurrent and chronic)[53,54].

At the moment no generally accepted international classification of HE exists, furthermore HE patients are often given multiple diagnoses showing the multifactorial nature of the disease, and in up to 20% of the cases the etiology remain unknown[12,41,55].

The severity of HE can be assessed by the Hand Eczema Severity Index (HECSI) by health personnel. This index scores the severity based on a clinical assessment of erythema, vesicles, fissures, scaling, papules and edema and the extent of the clinical symptoms for the different localizations on the hands[56]. Other scoring systems exists such as the Osnabrueck hand eczema severity index (OSHI) and the photographic guide, however standardization is needed to increase the comparability of HE studies[57–59]. In addition to severity assessment by a health personnel scoring indexes and questionnaires can be used to assess the self-reported severity of the patients.

The standard treatment of HE is depending on its type, but skin care and avoidance of triggers are key. Topical corticosteroids are used as first line therapy , however, some studies show that in over 60% of the cases the eczema persists or reoccur despite of the topical treatment[60].Currently only one systemic treatment (with alitretinoin) is approved for HE but the emergence of new more specialized treatments increase the demand for knowledge about the pathology of HE and its subtypes[61–64]. An increased knowledge of the subtype-specific biomarkers of HE could support the diagnosis and treatment of different subtypes of HE in the future.

2.3 The skin barrier

The skin is the body's largest organ and serves as the physical and immunological first line of defense in the contact with the outside world. Furthermore, an intact skin barrier is important to limit passive water diffusion through the skin, causing the skin to dehydrate (Figure 3).

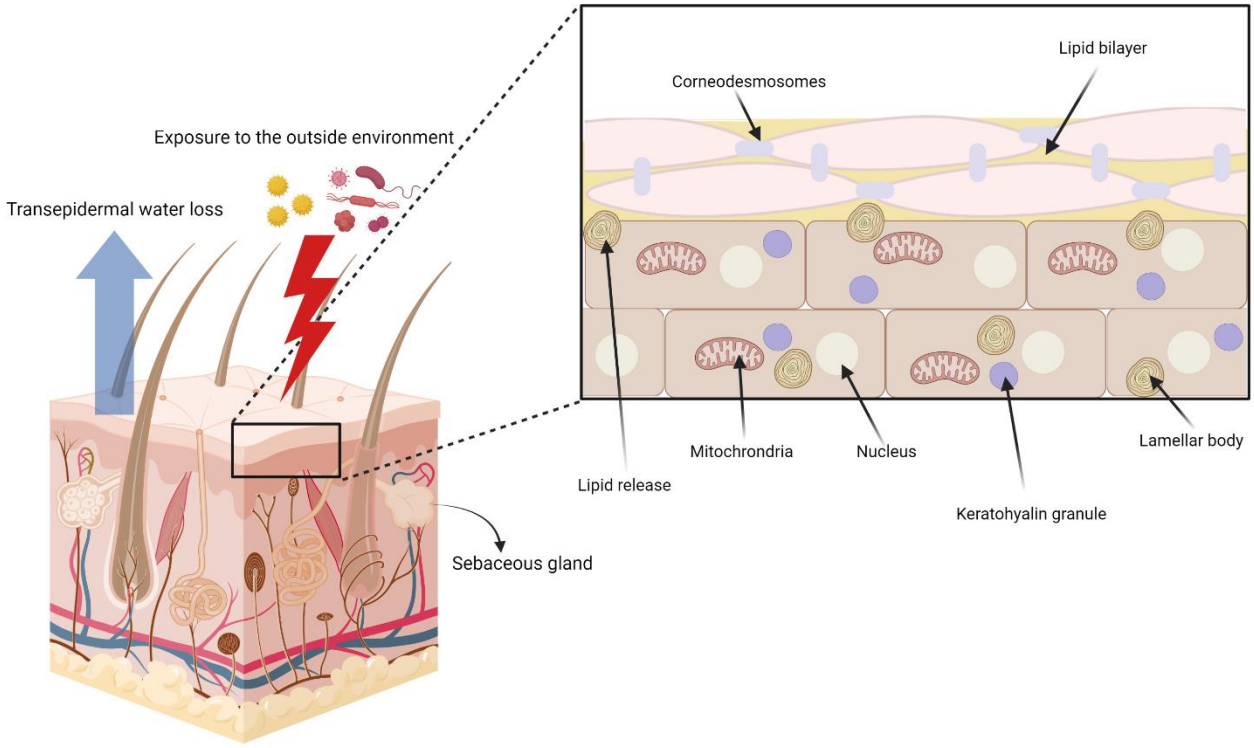

Figure 3 Schematic of the stratum corneum "brick and mortar model". The intercellular lipids are mainly produced in the stratum granulosum and are then transported to stratum corneum in lamellar bodies. Inspired by Pouillot et al.[35]. Created with BioRender.com.

The skin can be divided into three major parts: the lower layers called subcutis followed by dermis and the upper layer called epidermis. Epidermis can be further subdivided into the following five strata: basal, spinous, granular, lucid (only found in the thick skin of the palms and soles) and corneum, called stratum corneum (SC) (Figure4)[65]. This thesis will focus on the epidermis and especially the outermost layer SC, as these are the cells collected by tape stripping.

The main component of the epidermal layer of the skin is keratinocytes. These are very active cells involved in the homeostasis of the skin as well as being major players of the immune system[66].

The keratinocytes are continuously renewing during the process of cornification. During the cornification process keratinocytes from the basal layer moves upward through the skin changing its´ structure and molecular profile eventually becoming corneocytes that are shed from the surface

(Figure 4). This process takes about 1 month in healthy skin but in diseased skin hyperproliferation can occur leading to a faster turnover time[67,68]. As the keratinocytes proliferate the cell anucleates and the cytoplasm disappears, furthermore the cell membrane is replaced by a specialized barrier structure called the cornified envelope[35]. Due to the loss of cell components the corneocytes of SC are basically "dead" skin cells with no transcription or translation of proteins. Several studies of the outermost layers of SC, however, reveal RNA and proteins intact enough to perform molecular analysis[69–74]. Furthermore, it is today understood that the SC is indeed a metabolically active organ[A].

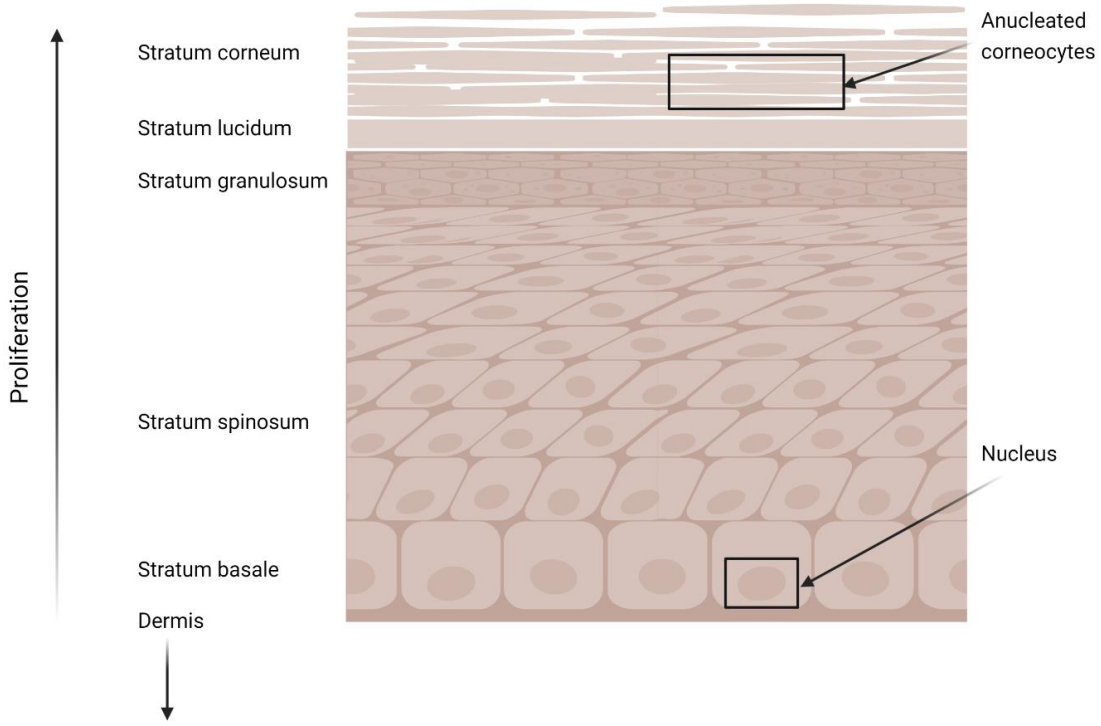

Figure 4. A schematic drawing of the 5 major strata of the epidermal layer. Created with BioRender.com.

In 1987 Elias proposed the "brick and mortar model" explaining the structure of SC[75]. In this model the flat and dead corneocytes are the bricks and the lipid matrix surrounding the corneocytes the mortar (Figure 3).

In the stratum granulosum the proliferating keratinocytes start to produce keratohyalin granules and lamellar bodies. The keratohyalin granules contain the intracellular components of SC including

[A]It should be noted that in some special disease cases such as skin melanomas or parakeratosis the cornification process is a bit different and here corneocytes of SC will have their nuclei retained explaining the mRNA retrieved by tape stripping in these samples[185,234].

filaggrin (FLG), whereas the granular bodies contain the extracellular components of SC such as the lipids (e.g. ceramides and free fatty acids) that are released in the transition from stratum granulosum to SC creating the lipid bilayer[35,76,77].

FLG is a key protein for maintaining a normal skin barrier. FLG comes from a large precursor protein called profilaggrin that consist of 10-12 repeats of linked FLG monomers. Once reaching the SC profilaggrin is cleaved by proteases releasing the FLG monomers. The FLG monomers bind to keratin filaments thereby creating a crucial corneocyte structure of keratin-FLG bundles. In the upper layer of SC, the keratin-FLG bundles are dissociated and the FLG monomers further cleaved to single amino acids called natural moisturizing factors (NMF). The FLG metabolites are important for several reasons including keeping the acidic pH of the skin and retaining water in the SC[76].

The tight structure of SC makes it difficult for substances from the outside world to penetrate the skinand it also regulates the transepidermal water loss, protecting the skin from dehydration.

2.3.1 The impaired skin barrier of atopic dermatitis

The most well-known genetic disposition to AD is mutations in the FLG gene affecting around 10% of the Northern European population[78–81]. Several mutations of the FLG gene is known and the outcome can either result in a depleted or reduced amount of FLG in epidermis[82]. The result can be an impaired skin barrier allowing substances to penetrate the skin more easily as well as drying out the skin due to water loss. Furthermore, many cytokines includingIL-4, IL-13, IL-17A, IL- 22, IL-31, and TNF-α have been shown to downregulate the expression of FLG showing that inflammation itself adds to the skin impairment[82,83]. Despite the major impact mutations in the FLG gene can have it should be noted that only around 30% of patients with AD have a FLG mutations and up to 80% of the FLG mutation carriers do not suffer from AD[3,84]. FLG mutations have also been associated with both the incidence and persistence of HE[9,84].

Though FLG mutations are an important risk factor for the development of AD, skin barrier impairment can be caused by any factor that changes the development of the tight SC including changes in keratin expression, enzymatic factors and changes in the tight junctions allowing substances to penetrate the skin more easily and water to evaporate out of the skin.

The importance of the impaired skin barrier in the development of AD can be seen in the non-involved skin areas, where studies have shown that AD patients have a distinct molecular pattern

with an increased expression of inflammatory markers and a lower expression of important structural components as compared to healthy skin[85,86].

2.4 Immunology of the skin

The skin, in addition to being a strong mechanical barrier, provides a complex immunologic first line of defense with active keratinocytes as well as innate and adaptive immune cells residing in the different skin layers (Figure 5)[87]. The innate immune cells include, but are not limited to, keratinocytes, macrophages, mast cells, and innate lymphoid cells that act fast upon entrance of unspecific foreign stimuli such as pathogen-associated molecular patterns motifs (PAMPs), which are small molecules motifs specifically expressed by microbes such as the bacterial lipopolysaccharides (LPSs). In contrast, the adaptive immune defense responds to already known antigens through antigen-specific receptors on T- and B cells resulting in a fast and efficient immune defense upon reactivation[87,88]. The innate and adaptive immune cells collaborate to make sure that foreign substances and pathogens do not enter the body and induce disease. However, in inflammatory skin diseases such as AD, the system becomes overreactive resulting in a chronic inflammatory cascade[87,89].

The first line of defense is comprised by the, both structurally and immunologically important, keratinocytes. The keratinocytes express several receptors capable of detecting exogeneous signals such as intruding allergens or pathogens as well as cellular stress such as wounding or DNA damage. One of the receptor families expressed by keratinocytes is the pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) that amongst other molecules detect PAMPs. Human keratinocytes express TLR 3, 4, 5 and 9 that upon activation initiate cellular cascades leading to the secretion of pro-inflammatory cytokines and chemokines such as TSLP, TNF and IL-1 family members such as IL-1α and IL-33[90]. TSLP and IL-33 are both known to polarize a T_H2 response[87,89,91]. The release of cytokines and chemokines also initiate recruitment of other immune cells such as neutrophils and macrophages that can help clear the infection by phagocytosis of the intruding substance as well as initiate an immunologic cascade through further secretion of cytokines[92]. Besides acting as pro-inflammatory initiators, the keratinocytes are also able to secrete

AMPs such as defensins and S100 proteins[B], which are important in inhibiting growth of pathogenic microorganisms[93,94].

Upon re-entrance of a specific exogenous substance, the adaptive immune system is activated. The adaptive immune system consists of highly specialized T- and B cells that have been primed in the lymph node to become effector cells. When an effector T cells binds its specific antigen, a fast and efficient immune response is initiated[87], a process which will be elaborated in the *Allergic- and irritant contact dermatitis* section.

2.4.1 Immunology of AD

The immunology of AD is very complex, and it involves a wide variety of immune components and pathways in an interplay not fully understood. Furthermore, the etiology of AD is still unknown, and researchers continuously discuss if the disease is caused by an initial skin barrier dysfunction leading to an increased allergen and pathogen penetration that initiate inflammation and increase IgE sensitization (the outside-in theory) or by initial systemic inflammation, eventually leading to an impaired skin barrier (the inside-out theory)[95–97]. Adding to the complexity, researchers have identified several endotypes of AD further dividing the immunopathology into several subtypes according to e.g. age, ethnicity and the presence of food-allergies[98–102]. This section will focus on the classical understanding of the immunologic cascade of AD.

The immunopathology of AD can be divided into an acute and a chronic phase according to the time of onset and the activated T-cell response (Figure 5). In the classical understanding, the chronic phase of AD is primarily driven by a T_H2 polarization that changes towards a primarily T_H1-driven response when the lesion becomes chronic (Figure 5). It should, however, be noted that several pathways are activated to some extent and depending of the endotype and chronicity, other pathways such as T_H17 and T_H22 may be more dominant and even worsen the chronic inflammation[36,103]. Furthermore, the inflammatory responses are enhanced by secondary bacterial infections by microorganisms such as *Staphylococcus aureus*[104].

The initial T_H2 response is activated by the encounter of foreign substances due to an impaired skin barrier as detected by the innate immune cells such as Langerhans cells and inflammatory dendritic epidermal cells. This induces the production of pro-inflammatory cytokines such as CCL17

[B]The S100 family consists of 21 proteins of which 13 are expressed in the epidermal compartment of the skin. The S100 proteins have various functions. While some S100 proteins act as antimicrobial peptides, other S100 proteins serve several other functions including being components of the cornified envelope[235].

(TARC), CCL18 and CCL22[105]. Furthermore, the keratinocytes are activated to secrete cytokines such as TSLP, IL-10, IL-25 and IL-33 that drive the key T_H2 polarization[106]. This initiates an inflammatory cascade where T_H2 T cells secrete pro-inflammatory cytokines such as IL-4 and IL-13 that act synergistically to induce many key features of the AD inflammation including production of IgE and recruitment of innate immune cells[107]. Another important hallmark of AD is the induction of pruritus. IL-31 released by T cells has been shown to activate the sensory nerves and induce pruritus, and the scratching of the skin further impairs the skin barrier and allows for exogeneous substances to enter the skin more easily[108,109]. Adding to this, released cytokines, such as IL-4, IL-13, IL17A and IL-22 can decrease the level of structurally important proteins such as FLG and loricrin (LOR) thereby adding to the skin impairment[110,111]. The inflamed skin of AD patients allows allergens and irritants to penetrate the skin more easily and thereby make patients susceptible to allergic- and irritative reactions as well as colonization and infection by microorganisms, further adding to the chronic inflammation of the skin[112]. Studies have also shown that the adaptive immune response of AD patients might be hyperreactive thereby producing an adaptive immune response against otherwise harmless substances from the environment.

The chronic AD lesions are predominantly driven by T_H1 polarized T cells producing IFNγ that can lead to apoptosis of keratinocytes[113]. Other important T cell subtypes include T_H17 and T_H22 T cells that are both present at some extent in both acute and chronic lesions; however, research is still needed to fully refine our understanding of the role of these pathways in AD[105].

Knowledge about the different pathways and their downstream molecules can help refine our understanding of the complex immunopathology of AD and thereby lead to a more personalized treatment[98].

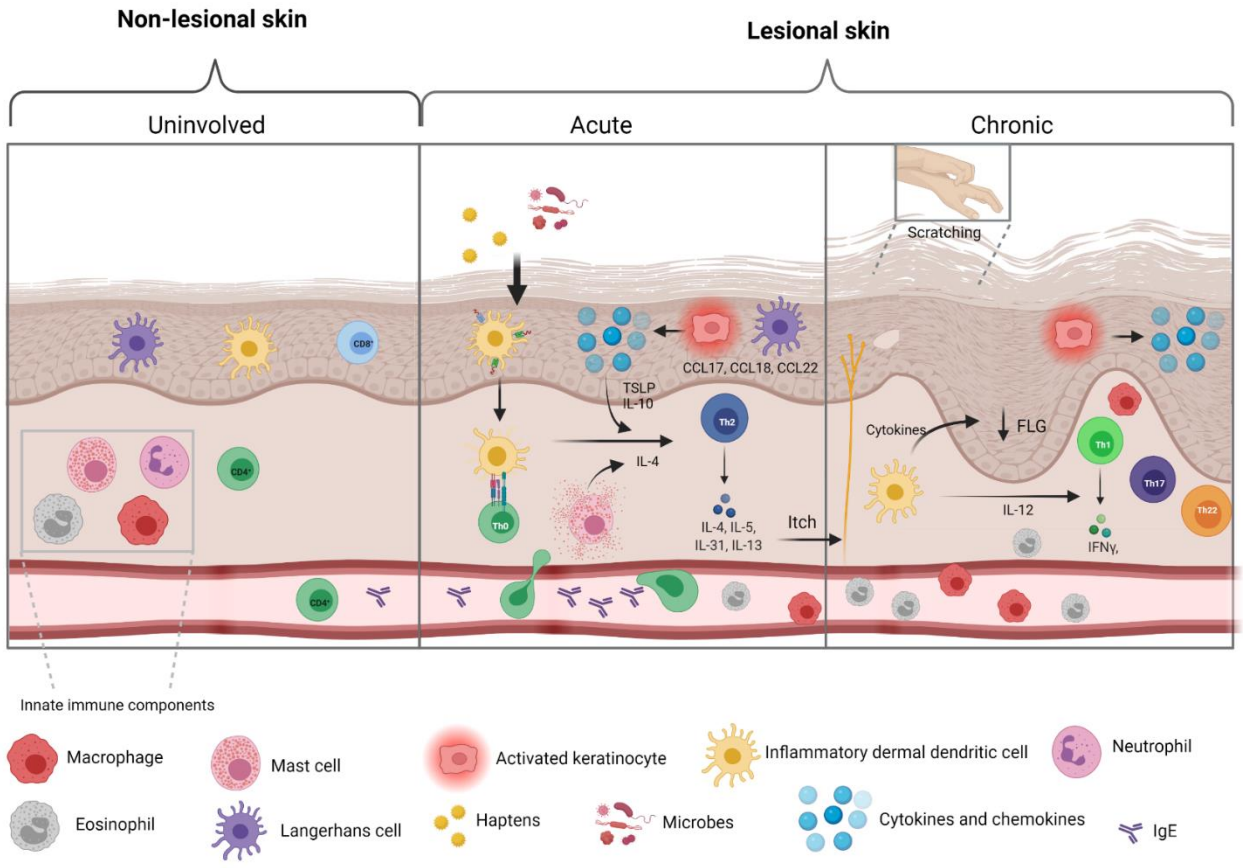

Figure 5 A schematic overview showing the classical understanding of atopic dermatitis. Inspired by Kader et al. 2021[105] and Leung et al. 2014[114]. Createdwith BioRender.com.

2.4.2 Immunology of allergic- and irritant contact dermatitis

Our skin is constantly in contact with the environment. Most substances are blocked from entering the skin by the tight skin barrier; however, in some cases, an allergen penetrates the skin eliciting an immunologic response.

ACD is caused by an immunologic reaction to a contact allergen penetrating the skin. A contact allergen is often a small molecular weight molecule, also called a hapten, that can enter the skin either due to an impaired skin barrier or due to its small size (<500 dalton) enabling it to cross the tight barrier of the stratum corneum[115]. ACD is a two-phase type IV hypersensitivity reaction consisting of a clinically asymptomatic sensitization phase and a symptomatic elicitation phase (Figure 6).

In the sensitization phase, a novel allergen penetrates the skin and is taken up by antigen-presenting cells (APCs), such as Langerhans cells, residing in the epidermis. Furthermore, immune cells, such as the keratinocytes, are activated to secrete inflammatory cytokines such as IL-18 and IL-1β important for the migration of APCs to the lymph node. The Langerhans cells also secrete IL-1β[116].

In the lymph node, the APCs present the antigen to a naïve T cell inducing the development of antigen-specific cytotoxic CD8+ T cells and helper CD4+ T cells as well as skin-resident memory T cells[117–121].

In the second phase, the elicitation phase, the allergen reenters the skin and an allergic reaction caused by the adapted T cells is initiated within hours to days upon re-exposure. Though both $CD4^+$ and $CD8^+$ are important for the inflammatory response of ACD, studies have shown that the inflammatory response of ACD is primarily driven by $CD8^+$ T cells that rapidly enters the skin upon re-encountering antigen releasing pro-inflammatory cytokines such as IL-1β and IFNγ[122–125].

ICD is caused by agents that cause damage to the skin cells. The irritant contact dermatitis does not cause an allergic response, and therefore, no priming of T cells will occur. Instead, the inflammatory response is driven by the innate immune system induced minutes to hours within exposure to the irritant. The inflammatory response upon exposure with an irritant will resolve once the exposure is removed and the skin has been restored[126].

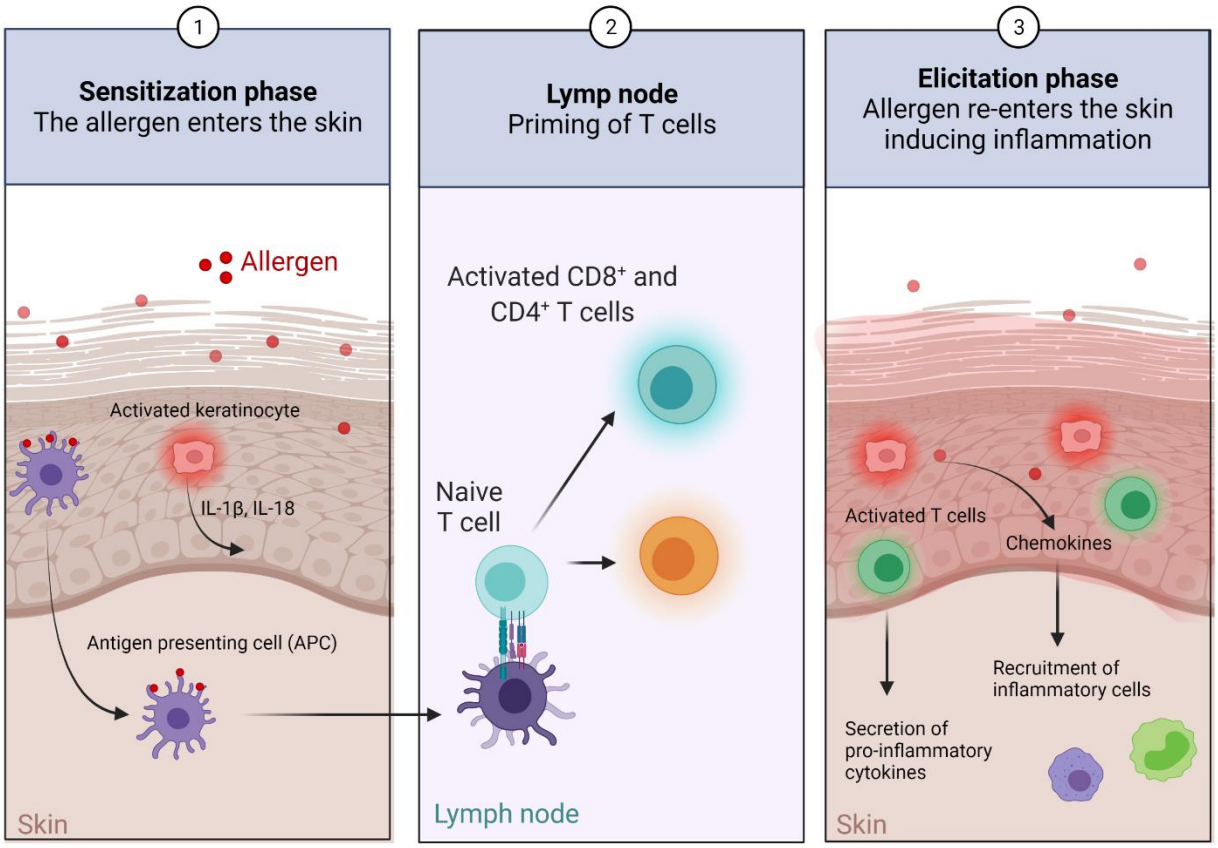

Figure 6. Allergic contact dermatitis is a delayed type IV sensitivity with a clinically asymptomatic sensitization phase followed by a symptomatic elicitation phase. Created with BioRender.com

2.4.3 Immunology of hand eczema

The immunology specific for HE is still widely unexplored. In 2014 Molin et al. showed a decreased expression of several barrier proteins in chronic HE patients as well as an increase in the antimicrobial peptides S100A7 and S100A8/A9 indicating a role of barrier dysregulation as well as microbial infections in the immunology of chronic HE[127]. Another study by Kumari et al. showed an increase of TSLP in chronic HE patients indicating the importance of the T_H2 polarization also known from AD[128]. Furthermore, the immunology is most probably related to the etiology and severity of the disease[61]. This is supported by studies showing an effect of the T_H2 inhibitor Dupilumap for the treatment of chronic atopic HE[61,129]. In addition, the different morphological subtypes of HE could be related to different pathogeneses of HE in the future.

2.5 Molecular investigations of the skin

DNA, RNA and proteins may all be considered biomarkers, and knowledge about them may refine the understanding of the immunology and subtypes of skin diseases.

The DNA can be used to investigate the presence of mutations associated with diseases, whereas the transcriptome and proteome can shed light on the gene activityand protein functionin the skin.

2.5.1 Whole transcriptome sequencing of the skin

The transcriptomecan be defined as the full range of RNA transcripts in a sample, including messenger RNA (mRNA) and non-coding RNAs. However, the term may also describe only the mRNAs in a sample[130]. In this thesis, the transcriptome refers to the full range of RNA transcripts.

The gene expression of a cell is rapidly modified upon cellular changes, such as disease, and it regulates the biological activities of the cells. Several methodscan be used to investigate the transcriptome, including microarrays and next generation sequencing (NGS)[131]. The microarray analyzes a predefined number of RNAs, whereas the full transcriptomic profile can be investigated by whole transcriptome sequencing (WTS)[132].

Several studies have investigated the transcriptome of the skin. These studies have increased the understanding of the immunology of both healthy and diseased skin, as well as identified subtypes of different skin diseases including AD[71,72,98,101,133–138].

Laboratory work

The first step is to collect a skin sample. Once a suitable skin sample has been obtained, the RNA must be extracted (Figure 7). For the extraction of total RNA, there are several things to consider when choosing an RNA extraction protocol. An important consideration is the expected quality of the RNA fragments. The quality of the RNA can e.g. be measured by the RNA integrity score[C] (RIN)[139]. For skin samples, the quality of the RNA depends on the skin layer and the sampling method. In the skin layers containing living cells (epidermis and dermis), the expected quality of an RNA sample is high. However, in dead skin cells of SC, the RNA is highly fragmented, either due to cornification processes or by degradation by ribonucleases (RNases) on the outer surface of the skin. Today, several commercial kits are available for the extraction of both high- and low-quality RNA samples. Therefore, the quality of the RNA is no hindrance for obtaining an RNA pool suitable for WTS.

The first step of the WTS is to convert the RNA fragments into cDNA. This step is performed as sequencing platforms require a DNA library. Furthermore, the double stranded cDNAs are more stable than the single stranded RNA fragments. For the sequencing of total RNA, random hexamer primersamplify the cDNA.

Approximately 95% of the total RNA is rRNA. Therefore, it is important to reduce the amount of rRNA before sequencing of total RNA. If the rRNA is not depleted, it will constitute most of the data output (sequencing reads) and, thereby decrease the data output of other important RNAs[132]. Another method is to use polyA cDNA synthesis, but this will only result in mRNA and hence, non-coding RNAs will not be investigated. Furthermore, polyA cDNA synthesis is less suitable for degraded low-quality RNA than random hexamer cDNA synthesis. Another advantage of using random hexamers is the possibility to investigate non-human RNAs, e.g. RNA from microorganisms. However, this can be at the expense of the sequencing depth for the human genes.

In order to run multiple samples in one sequencing run, the individual samples must be barcoded. This is done by ligating sequencing adapters with sample-specific index sequences to the ends of the cDNA fragments. Once the cDNA pool has been barcoded, it is amplified to increase the number of transcripts followed by a quality check to ensure that the concentration and fragment length of the library is suitable for sequencing.

[C]The RIN score is given as a number from 1-10 with 10 being the highest and 1 being the lowest quality.

For Illumina sequencing, the sequencing is performed on a highly specialized flow cell with nucleotides in the bottom that hold the cDNA strings in place during the sequencing by complimentary adaptor sequences.

The Illumina sequencing platform is based on a concept called sequencing by synthesis. The sequencing is performed in three steps called bridge amplification, clonal amplification and sequencing by synthesis[140,141].

Bridge amplification is performed to make clusters of amplified cDNA fragments, thereby increasing the signal intensity. It is performed by DNA polymerases and results in synthesis of a reverse strand. By repeating this procedure, distinct clonal clusters of forward and reverse strands origination from single fragments are made.

The sequencing itself is performed by adding single nucleotides with fluorescent markers one base at a time, taking a digital image between each cycle. In this way, a complimentary sequence of n nucleotides can be obtained, where n is the number of cycles.Sequencing can be performed as single end or paired end. The single end sequencing sequences from only one end of the fragment, whereas paired end sequencing sequences from both ends of the fragment giving the opportunity to detect insertion- and deletion mutations, as well as better quality and alignment of fragments.

Data analysis

Post-sequencing data analysis includes filtering out low-quality reads, trimming of reads (removal of index and adapter sequences together with removal of low-quality nucleotides at the read ends), alignment to a reference genome, and the final gene expression data analysis. The first step is to assess the quality of the reads. Low-quality reads can have several causes, including poor RNA quality, and sequencing technical issues(e.g. clusters with low diversity making it difficult to assess the intensity of the single clusters).

When the quality has been assessed, the sequence data can be trimmed to get rid of adaptors and hereafter aligned to a proper reference genome. Several algorithms can be used to align the reads to a reference genome. In the studies included in this thesis, the Spliced Transcripts Alignment to a Reference (STAR) alignment tool was used. This tool has been shown to outperform previous tools in both speed and accuracy[142,143]. After alignment the read counts must be normalized before differential analysis can be properly performed. This is due to differences in the library size

(sequencing depth) and composition between samples. Several software packages are available to conduct the normalization and differential analysis including *DEseq2*and *EdgeR*[144,145].

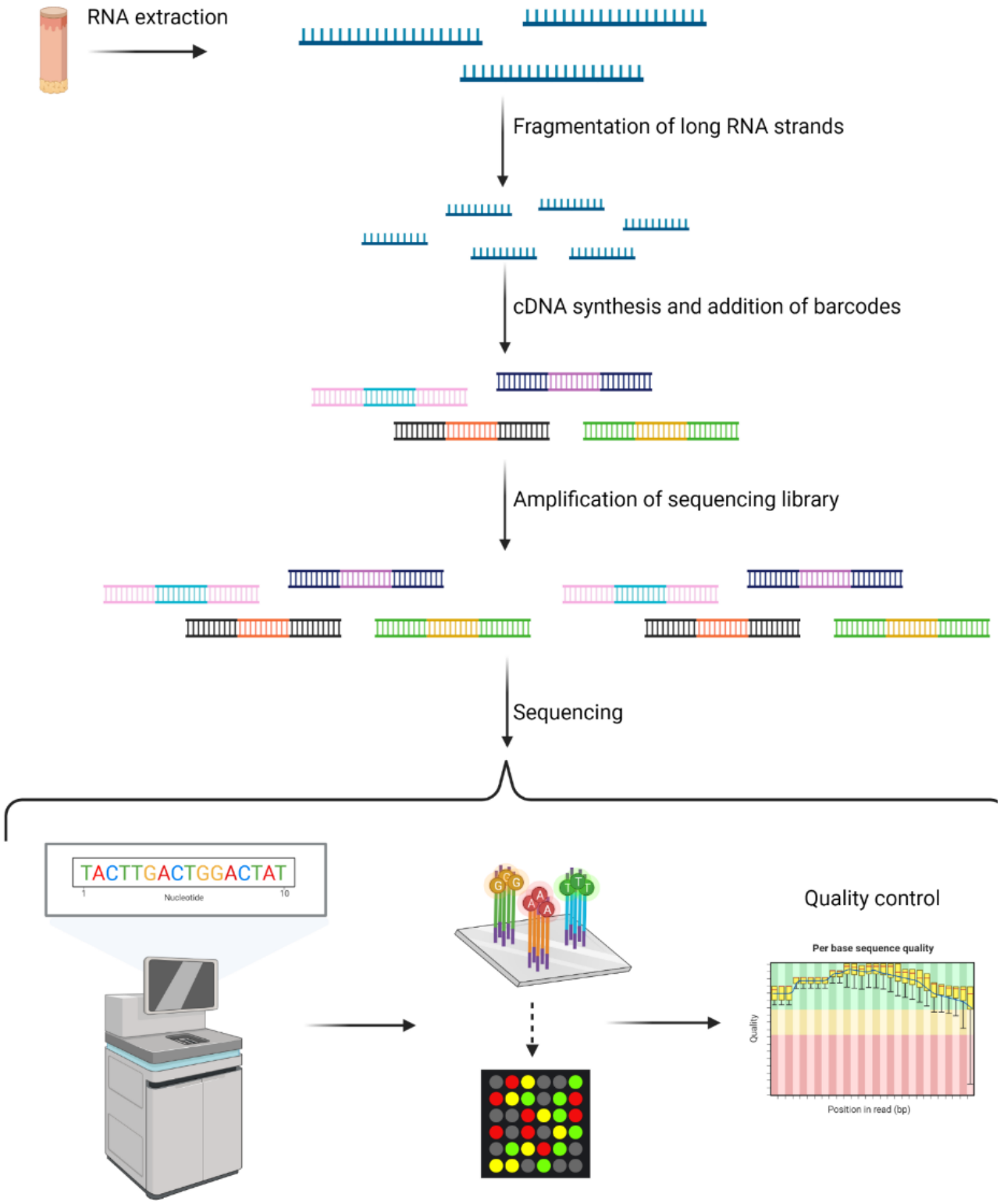

Figure 7. A schematic overview of the sequencing procedure. Created with BioRender.com

2.5.2 Proteomics of the skin

Proteins are the functional molecules of a cell, and the protein expression is thereby responsible for the phenotype of the cells. The investigation of the global protein expression of a sample is called the proteome, and it can deepen our understanding of the molecular mechanisms at a given state[146].

Proteins are structurally more complex than RNAs with many different isoforms and modifications, complicating the proteomic analyses. Many methods exist to quantify the protein level of a sample. These include antibody-based methods such as immunohistochemistry and enzyme-linked-immunosorbent assay (ELISA). These methods, however, are based on prior knowledge about a sample, and only few proteins can be investigated at a time. High-throughput methods include liquid chromatography mass spectrometry (LC-MS), that enables a large-scale investigation with the level of detail important for the detection of the proteins many features[147,148].

Within the last decades, large-scale proteomic investigations of skin diseases have become more common due to the increased availability and precision of the high-throughput techniques as well as the development of down-stream bioinformatic tools. These studies have made important contributions in the understanding of the complex mechanisms of skin diseases such as AD[148–157].

Liquid chromatography mass spectrometry (LC-MS)

LC-MS enables the high-throughput identification as well as quantification of both known and unknown compounds in a mixture. This is done by combining liquid chromatography with mass spectrometry (Figure 8).

Once a proper sample has been obtained, the proteins must be extracted and fragmented, e.g. by enzymatic digestion, to get peptides suitable for LC-MS[D]. The resulting peptide extract is a mix of a wide variety of peptides originating from the proteins originally in the sample.

To separate the single peptides from each other before mass spectrometry, the sample is injected into the LC which is interfaced with the MS. In the LC, the mix travels through a column with a gradient.

This gradient separates the peptides based on a physical property such as polarity or molecular size. In this way, the peptides are separated into pure peptides that reach the MS at different time points.

[D] Proteins and peptides are both composed of amin acids linked by peptide bonds. When a chain is 2-50 amino acids long it is referred to as a peptide, whereas a chain longer than 50 amino acids is called a protein.

Within the MS, the single peptides are ionized and the mass to charge (m/z) ratio detected. This ratio can be used to detect the different compounds, as each peptide, in theory, has a distinct m/z ratio. Often MS is run in tandem mode (MS/MS) allowing for a more precise m/z ratio, as the peptides are fragmented and detected twice.

MS can be run in either data-dependent acquisition (DDA) or data-independent acquisition (DIA) mode. In DDA mode, the first MS round identifies the most abundant peptides, which are then further fragmented allowing for a more precise detection during the second round of MS. In DIA mode, no selection is made in the first MS round.Instead, all ions are quantified[158]. The mode of choice depends on several things. If the endpoint is to quantify an already known protein DDA offers a more sensitive quantification. In contrast, DIA is the best choice for explorative proteomics as all peptides in a mix are quantified.

LC-MS on skin samples may be tricky, as major skin components such as keratins, can make it difficult to detect low expressed peptides. This can be circumvented by choosing a proper sample and extraction strategy before performing LC-MS.

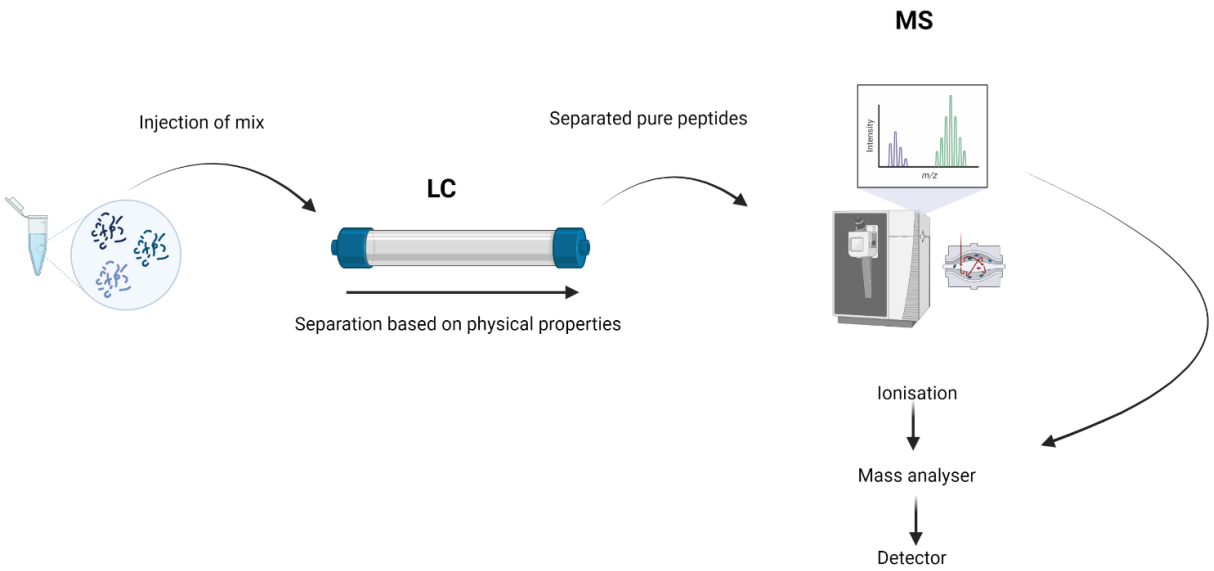

Figure 8. Schematics of the liquid chromatography (LC) mass spectrometry (MS) set-up. Created with BioRender.com

2.6 Skin sampling by tape stripping

Since the 1950´s, skin tape stripping has been used for dermatological investigations[159–161]. The tape strip procedure captures dead skin cell from SC using adhesive tape strips or disks. As the procedure does not reach the deeper layers of the living skin, it does not cause scarring and does not cause any pain for the patient[162,163]. The non-invasiveness of the method makes it very suitable for obtaining skin samples from sensitive skin areas such as the face, hands and it has even been used on pre-mature newborns[15,71,164,165].

The current standard to obtain a skin sample is a skin biopsy. The skin biopsy collects a full skin depth sample and can therefore be used to investigate all layers. The skin biopsycomes with a risk of infections and scarring. Therefore, the skin biopsy is not preferable for some research purposes sampling of sensitive skin areas such as the hands and face and on pediatric patients[14].

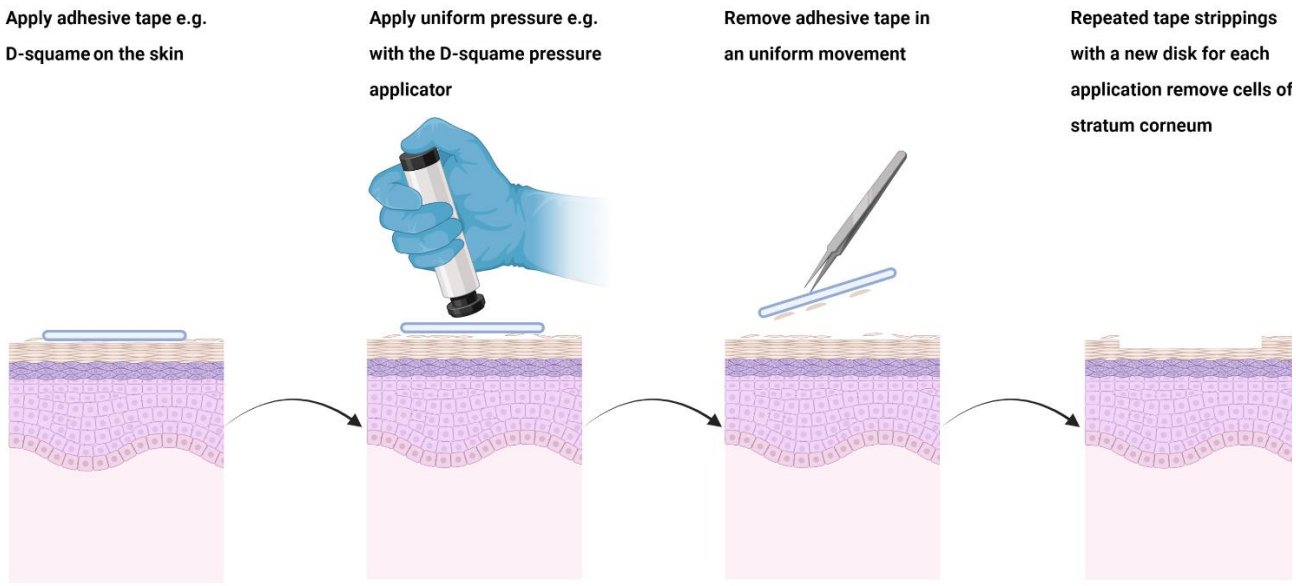

Figure 9. The tape stripping procedure captures corneocytes from stratum corneum using adhesive tapes. Created with BioRender.com.

For histologic purposes, the freshly obtained skin biopsy can easily be stored in formalin. For the molecular investigations of RNA, formalin fixation is not preferable as it degradesthe RNA[166]. Therefore, snap freezing the biopsy using a -80°C freezer or liquid nitrogen is often preferred, when working with skin biopsies. Other methods exist, including preserving buffersfor RNA that delays the degradation of the RNA and thereby delays the time from sampling to the need of a cold long-time storage facility.

A tape strip sample is obtained by consecutive applications of adhesive disks or tape on the same skin area, using a new disk/tape for each application. To make sure that uniform pressure is applied, a pressure applicator can be used (Figure 9). The number of tape stripping's needed to completely remove SC depends on the tensile strength of the tape andthe skin barrier of the individual[162]. Other factors include the pressure of the application, how many furesare present on the skin site, having an effect on the evenness and surface area of the application,and the integrity of the skin[167].

In the 1950's,Pinkus et al. began investigating the use of the tape strip procedure for the removal of SC[159–161]. In the beginning, the method was used to investigate the renewal of SC, but with the development and accessibility of new methods for the investigation of molecular components such as proteins and RNA, investigations of the cells collected by tape stripping became a possibility. In 2011, the American company "Dermtech Inc." patented a tape stripping technique for the non-invasive detection of melanomas.From here on, the method has become increasingly popular for the investigations of several skin diseases[168–170]. The tape strip technique has been used for many purposes includingdetecting inflammatory cytokines, natural moisturizing factors and antimicrobial peptides[163,165,171–173]. Furthermore, tape stripping has served as a model for disruption of the skin[174–180], investigation of the penetration depth of topically applied substances[181,182], detection of malignant melanoma[183–185], the investigation of cutaneous pathogens[186], and skin morphology using atomic force microscopy[165,187–189]. More recent research has focused on RNA and protein purification to detectspecific biomarkers of inflammatory skin diseases from tape strip samples[63,71,98–101,150,151,190–194].

The most used tape for the investigation of skin diseases today is the D-squame® sampling disc that comes in two pre-cut sizes and with additional equipment such as a standard pressure applicator (225g/cm^2) and a machine for the measurement of optical absorption for fast and easy protein mass quantification. The standardization of the sampling technique enables the comparison between

studies. Throughout this thesis, tape stripping will refer to the procedure of tape stripping using D-squame® adhesive disks.

In most studies the tape strips are stored at temperatures between -20°C and -80°C making the storage and transportation of the samples demanding. Storage at room temperature would enable transport by regular post and would not only simplify the method in a clinical setting but would also open for out-patient sampling. However, it still needs to be investigated which methods can be used to assess the molecular markers from tape strips stored at room temperature, as well as the differences related to different storage temperatures.

3 Objectives

The aim of this thesis was to investigate if the tape stripping technique could be used to assess the transcriptome of dermatitis patients. As the tape strip technique is a non-invasive alternative to skin biopsies at sensitive skin areas, we wanted to test the method for skin sampling in HE patients. Furthermore, we investigated if the tape strip samples could be used for investigations of the proteome and the metatranscriptome.

The specific aims of this thesis were:

- To investigate the differences and similarities between the transcriptome of SC corneocytes, obtained by tape stripping,and the full epidermis, obtained by skin biopsies,for both AD patients and healthy controls (*Manuscript I*).

- To investigate if tape strip samples can be used to assess the skin transcriptome after storage at room temperature for up to three days (*Manuscript I*).

- To investigate if the transcriptome and proteome of the palmar and dorsal aspects of the hands of HE patients and healthy controls can be investigated by tape stripping (*Manuscript II and III*).

- To investigate if RNA and protein level differences between subtypes of HE can be detected by tape strip samples (*Manuscript II and III*).

4 Materials and Methods

In this section, a short summary of materials and the applied methods is given. Detailed descriptions of the materials and methods are given in *manuscript I-III*.

4.1 Study population and skin sampling

This thesis is based on two study groups. The first study group (*manuscript I*) was established in 2017 as part of this thesis and included nine AD patients and three healthy control subjects. All AD patients fulfilled the HanifinRajkacriteria, and the severity of the eczema was assessed using the EASI score. AD patients were recruited from the AD clinic at Herlev-Gentofte Hospital, University of Copenhagen, Denmark. From all patients, a lesional and non-lesional tape strip sample, as well as a lesional skin biopsy were obtained. From the healthy control subjects, a tape strip and a skin biopsy sample were taken. Tape strip samples were stored at room temperature for up to three days, whereas biopsies were stored at -80°C.

The patients in the second study group (*manuscript II and III*) were recruited from the Department of Dermatology and Allergy, Herlev-Gentofte hospital, Denmark, between March 2019 and September 2020. The patients were recruited as part of a larger study of 110 eczema patients and 40 age matched healthy controls. Patients had either AD (diagnosed by theHanifinRajka criteria), HE (assessed by a medical doctor at the department) or both AD and HE. The AD severity was assessed by EASI, and the HE severity was assessed by HECSI. For *manuscript I and II*patients with current HE (with and without concurrent AD) and no use of systemic treatment were included. Tape strips were obtained from lesional and non-lesional skin of the patients and from healthy skin of the control subjects.

For *manuscript II*, 30 HE patients and 16 healthy controls were included. Tape strips were stored at room temperature and RNA extracted within three days from sampling.

For *manuscript III*, 34 HE patients and 13 healthy controls were included. Tape strip samples were stored at -80°C, and the proteins were extracted.

A total of 28 patients and 11 healthy controls were overlapping between the studies.

4.2 Ethical statement

From all participants oral and written consent were gathered before inclusion, and all studies followed the Helsinki declaration.

All studies were approved by the local ethics committee (H-16050507) and the Danish Data Protection Agency (HGH-2017-073)

4.3 RNA extraction and sequencing

For *manuscript I*, RNA was extracted using the ExiqonmiRCURY™ RNA Isolation Kit - Cell and Plant (Exiqon, now Qiagen Denmark, Copenhagen, Denmark). This kit was unfortunately taken out of production when the company merged with Qiagen. For *manuscript II*, RNA was therefore extracted using the miRNeasy Micro Kit from Qiagen (Qiagen Denmark, Copenhagen, Denmark). For both studies, the first two consecutive tape strips were used.

For both studies, library build was performed with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian kit (Takara Bio Europe,Saint-Germain-en-Laye, France) without fragmentation. This kit uses random hexamer primers to capture all RNA transcripts.

For *manuscript I*, 75 bpsingle-end sequencing was performed with a NextSeq500 (Illumina Denmark, Copenhagen, Denmark). For *manuscript II*2x 100 bppaired-end sequencing (2 x 100 bp) was performed on a NovaSeq 6000 (Illumina, California USA).

For both studies, readswere aligned usingSTAR version 2.5.3a with default settings. For *manuscript I*,microbial reads were aligned using Kraken[195].

4.4 Protein extraction and LC-MS

Proteins were extracted from two consecutive tape strips (tape 5+6). In short, proteins were lysed directly on the tape strips. After a short centrifugation, the tape strip was removed from the buffer, and the remaining material was boiled and sonicated. Overnight enzymatic digestion using trypsin and lysine was performed. The following day samples were stage-tipped using a wash buffer containingtrifluoroacetic acid (TFA). Theproteins were eluted using an elution buffer containing acetonitrile (ACN) and ammonium hydroxide. After elution, the samples were dried using a vacuum concentrator and resuspended in buffer directly into a MS-plate. LC-MS was performed on

an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an EASY nLC 1200 ultra-high-pressure system (Thermo Fisher Scientific).

A full protein extraction protocol as well as a technical description of the LC-MS run can be found in *manuscript III*.

4.5 Statistical analyses

For *manuscript I and II*, differential analysis (DESeq2) and Wilcoxon tests were conducted in R (R core team, version 4.0.4[E], http://www.R-project.org/). Gene ontology (GO) analysis was performed using Ingenuity Pathway Analyser (IPA, Qiagen, Redwood City, California) (*manuscript I*) or STRING (Version 11.0, https://version-11-0.string-db.org/) (*manuscript II*).

For *manuscript III*, the differential analysis was conducted inPerseus (version 1.6.15.0 , Maxquant), and the secondary dataanalyses were conducted in R (R core team, version 4.0.4, http://www.R-project.org/). GO analysis was performed in STRING (Version 11.0, https://version-11-0.string-db.org/).

For all manuscripts, the data visualization was performed in Qlucore Omics Explorer v. 3.6 (Qlucore AB, Lund, Sweden), R or with BioRender.com.

A detailed description of the statistics can be found in the individual manuscript.

[E] For *manuscript I* R version 3.6.0. was used.

5 Results

In this section the key findings of each manuscript are summarized. The original manuscripts are included at the end of the thesis (section 10).

5.1 Manuscript I: *The stratum corneum transcriptome in atopic dermatitis can be assessed by tape stripping*

In this study, we investigated if RNA obtained from tape strip samples, stored for up to three days at room temperature, could be used to investigate the transcriptome of healthy subjects and AD patients. Furthermore, we sought to assess the gene expression differences between RNA from tape strip samples and skin biopsies (Figure 10).

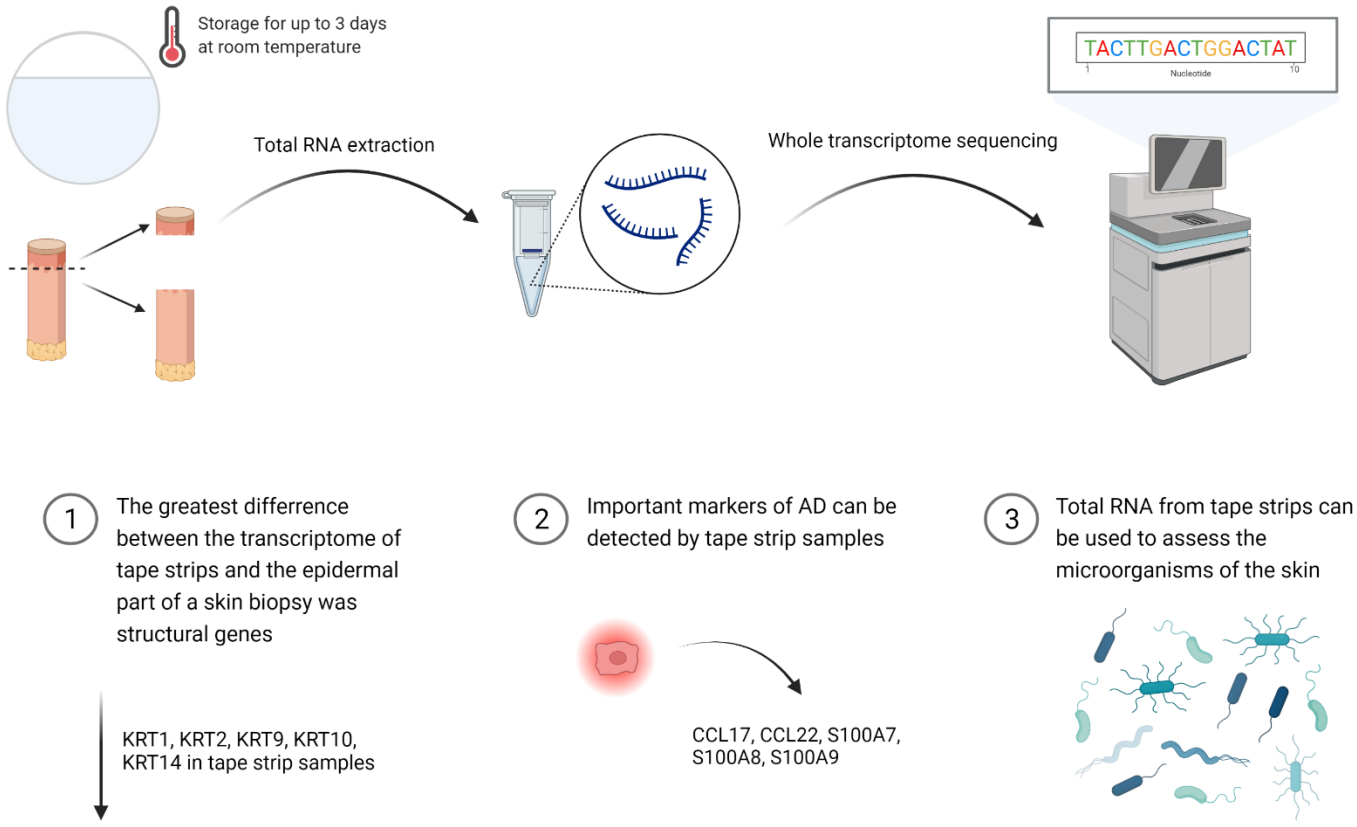

Figure 10 Overview of manuscript I. Created with BioRender

The study included nine Caucasian AD patients and three healthy controls. From each patient, a tape strip skin sample was obtained from a lesional and a non-lesional skin area. Furthermore, a skin biopsy was obtained from a lesional skin area adjacent to the one where the lesional tape strip sample was taken. From the healthy controls, both a tape strip and a skin biopsy were taken.

As the tape strip samples only collect corneocytes from the uppermost layer of the skin[162], SC, the skin biopsies were split into dermis and epidermis, allowing for the comparison between SC tape strip and skin biopsy epidermis samples. From all samples, the total RNA was extracted, and WTS performed.

We found that WTS could be carried out on RNA collected from the two most superficial tape strips. The tape strip samples were stored for up to three days at room temperature from sample collection.

With the use of the random hexamer primers, we were able to investigate non-human RNA and assess the abundance of microorganisms on the skin. Whereas a limited number of microorganisms were detected from the skin biopsy samples, the tape strip samples showed potential for investigations of the metatranscriptome.

As expected, the RNA from tape strip samples were of lower quality than that of skin biopsy samples but known markers of the classical T_H2AD pathway could be assessed by tape strip samples. These markers included *CCL17, CCL22,*and *S100A7-S100A9*.

The global gene expression differences between SC tape strip samples and epidermis skin biopsy samples were primarily driven by structural genes including several keratins. Due to these global differences, care must be taken when comparing study results obtained with different sampling methods.

The study showed that RNA from tape strip samples stored for up to three days at room temperature could be used to assess the transcriptome of healthy and AD skin. Furthermore, we showed that the global gene expression differences between corneocytes as obtained by tape stripping and living skin cells from the epidermal compartment of a skin biopsy were mainly caused by structural genes.

5.2 Manuscript II: *The transcriptome of hand eczema assessed by tape stripping*

In this study, we investigated if the gene expression profile from material obtained with tape strips could be used to assess the molecular profile of HE. Furthermore, we investigated the gene expression differences between different localizations on the hands (dorsal and palmar aspects) as well as the gene expression differences according to different etiologies and subtypes of HE (Figure 11).

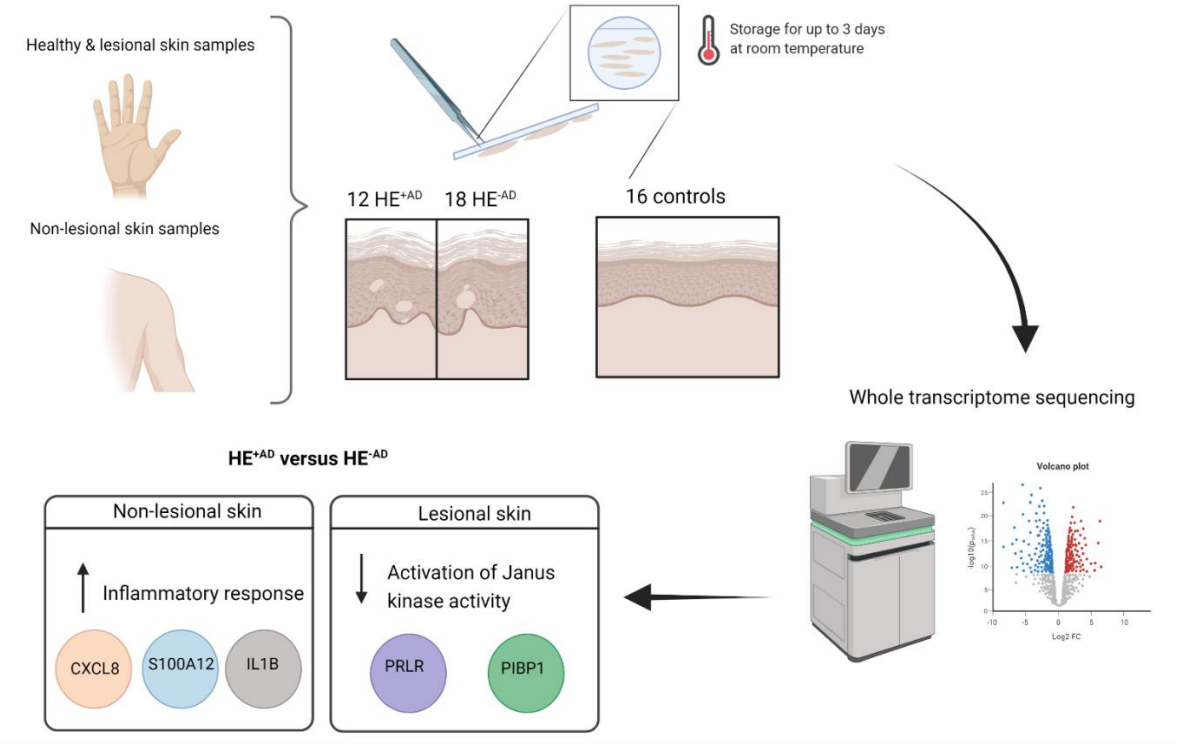

Figure 11 Overview of manuscript II. Created with BioRender.

The study included 30 Caucasian HE patients (12 with and 18 without AD) and 16 healthy controls. From the patients, a tape strip sample was taken from a lesion on the hand where the eczema was worst, as well as a non-lesional sample from the upper arm. From the healthy controls, a tape strip sample was taken from the hand. The tape strip samples were stored for up to three days from sample collection before the total RNA was extracted, and WTS performed.

We successfully performed WTS from RNA extracted from tape strips for both healthy and HE skin samples. The inter-localization differences were larger for lesional skin samples than for healthy skin samples, both for the number of protein coding reads as well as the number of differentially expressed genes between the dorsal and palmar aspects of the hands.

By investigating the overall transcriptomic differences between the different skin areas (healthy, non-lesional, and lesional), the greatest difference was observed between lesional and healthy/non-lesional skin, whereas the healthy and non-lesional skin areas were more similar although the non-lesional samples originated from the upper arm and not the hands. Of the top 25 most differentially expressed genes between lesional and healthy skin, 10 were common between all skin contrasts (non-lesional vs healthy, lesional vs non-lesional, and lesional vs healthy). These genes enriched for inflammatory processes, showing the general barrier inflammation of both lesional and non-lesional skin of HE patients.

The difference between HE with AD (HE^{+AD}) and HE without AD (HE^{-AD}) was most prominent for non-lesional skin areas, with an increased expression of inflammatory markers for the non-lesional skin of HE patients with AD. However, by investigating the difference between non-lesional and healthy skin for the two subtypes (HE^{+AD} and HE^{-AD}), respectively, a common inflammatory response was found, reflecting the general inflammation of non-lesional HEskin despite AD status. In addition to this, known AD inflammatory markers including *CCL17* were detected for the difference between non-lesional and healthy skin of the HE^{+AD} patients.

In this study, we also investigated the transcriptome differences between the etiologies of HE (ACD, ICD and AD). The largest difference was found between AD and ICD, whereas no skin relevant difference was found between AD and ACD.

Six genes were differentially expressed between ACD and ICD, of which several showed potential for the distinction between the two etiologies. However, the biomarkers could not differentiate between ACD/ICD and the mixed etiologies or AD.

In this study, patients with several clinical subtypes of HE were included. Differential analysis was done on clinical subtypes with three or more patients (chronic, fissured and vesicular eczema). The tape strip samples were able to detect transcriptome differences between the two subtypes (248 DEGs). However, more research including a larger patient group is needed in order to investigate this further.

This study showed that tape strip samples from the hands can be used to assess the transcriptome differences between localization, skin areas (healthy and lesional) as well as subtypes of HE from the hands.

5.3 Manuscript III: *The proteome of hand eczema assessed by tape stripping*

In this study, we investigated if the LC-MS could be performed on tape strip samples from the dorsal and palmar aspects of the hands of HE patients as well as healthy controls (Figure 12). The study included both HE patients with AD (HE^{+AD}) and without AD (HE^{-AD}). Furthermore, the proteomedifferences between localizations on the hands as well as subtypes of HE (HE^{+AD} vs HE^{-AD}) were investigated.

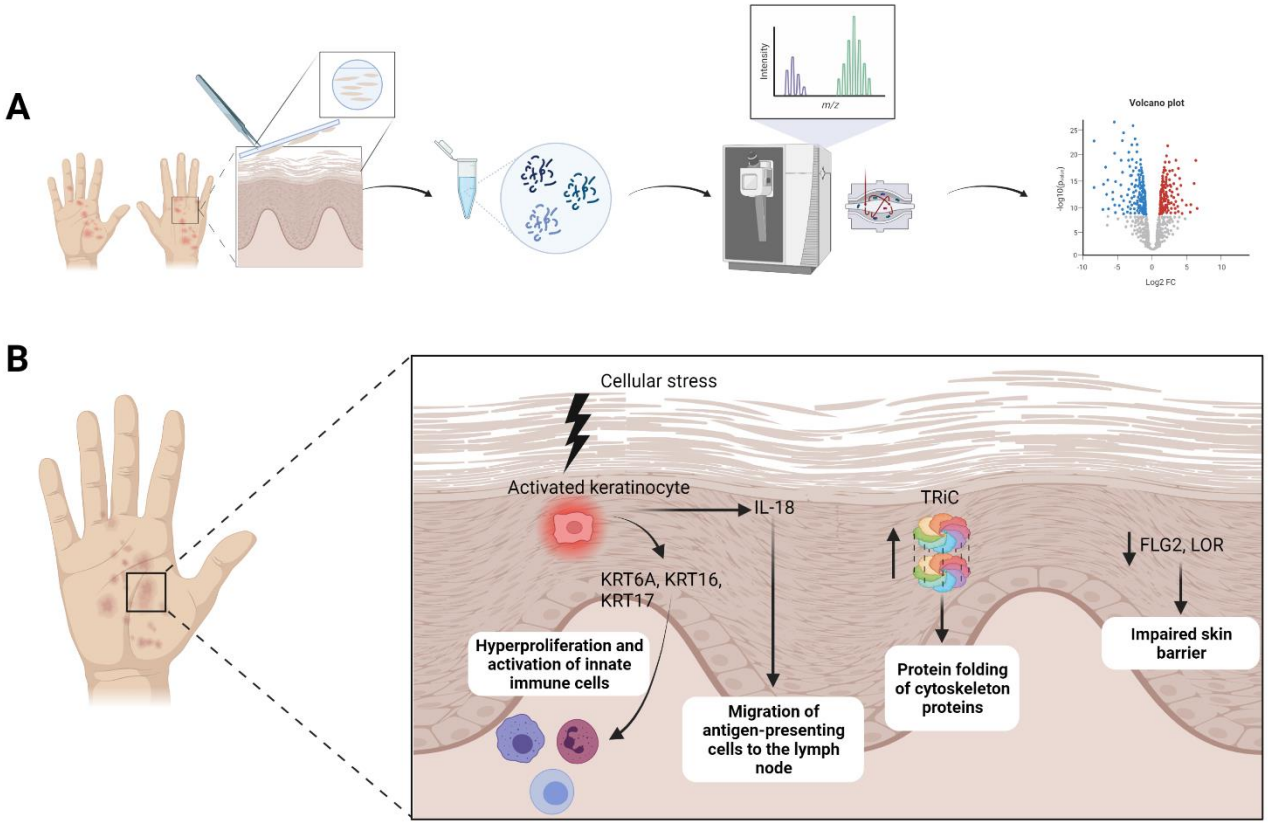

Figure 12 Overview of manuscript III. Created with BioRender.com.

The study included 34 Caucasian HE patients (16 with AD and 18 without AD) and 13 healthy controls. From the patients, a lesional tape strip sample was obtained from an active lesion on the hand, where the eczema was worst. Furthermore, a non-lesional tape strip sample was obtained from the upper arm. From the healthy controls, a tape strip sample was obtained from the hand.

For all samples, proteins were extracted, and LC-MS performed, followed by differential analysis.

We identified 2,919 proteins, of which 1,515 had quantitative values in >70% of the samples in at least one skin area group (healthy, non-lesional, and lesional). This is, to our knowledge, the highest

detected amount of proteins from tape strip samples. High abundant proteins included several epidermal-specific proteins such as S100A7 and KRT14.

Looking at the differences between the dorsal and palmar aspects of the hands, we found no difference in the number of detected proteins for neither healthy nor lesional skin. For both healthy and lesional skin, the proteome difference between the localizations on the hands included an increase of the palmar-specific KRT9[196] for the palmar aspects of the hands. For the lesional skin samples, increased expressions of KRT6A, KRT16 and KRT17 were found for the palmar aspect of the hands. These keratins are not only known for their role as alarmins but are also associated with palmoplantar keratoderma and hyperkeratotic HE[197,198].

The greatest difference between the skin areas (healthy, non-lesional and lesional) was found between the lesional and healthy/non-lesional skin whereas the healthy and non-lesional skin areas had more similar proteome profiles.

Several biomarkers related to the immune system were found to be different between healthy and lesional skin areas. These included an upregulation of HLA proteins, important for antigen presentation and thereby immune activation. Furthermore, a higher expression of the T-complex protein Ring Complex (TRiC), involved in protein folding of misfolded proteins, was found in the lesional skin.

The difference between healthy and non-lesional skin also included a higher expression of inflammatory markers in non-lesional skin.

The proteome difference between HE with and without AD was largest for the lesional skin areas. This included a higher expression of FLG2 and LOR and a lower expression of KRT16 for HE^{+AD}. No differentially expressed proteins (DEPs) were found for the non-lesional skin areas.

This study shows that the tape strip samples from the hands can be used to detect proteome differences between different localizations, skin types (healthy, non-lesional and lesional) and subtypes of HE.

6. Considerations on methodology

The following section elaborates on the methodological considerations not covered or only briefly commented in the original manuscripts (I-III).

6.1 Study populations and skin areas

6.1.1 Manuscript I

In this study, all included patients had the diagnosis AD. The AD diagnosis was based on the HanifinRajka criteria; it is well known that the result depends on the tool used for diagnosis[199]. In this case, patients were seen in our out-patient clinic, and the diagnosis was made by experienced dermatologists.

However, data to further subtype the disease was not collected. Several studies have shown that the subtype of the eczema is important for the immunology[98,100,101]. The main aim for this study was to investigate the feasibility to obtain valid skin samples by tape stripping and compare it to that obtained by skin biopsies. Therefore, a simple patient categorization was preferable.

Patients could continue with their current treatments, including both systemic and topical treatments. This can impact the obtained gene expression pattern.However, in this study we did not explore the relation between eczema severity and individual biomarkers. Therefore, the continued treatment was not expected to impact the results. For the assessment of the difference between tape strip SC samples and biopsy epidermal samples, differences in subtypes and treatment was of no concern as the samples were taken from the same patients, and a paired analysis was therefore conducted. In order to enable a good tape stripping, all study subjects were instructed to abstain from applying topical moisturizers for 24 hours before inclusion in the study.

For an in-depth study of subtype-specific gene expression profiles, a more well-described patient group with at least no use of systemic treatments is preferable, as in *manuscript II and III*.

For this study (*manuscript I*) only three healthy subjects were included. To investigate the difference between healthy and AD skin, more healthy subjects would be needed. In this study, the healthy subjects were primarily used to validate if the gene expression profile of tape strip samples could differentiate between healthy and AD lesional skin sites, which was clearly shown.

6.1.2 Manuscript II and III

For this study, the HE^{+AD} group included both patients with current (n=16) and previous AD, only (n=2), whereas the HE^{-AD} group included patients with no history of AD. This is a common way to classify HE, but it can be discussed if a previous AD diagnosis remains relevant for current HE[12,53]. Several studies, however, have shown that the skin of adult AD patients is generally impaired as compared to healthy skin[85,86].

Patients were recruited based on the presence/absence of AD. This means that many patients had a mixed or unclassifiable etiology, which would complicate differential analysis. Therefore, differential analysis was only performed on the subgroup of patients having a single etiology. The heterogeneous etiologies resemble what is seen in the clinic. However, for an in-depth investigation of the gene expression of the different etiologies, more homogenous groups with more patients should be included.

We used a Danish proposal for the classification of HE. The out-come of the analysis will depend on the chosen classification[41]. So far, no common system exists, but may be on the way in a new version of the European Hand Eczema Guideline, expected later in 2021. The different proposals for classification systems of HE have so far been reasonably similar, especially concerning the etiological subtyping, which makes the results of general importance[12,200].

In this study, the use of topical corticosteroids was not an exclusion criterion. However, all included subjects were asked to abstain from the use of all topicals for 48 hours before the study day. Despite the short wash-out period, the general use of topical corticosteroids could alter the molecular print of eczema; however, many patients are not prepared to go for long periods of time without the use of topicals[201–203]. For this study, we sought to investigate patients in a real-life clinical setting. Therefore, no longer wash-out period of topicals was required. It can, however, not be ruled out that some patients might have showed another gene- and protein expression after a wash-out period, as their eczema would most probably be more severe.

Skin areas

In *manuscript II and III*, the lesional and healthy control samples were taken from the hands, whereas non-lesional samples were taken from the upper arm. This was done as it can be difficult to define a clear non-lesional skin area on the hands of a HE patient, however, due to anatomical differences, the optimal study design would be to have non-lesional samples from a skin area close to the lesional skin site[204]. We found no statistically significant difference between the number of protein coding reads from the different skin areas. Therefore, the obtained differences were not technical but biological. The distant area for non-lesional samples, however, made these samples more comparable among all patients.

6.2 Storage of tape strips

In *manuscript I and II*, the tape strips were stored and shipped at room temperature for up to three days before RNA extraction. In previous studies, tape strips were stored at -20°C or even down to -80°C[191,205,206]. The room temperature storage allows easily transportation of samples, e.g. by mail[F] from the clinic to the laboratory, but it also allows for outpatient sampling.

This study was done in collaboration with the Section of Forensic Genetics, Department of Forensic Medicine at the University of Copenhagen. The protocol was based on their experience with RNA and tape strip samples. Furthermore, the department has experience in high-throughput DNA and RNA analyses of low-quantity and low-quality samples[207]. The rationale for keeping the tape strips at room temperature is that drying up the cells and keeping them at a stable temperature in a sun unexposed environment protects nucleic acids(RNA and DNA) that can be degraded if it comes in contact with water, as can be the case when thawing the samples after cold storage. For this study, the RNA extraction protocol was based on an unpublished in-house protocol for tape stripping of skin samples.

The idea of storing tape strips at room temperature is not novel. The American company *Dermtech Inc.*has patented a technique, where tapes for the detection of melanoma can be stored at room temperature[168,208]. The *Dermtech*study shows no differences in total RNA yield among different

[F] Unpublished data from the Section of Forensic Genetics, Department of Forensic Medicine show no difference in gene expression of selected genes after storage at room temperature for 0, 7 and 14 days respectively. This enables transport by external mail.

storage temperatures[209]. It should, however, be noted that they use another tape[G], and that their studies were conducted on other skin types (melanoma and healthy skin) for RT-PCR purposes. Future studies could investigate if storage at other temperatures improves sampling quantities with the D-squame tape strips.

In *manuscript I*, RNA was extracted from all samples, except one, within 48 hours, and we could therefore not assess the effect of storage time in this manuscript.

In *manuscript II*, the samples had different storage times (Table 1). Therefore, we were therefore able to investigate the impact of the time at room temperature before RNA extraction.

Table 1. Storage times of tape strip samples before RNA extraction in manuscript II

	Healthy (n)	Patients (n)
<24 hours	1	7
24-48 hours	7	13
49-72 hours	8	10

No correlation was observedbetween the storage time before RNA extraction and the exclusion of samples from the study due to low quality (data not shown). Furthermore, when investigating the principal component analysis (PCA), no overall clustering due to with storage time was found. Patient and healthy samples grouped together (Figure 13).

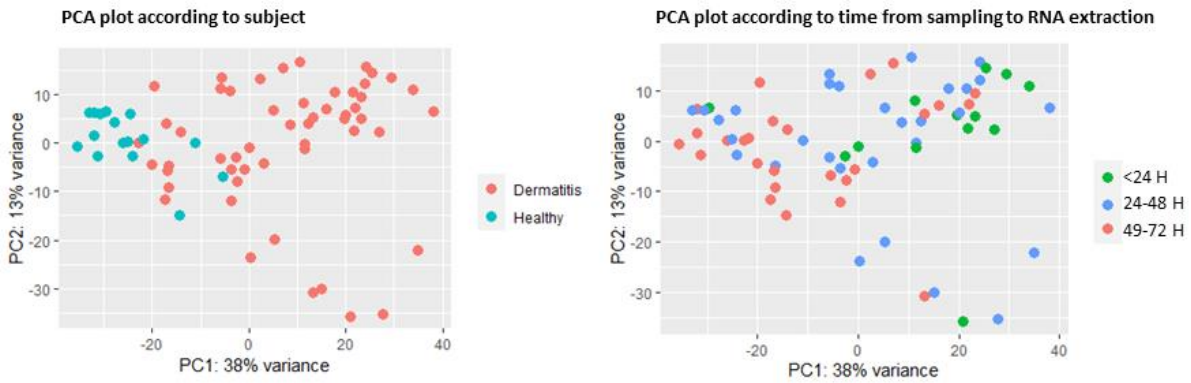

Figure 13

[G] The tape used in the sampling kit from Dermtech is rubber-based and have a soft backing, which increases the tensile strength of the tape as compared to the D-squame tape strips[168].

6.3 RNA extraction and sequencing strategy

In*manuscript I*, we used the same protocol for RNA extraction, library build, sequencing, and downstream data analysis for both tape strip and skin biopsy samples[H] (epidermis and dermis). To consider the poor quality of RNA samples from tape strip samples (mean RIN 2.5), the protocol was optimized for low input and poor quality RNA samples. In our study, we observed relatively low quality scores for the skin biopsy samples (mean RIN 4.3) Besides RNA extraction, the library build was also optimized for low input and low quality RNA (SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian kit (Takara Bio Europe, Saint-Germain-en-Laye, France)). Furthermore, as the RNA from the tape strips was fragmented, we skipped the optional RNA fragmentation step.

The RNA sequencing strategy applied in *manuscript I* allowed for a direct comparison between the skin sampling techniques.

[H]The RNA extraction protocol differed in the homogenization step, where skin biopsy samples were homogenized with a tissuelyser before lysis.

7. General discussion

7.1 High-throughput examination of skin cells from stratum corneum

Several studies, including those in this thesis, show that skin cells from SC obtained by tape strip samples can be used to investigate the transcriptome and proteome of the skin, but the origin of the obtained nucleotides and amino acids are still discussed[63,71,98–101,150,151,190–194,210,211].

The corneocytes obtained by tape stripping are anucleated cells with no cellular organelles. Whereas some proteins, including keratins, occur naturally in SC, most RNA and proteins extracted from tape strip samples must originate from the deeper skin layers[I212–214]. Most studies do not comment on the origin of the RNA from tape strip samples. Wong et al. suggested in 2006 that the RNA originates from cells other than corneocytes such as hair follicle-associated keratinocytes. This theory was based on the fact that RNA yield differed according to anatomical localization, whereas the obtained amount of SC (as measured by the transepidermal water loss) was consistent with the number of consecutive tapes used[190]. This theory, however, is not supported by the fact that we could extract RNA and proteins from tape strips taken from the palmar aspect of the hands, where no hair is found *(manuscript II and III*). A recent study from 2021 suggested that the RNA obtained from the skin surface comes from lipids on the skin and are thereby protected from RNAses on the skin surface. However, it must be emphasized that this study wiped lipids of the face and tape stripping was not performed[215]. This method, however, may very well only be suited to take samples from oily skin areas such as the face. It could also be that the RNA has not been fully degraded during the cornification process, thereby lying freely in the cells. This would be in line with our findings that the RNA fragments are very short.

As immunologic markers from all the epidermal strata are represented in the obtained transcriptome/proteome of tape strip samples, it can be difficult to further elucidate on the origin of RNA/protein from tape strips based on molecular studies.

In *manuscript I*, we showed a global difference between skin biopsies and tape strip samples. This difference was mainly due to structural genes, which might be ascribed to the spatial distribution of the cells, such as keratins. A study by Kim et al. showed a positive correlation between tape strip and biopsy samples for structural epidermal differentiation genes including FLG and CDSN, so the

[I]As elaborated under det *Skin barrier* section in some special disease corneocytes of SC will have their nuclei retained explaining the mRNA retrieved by tape stripping in these samples[185,234].

global difference observed in our study may only be relevant for structural genes not found in the uppermost layers of epidermis[216].

As expected, we only found few dermal markers (mainly collagens) from tape strip samples. Therefore, the identified RNA and proteins, most likely comes from the epidermal layer of the skin.

Despite the unknown origin of the RNA and proteins, several studies, including ours, have convincingly showed that robust transcriptome and proteome results can be obtained by tape stripping, and that known markers of healthy and diseased skin can be assessed by tape stripping.

7.2 Investigation of the microorganisms of the skin by tape stripping

As briefly described in the *Atopic dermatitis*section (Section 2.1), the microorganisms of the skin play a major role in both healthy and inflamed skin.

In *manuscript I*, the extraction of total RNA in combination with the use of random hexamere primers for the library build allowed us to make a concomitant exploration of the microorganisms of the skin from the SC tape strip samples. For the epidermal part of the biopsies, no skin-relevant microorganisms were detected, whereas the tape strip samples showed an abundance of skin-relevant microorganisms of both healthy and lesional skin, including an increased abundance of both *staphylococcus epidermidis* and *staphylococcus aureus* for the lesional skin samples[217]. That biopsy samples are not preferable for investigating the skin microbiota, is in line with previous studies showing that skin swabs are superior in detecting the skin microbiome compared to skin biopsies[218]. Furthermore, another study showed that tape strip samples collected viable bacteria from the skin, comparable to those detected by the standard swabbing technique[219].

The co-examination of human and microbial RNA from tape stripping opens for studies investigating the immunologic and microbial interplay from the same skin area. Furthermore, this interplay can be investigated for sensitive skin areas including those of the hands and newborn babies.

7.3 The molecular markers of HE assessed by tape strip samples

In *manuscript II and III*, the transcriptome and proteome of HE was investigated. Though mRNA has the genetic code for the synthesis of proteins, mRNA is not translated into proteins in a 1:1 manner. This can be due to transcript degradation, different modifications and programmed destruction of the synthesized proteins. mRNA can therefore not predict the protein outcome

alone[148]. Recent studies have found correlations between the amounts of mRNA and protein in a sample, and the use of mRNA to predict a functionally molecular outcome is therefore theoretically possible[220,221].

The study groups of *manuscript II and III* had some overlap (28 patients and 11 healthy controls). Therefore, it would be possible to investigate the correlation between mRNA and proteins from these studies. However, due to the storage and tape strip number bias[J], this discussion will focus on the more general transcriptome and proteome findings and not the correlation of mRNA and proteins of individual biomarkers.

Identification of known markers of dermatitis

All the studies included in this thesis successfully identified known molecular markers of the different immunologic pathways of AD (T_H2, T_H22, and T_H17)from lesional skin sites. The RNA with higher levels in lesional skin sites than inhealthy skin included RNA coding for the more general inflammatory *S100A* proteins,*IL-4*, *CXCL8/IL-8*,*IL-13*, and*CCL17/TARC*which is highly associated with AD[222,223]. The markers found to be higher expressed at the protein level in lesional skin than healthy skin included the alarmins KRT6A, KRT16 and KRT17, and the IL-1 family member IL-18. Furthermore, the proteome data showed a higher expression of all subunits of the TRiC complex, which to our knowledge has not been shown in HE or AD skin before. This shows that the tape strip samples can not only detect known markers of skin diseases but can also be used to investigate new markers. For the RNA samples, more interleukins were found compared to the protein samples. On the other hand, more keratins were observed in the proteome samples. This difference can either be ascribed to differences in the degradation of RNA and proteins during cornification or by differences in molecular half-lifes, or it may be due to technical differences. It should be noted that we got data from approximately 19,000 mRNA transcripts compared to 2,919 protein transcripts (1,515 after filtering).

Differences between palmar and dorsal aspects of the hands

In *manuscript II and III*,tape strip samples were taken from the palmar and dorsal aspects of the hands. Even though the skin on the palmar aspects of the hands is thicker than that on the dorsal, we did not find any statistically significant differences in the amount of RNA/proteins in any of the

[J]In *manuscript II* RNA was extracted from the two first tape strips obtained, and the tape strips was stored and shipped at room temperature. Due to practicalities the tape strips from *manuscript III* were extracted from tape strip 5 and 6, and the tapes had been stored at -80°C.

studies. On both the RNA and protein levels, the differences between the palmar and dorsal aspects of the hands were larger in the patients (lesional skin) than the healthy controls. On the protein level, the difference between the localizations included a higher expression of KRT9 in the skin of the palm of healthy and lesional skin. This keratin is known to be associated with the skin on the palm and soles, where it contributes to the mechanical resilience needed for these skin areas[196]. Furthermore, we found a higher expression of the alarmins KRT6A, KRT16 and KRT17 in the palm. These proteins recruit cells of the innate immune system and their expression in the palms of hyperkeratotic HE and palmoplantar keratoderma is increased[197,198,224,225].

The difference between non-lesional and healthy skin areas

In both RNA and protein samples, we found a larger difference between lesional and non-lesional/healthy skin areas, whereas the non-lesional and healthy skin had similar molecular profiles. This has previously been shown to be the case in psoriasis. However, in AD, it has been shown that the molecular profile of non-lesional skin becomes more alike the one found in lesional skin with increased severity (as measured by SCORAD)[226,227]. To our knowledge, this has not previously been observed in HE.

In *manuscript II*, we investigated if the similarity between non-lesional and healthy skin was due to the non-lesional skin of HE without AD being "healthier" than the non-lesional skin of HE with AD. However, this theory was, not confirmed as the immunologic differences between non-lesional and healthy skin areas were similar for HE with and without AD. This result shows that the non-lesional skin of HE is impaired independent of the AD status. However, more studies are needed to investigate this further.

For the difference between non-lesional and healthy skin of HE with AD we, however, found a higher expression of both CCL17/TARC and CCL27/CTACK in the non-lesional skin areas, showing the AD print in non-lesional skin of the HE^{+AD} patients. It should be noted that the patients included in our study in general had a high HE severity score (HECSI), which might affect this finding.

The molecular difference between HE with and without concomitant AD

On both the RNA and protein levels, we found molecular differences between the skin of HE patients with and without AD (HE^{+AD} and HE^{-AD},respectively). Interestingly, on the RNA level, the difference between the two subtypes were largest in the non-lesional skin areas, whereas the

difference was largest in the lesional skin areas at the protein level. Whether this reflects the proteins being better at reflecting an immunologic difference than RNA is still to be investigated. However, based on the differentially expressed markers we believe this is a technical difference.

At the protein level, we found a higher expression of structural proteins such has FLG2 and LOR as well as the immunologic proteins SERPINB6, SERPINB9 and SPINK5 in the HE^{AD+} group. The higher expression of FLG2 is in contrast with previous studies showing a decreased amount of FLG2 in AD skinand an association between the loss of FLG2 and the persistence of AD[228,229]. If this difference is due to the samples being from different skin areas, and that FLG2 plays a different role in the skin of the hands is still to be investigated. Our findings indicate a lower level of LOR in the hands of HE patients with no AD, and it will be interesting to see if this finding can be reproduced in future studies.

At the RNA level, we found an increased expression of *S100A12*, *MMP9, CXCL8/IL-8* and *IL-1B*in HE^{+AD}. These are all involved in the immunologic cascade, and as the difference is on non-lesional skin areas, it again reflects the different skin barrier in AD and healthy skin.

Though the findings on mRNA and protein level are difficult to compare, both studies show the potential to differentiate subtypes of HE with the tape strip samples.

7.4 The molecular markers of etiological- and clinical subtypes of HE assessed by tape strip samples

In *manuscript II*, we assessed the transcriptome differences of etiological- as well as clinical subtypes of HE. As elaborated in the *Considerations on methodology* section (Section 6), the patients were not stratified for etiology or clinical subtype during the recruitment. Therefore, the patient groups with single etiologies and clinical subtypes became relatively small. This, however, reflects the complexity of the clinic, making real-life clinical investigations like this very important.

RNA from the tape strip samples showed promising results in identifying molecular markers that distinguish ICD from ACD. The genes did not include markers known to be involved in dermatitis, and their role in contact dermatitis should be investigated further.

For the clinical subtypes of HE, we assessed the difference between chronic fissured and vesicular eczema. A total of 240 genes were upregulated showing the potential of making molecular subtyping based on RNA from the tape strips. However, for both the etiology and clinical subtypes, more studies are needed to validate our findings.

8. Conclusion

In this thesis, it was shown that though a global difference between skin biopsies and tape strip samples exist,tape strip samples could be used to assess the transcriptome and proteome of different skin areas (healthy, non-lesional and lesional). Furthermore, it was concludedthat tape strip samples could be stored at room temperature for up to three days before RNA extraction. In addition, in *manuscript I*, it was found that RNA from tape strips could be used to assess the human and microbial transcripts of the skin using the correct extraction and library build strategy.

In *manuscript II and III*, it was concluded that tape strips could be used to assess the molecular markers of the palmar- and dorsal aspects of the hands, as well as the molecular markers of different subtypes of HE. Furthermore, the potential of the tape strip samples to identify novel markers of HE was shown. The findings are summarized in figure 14.

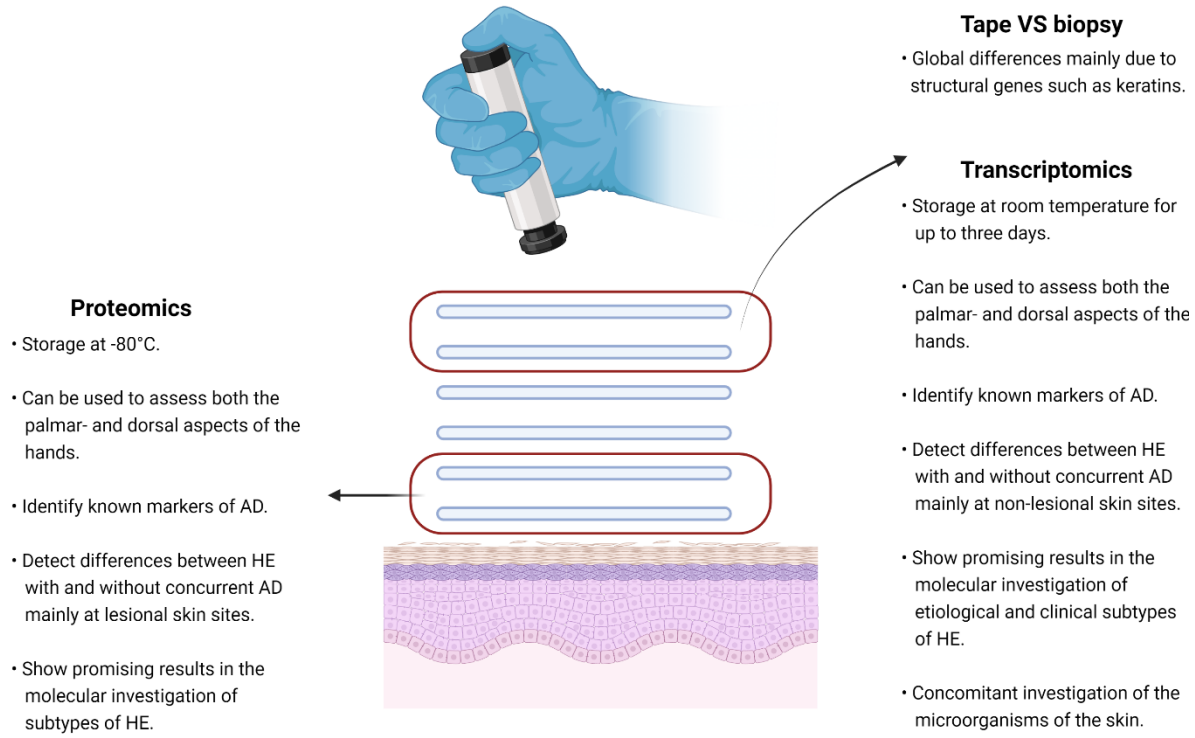

Figure 14

9. Future perspectives

The immunology of skin diseases is very complex. Though much research has already been done, the emergence of new treatments poses a need to refine our understanding of the specific subtypes of AD and HE. High-throughput investigations of the full gene- and protein expression of a disease can shed light on the immunology by connecting the phenotype of a disease to a molecular pattern.

Molecular investigations of the subtypes of very heterogeneous diseases such as HE require large study groups. Though skin biopsies enable the research of all skin layers, it can be difficult to recruit many study participants. Furthermore, there is a risk of recruitment bias towards patients with severe eczema, especially if many skin samples are to be taken. Some skin areas are also very difficult to obtain skin biopsies from in a research setting. These include the hands, face and samples from children. The non-invasive tape strip technique causes no harm to the patient and no specially trained personnel is needed to take the samples. Depending on the number of consecutive tape strips to be obtained, the sampling method is also very time efficient in a busy clinic. However, with the drawback that recent application of topicals can make adherence of the tape disk difficult.

As the SC has a fast regeneration, the tape strip sample enables follow-up studies on the same skin area[67,68,230]. This combined with the storage at room temperature and easy sampling procedure opens for large-scale, high-throughput studies with out-patient sampling. The studies in this thesis investigated the molecular markers from the first two tape strips, and therefore this procedure would be very applicable in an out-patient sampling setting.

AD often starts in early childhood, and studies investigating the skin of children before or right after the eczema starts will be very valuable in understanding the initiation of the disease. The tape strip sample with room temperature storage would enable researchers to set up large studies where parents could sample from the children's skin at home. This combined with clinical follow-ups by a medical doctor, e.g. upon the first sign of skin disease, would be very valuable. Furthermore, the COVID-19 pandemic significantly increased the prevalence of HE in children in Denmark, and it would be very interesting to explore the pathology of the HE in children, which could prove to be difficult if skin biopsies were to be taken[231,232].

The studies included in this thesis we showed that a reliable molecular print of the skin could be obtained from even a few tape strips. However, it was also shown that a global difference between the gold standard skin biopsy and the tape strip samples exists. Only a few studies have investigated

these differences, and even though these studies have shown the tape strip to be a very reliable skin sampling method, it is still not known exactly from where the nucleotides and amino acids from SC derive[69,71,233]. We investigated both the transcriptome and proteome of HE and for future studies it will be very relevant to explore the correlation between mRNA and proteins from tape strips in more detail.

In *manuscript I and II* tape strips were stored at room temperature for up to three days before RNA extraction. Even though, the obtained RNA could be used to perform RNA-seq, it would be very valuable to explore if the storage of tape strip samples could be optimized to get a larger RNA yield and obtain longer RNA fragments. Therefore, studies storing tape strips at different conditions, including RNA preserving buffers, at different storage lengths should be performed in the future to potentially improve on the method.

Most studies today use the easily available D-squame™ tape strip disks.These disks are, however, not sterile, and other alternatives with greater tensile strengths are on the market[162]. Though not as easy to implement in a clinical study an investigation of the transcriptome and proteome differences according to tape type would be very valuable.

The studies included in this thesis show the great potential of the non-invasive tape strip technique to refine our understanding of skin disease from even sensitive skin areas such as the hands. The convenience of the method presented in our papers will enable researchers to plan large studies with patient groups that has otherwise been difficult to recruit. This can lead to the discovery of new disease subtypes enabling a more targeted treatment in the future.

10. Manuscripts

In this section the manuscripts included in this thesis can be found. Supplementary material can be acquired upon request.

10.1 Manuscript I: *The stratum corneum transcriptome in atopic dermatitis can be assessed by tape stripping*

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Original Article

The stratum corneum transcriptome in atopic dermatitis can be assessed by tape stripping☆

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ABSTRACT

Background: Skin biopsies represent a gold standard in skin immunology and pathology but can cause pain and induce scarring. Non-invasive techniques will facilitate study recruitment of e.g. patients with paediatric atopic dermatitis (AD), hand eczema or facial dermatitis.
Objective: By RNA sequencing, we examined whether the stratum corneum transcriptome in AD skin can be assessed by tape stripping, as compared to the epidermal transcriptome of AD in skin biopsies. To make the procedure clinically relevant tape strips were stored and shipped at room temperature for up to 3 days.
Methods: Nine adult Caucasian AD patients and three healthy volunteers were included. Tape samples were collected from non-lesional and lesional skin. Biopsies were collected from lesional skin and were split into epidermis and dermis. Total RNA was extracted, and shotgun sequencing was performed.
Results: Shotgun sequencing could be performed on skin cells obtained from two consecutive tape strips which had been stored and shipped at room temperature for up to three days. The most prominent differences between the tape strip and biopsy derived transcriptome were due to structural genes, while established molecular markers of AD, including CCL17, CCL22, IL17A and S100A7-S100A9, were also identified in tape strip samples. Furthermore, the tape strip derived transcriptome showed promise in also analysing the skin microbiome.
Conclusion: Our study shows that the stratum corneum (SC) transcriptome of AD can be assessed by tape stripping the skin, supporting that this method may be central in future skin biomarker research.
NCBI GEO data accession: GSE160501

1. Introduction

Non-invasive tape stripping has been used to collect skin cells from the stratum corneum (SC) for several experimental purposes, e.g. detection of inflammatory cytokines [1–3]. More recent research has focused on RNA purification for detection of specific biomarkers of inflammatory skin diseases from tape strip samples [4–9].

RNA sequencing provides insight into the global gene expression pattern in a sample. RNA can be purified from tape strips [4,6,10–13], but traditionally only already known biomarkers have been investigated by e.g. qRT-PCR, whereas full sequencing of the transcriptome is expected to provide further insight and potentially allow for better disease subcategorization.

Atopic dermatitis (AD), a common skin disease of childhood and adulthood [14], is caused by a complex interaction between environmental and genetic factors that lead to skin barrier dysfunction and increased type 2 immunity. New endophenotype

Abbreviations: AD, atopic dermatitis; DEG, differentially expressed genes; EASI, eczema area and severity index; FDR, false discovery rate; GO, gene ontology; GTF, gene transfer format; IPA, ingenuity pathway analysis; L, lesional; NL, non-lesional; PCA, principal component analysis; RIN, RNA integrity number; STAR, spliced transcripts alignment to a reference; SC, stratum corneum.

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0923-1811/

classifications of AD would allow for more personalized and efficient treatment [15–19].

In this study, we examined if the transcriptome could be sequenced from skin cells from SC obtained by tape stripping and compared the results to those of concomitant analysis of skin biopsies. To make the procedure clinically relevant tape strips were stored and shipped at room temperature for up to 3 days improving the easiness of shipping for this sample method.

2. Materials and methods

2.1. Study participants

Nine Caucasian patients with chronic AD (56 % males, mean age 44.7 (20–64)) and three Caucasian young healthy volunteers (67 % males, mean age 22.7 (22–23)) with no previous history of skin disease were included. The patients were recruited from the AD clinic at Herlev-Gentofte Hospital, University of Copenhagen, Denmark. Oral and written consent was obtained from all trial participants before inclusion. The study was conducted according to the Declaration of Helsinki principles and after permission had been given by the Ethics Committee of Copenhagen (H-16050507) and the Danish Data Protection Agency (HGH-2017-073).

All patients fulfilled the Hanifin and Rajka criteria, and dermatitis severity was scored by the Eczema Area and Severity Index (EASI) (67 % had hand eczema, the overall mean EASI 10.2 (1–23.1)) [20,21]. A table of the demography of the study participants can be found in Table A. 1.The patients completed a questionnaire where they reported their use of topical and systemic treatments.

Patients could continue ongoing systemic and topical immunosuppressive treatment; however, all study participants were asked not to use topical emollients 24 h prior to the study day.

2.2. Skin samples

Skin samples were obtained by tape stripping and 4 mm skin biopsies. From AD patients, tape- samples were collected from both lesional (L) and non-lesional (NL) skin areas. Furthermore, a biopsy was collected from lesional skin areas. Lesional samples were obtained from an area with most prominent dermatitis and with an area large enough to include both a biopsy and tape sample. Patients had samples taken from their dorsal hands (n = 6) or other body sites (n = 3, back and arms) according to the dermatitis localization. Non-lesional tape samples were taken from a skin area where the patient had no visible dermatitis.

From the healthy volunteers, both tape strips and biopsies were obtained from the ventral side of the forearm.

Tape stripping was performed as previously described [22]. Two D-squame standard sampling discs (Monaderm, Monaco, France) were applied consecutively to the skin area, using a new piece for each application. For RNA extraction, the two tapes were pooled for analysis when loaded on the column.

Tape strip samples were stored dry at room temperature (approximately 21°C with no major temperature changes) in separate RNA free Eppendorf® LoBind microcentrifuge tubes (Sigma-Aldrich Denmark, Søborg, Denmark) ensuring no contamination of the samples. RNA from tape samples was extracted within three days from the time of sampling.

Before the skin biopsies were taken the skin were locally anesthetized with lidocaine-adrenaline (SAD 20 mg/5mikrog/mL). Skin biopsies were immediately put on dry ice and kept at −80 °C. Before extraction of RNA from the skin biopsies, these were divided into epidermis and dermis by incubation in a 3.8 % ammonium thiocyanate solution (VWR International, Søborg, Denmark) in Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich Denmark, Søborg, Denmark) for 30 min at room temperature followed by manual separation [23,24]. RNA was extracted immediately after division of the biopsies.

2.3. Full transcriptome sequencing

Total RNA was extracted using the Exiqon miRCURY™ RNA Isolation Kit - Cell and Plant (Exiqon, now Qiagen Denmark, Copenhagen, Denmark) following the manufacturer's recommendation, with additional heating of the lysis buffer to 56 °C for 5 min before adding 70 % ethanol (Qiagen Denmark, Copenhagen, Denmark). This has previously been suggested to improve the extraction and recovery of RNA from challenging samples [25]. Library preparation was performed with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian kit (Takara Bio Europe, Saint-Germain-en-Laye, France) following the manufacturer's recommendation without fragmentation. To capture all RNA transcripts, the kit uses random hexamer primers for amplification. The protocol included removal of ribosomal RNA. For comparability, the same protocol was used for all sample types.

Single-end sequencing (75 bp) was performed with a NextSeq 500 (Illumina Denmark, Copenhagen, Denmark) with an average sequencing depth of 89.9 M reads per case. Following quality assessment, alignment, and exclusion of non-coding transcripts an average of 3.5 M protein-coding reads per case was included in the analysis.

BCL files were converted to *FASTQ* files with the *bcl2fastq* software (Illumina, California, USA). To allow short fragments to be analysed, the automatic adapter trimming of the NextSeq 500 was disabled, and manual adapter removal performed. Adapters as well as consecutive stretches of low-quality bases (Q < 30) from the 5′ and 3′ termini were removed using *AdapterRemoval version 2.1.3* [26]. A length threshold of 20 bp was used for read removal. Reads were aligned using *STAR* (Spliced Transcripts Alignment to a Reference) version 2.5.3a with default settings [27] where reads with multiple alignment were discarded. For alignment, the Gene transfer format (GTF) annotation file for the hg38 reference genome (from the GENCODE consortium release 27) were used [28]. RNA fraction types were identified and labelled according to the transcript biotypes in GENCODE [29] and Ensembl.

2.4. Microbial transcripts

Due to the extraction and library build strategy, we were also able to investigate the microbial representation of reads of the samples used for the transcriptomic investigation. Reads were screened to match microorganisms using Kraken, a program that assign taxonomic labels to DNA sequences to assess the presence of microorganisms in a sample [30].

2.5. Statistics

The statistical analysis was conducted in R (R core team, version 3.6.0, http://www.R-project.org/), and data were visualized in Qlucore Omics Explorer v. 3.6 (Qlucore AB, Lund, Sweden), including principal components analysis (PCA), heatmaps and unsupervised hierarchical clustering.

Analysis of methodological differences (tape vs. biopsy) of RNA fraction characteristics and microbial reads were conducted by a paired Wilcoxon signed rank test and a significance threshold of α=0.05.

2.6. Normalization and differential gene expression analysis

For the statistical analysis, genes where all samples had 1 or 0 read counts were excluded. Normalization and differential expression analyses were carried out with DESeq2 package

(version 3.10) distributed by Bioconductor [31]. Data were transformed by the variance stabilizing transformation (vst) function of DESeq2, with "blind" set to "FALSE". The statistical significance was adjusted for multiple testing by estimating false discovery rates (FDR) and benjamini-Hochberg correction. PCA and heatmaps were generated using the vst transformed data in Qlucore.

A gene is considered differentially expressed if an observed difference in normalized read counts between two experimental conditions is statistically significant ($p < 0.05$) and has a relevant effect size (>2-fold).

For the statistical analysis, one non-lesional tape sample was excluded due to low number of reads (<0.5 M), and another lesional and non-lesional tape sample were excluded due to contamination of the samples.

2.7. Ingenuity pathways analysis

To investigate the underlying molecular mechanisms, Ingenuity pathway analysis (IPA, Qiagen, Redwood City, California) was conducted. Core analysis with a threshold log2fold change of -1.5 to 1.5 for the intra-sample (lesional vs non-lesional/healthy within a sample type) and -2 to 2 for the analysis of sample methods (biopsy vs tape) was conducted.

3. Results

3.1. Full transcriptome sequencing was successfully performed on RNA from tape strips

We successfully extracted RNA for transcriptome sequencing from two tape strips (tape 1 + 2) stored and shipped at room temperature (1–3 days). No difference in the obtained transcriptome in regard to time from tape stripping to RNA extraction was observed (data not shown). Despite poor RNA quality (mean RNA integrity number (RIN) 2.5, range 1.8–3.1) as compared to skin biopsy samples (mean RIN 4.3, range 1.9–6.1), both the number and quality of reads from the tape-derived RNA was high. A schematic overview of the different RNA fractions can be seen in Fig.1 (a full overview of the different RNA fractions can be found in Table A.2).

Overall, the percentage of uniquely mapped reads was significantly higher in tape strip samples compared to biopsy derived dermis ($p < 0.05$) and epidermis ($p < 0.01$) RNA, while the percentages of exonic reads of the uniquely mapped reads were lower for the tape as compared to those of dermis and epidermis ($p < 0.01$). The total number of exonic reads was in average 2.2 million for all tape samples and 5.5 million and 5.1 million for the epidermal and dermal samples, respectively. The percentage of protein coding reads was similar in dermis and tape samples, but statistical significantly higher for the epidermis samples ($p < 0.01$). No difference was observed between the average mapped read length (67–69 bp) ($p > 0.05$) for all sample types (Table A.2).

These findings showed that the RNA from SC obtained by tape stripping could be used to assess the transcriptomic profile of the cells in the SC.

3.2. Differences between the tape strip and biopsy derived transcriptome

When examining the PCA plot based on the 4454 most variable genes across all samples (σ/σ max >0.245), PC1 (66 %) separated the samples by sampling method (tape stripping vs. skin biopsy both epidermis and dermis), whereas PC2 (10 %) separated the samples based on disease status (healthy vs. AD skin (Fig. 2a)). The difference between the sampling methods is also reflected in the 2-way unsupervised hierarchical clustering of the same data (Fig. 2b). Among the top DEGs between tape strip and biopsy samples were structural genes from all layers of the epidermis, such as *KRT1, KRT2, KRT9, KRT10, KRT14* (lower expression in tape strip samples) and genes related to diverse, more general cellular mechanisms as well as IL8 and IL36A (higher expressed in tape strip samples) (Table A.3). When the data were divided by sampling method, the PC1 separated the samples according to disease status (Fig. 2c-e). Furthermore, when investigating the lesional and non-lesional tape samples, PC1 clearly separates the samples by histology (Fig. 2f).

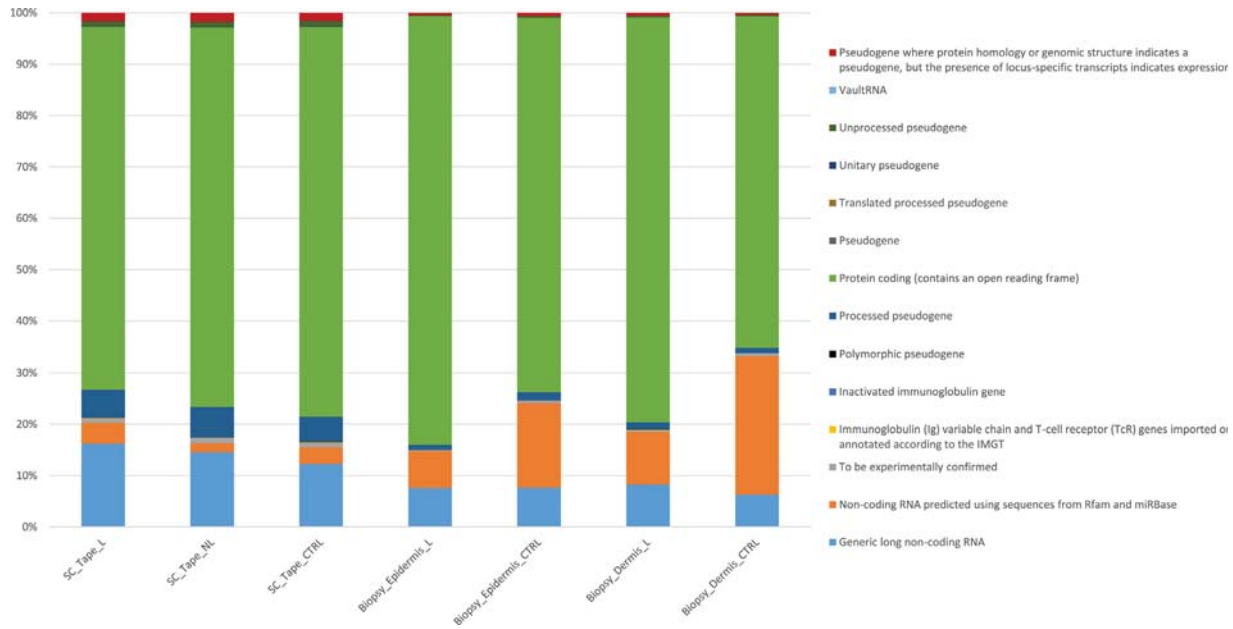

Fig. 1. Stat chart showing the different RNA fractions of the exonic reads found for tape strip and biopsy samples. The charts are divided by sampling method and skin area. For all sample types the largest fraction of the exonic reads is the protein coding (having and open reading frame (ORF)). Interestingly, the tape strip samples have a larger fraction of generic long non-coding RNA.

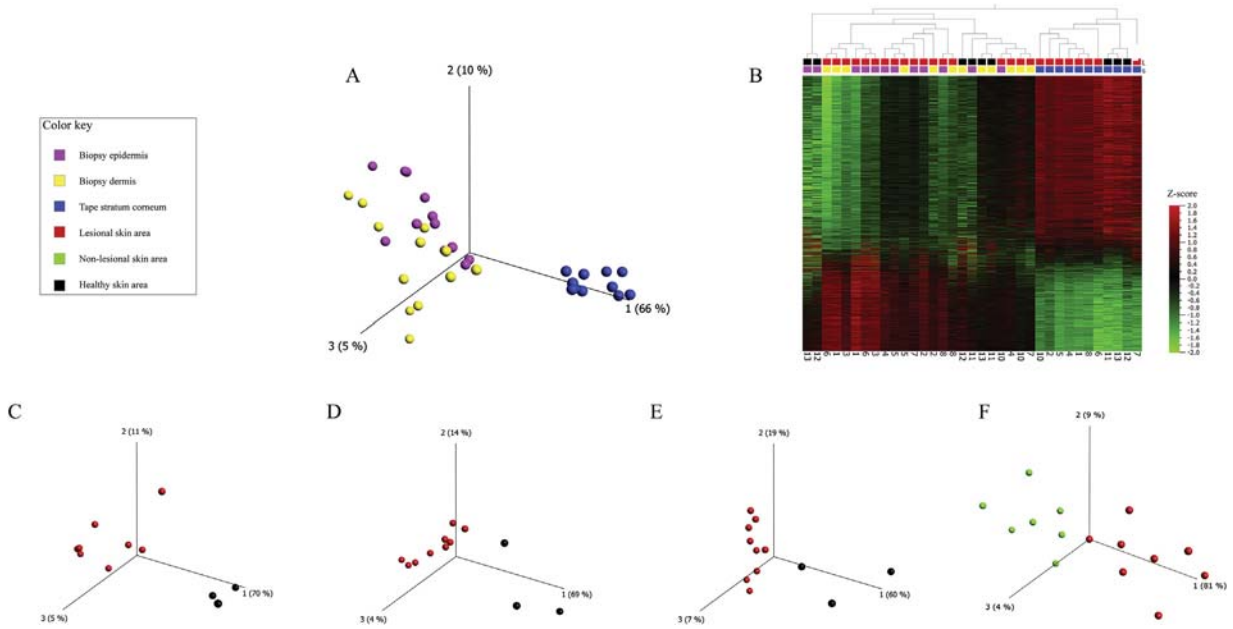

Fig. 2. A) Principal component analysis (PCA) plot based on the 4454 genes with the largest variation among all lesional and healthy samples. The figure shows that the main variation in the dataset (66 %) can be explained by the sampling method. B) Unsupervised hierarchical clustering and heat-map based on the 4454 genes with the largest variation among all samples. The heat-map shows clear clustering according to sampling type (tape strip vs. biopsy samples) and secondary clustering according to skin area (lesional vs. healthy). C) PCA plot based on the 765 genes with the largest variation among lesional and healthy tape samples. The plot shows that the tape samples clearly (70 %) separate atopic from healthy skin areas. D) PCA plot based on the 266 genes with the largest variation among lesional and healthy epidermal biopsy samples. The plot shows that the main variation (69 %) can be explained by skin area (lesional vs. healthy). E) PCA plot based on the 1077 genes with the largest variation among lesional and healthy dermal biopsy samples. The plot shows that the main variation (60 %) can be explained by skin area (lesional vs. healthy skin). F) PCA plot based on the 108 genes with the largest variation in a paired analysis between lesional and non-lesional tape samples. The plot shows that the main variation (81 %) can be explained by the skin histology.

3.3. Successful separation of biopsy samples into epidermis and dermis

In a paired analysis followed by hierarchical clustering of the epidermal and dermal part of the biopsies from lesional and healthy skin respectively, we observed a clear separation of the two layers with enrichment for epidermis-specific genes such as keratins, FLG and LOR for the epidermal part and enrichment for fibroblast-specific genes such as collagens for the dermal part (Figure A.1). Therefore, the downstream analysis was carried out with the epidermal part of the biopsies.

Furthermore, in silico rRNA depletion were performed and the data analysis included the protein coding subset of the transcripts.

3.4. Skin relevant molecules can be detected and assessed by tape stripping

We investigated the gene expression level of well-known markers of AD [32,33]. Molecular markers of: Th_2 (*MMP12, IL13, CCL11, CCL17, CCL22, IL31, IL5, IL10, IL4,* and *CCL13*), Th_1 (*CXCL10, MX1, IL1B, CCL2, IL13RA2,* and *CXCL9*), Th_{22} (*IL22*), Th_{17} (*IL19, CXCL1, PI3, IL17A, CCL20,* and *LCN2*) as well as IL-17/IL-22 induced inflammatory markers (*S100A7-S100A9*) was all found in both SC tape samples and epidermal biopsy samples. Furthermore, molecular markers of terminal differentiation *(LOR, IVL, PPL, FLG, FLG2,* and *SPRR4*) and proliferation (*ki67/MKI67* and *KRT16*) as well as *POSTN, NTRK2,* and *CXCL8* were also found in tape samples (Figs. 3 and 4 (heatmap) and A.2 (scatterplots)).

To further address if tape strip samples can be used to assess the disease state, we investigated the top upstream regulators of the gene expression pattern of the lesional/healthy contrast by IPA. This analysis links DEGs in the dataset to experimentally identified upstream regulators of gene expression patterns. The top 5 upstream regulators of DEGs between lesional and healthy skin for both the tape strip and the epidermal biopsy samples had known regulatory effects of molecules already known to be involved in skin development or immunological pathways related to dermatitis (Table A.4).

3.5. AD relevant pathways can be assessed by both SC tape strip and epidermal biopsy samples

IPA analysis revealed that the top canonical pathways of the gene expression pattern difference between lesional and healthy skin, for both SC tape strip samples and epidermal biopsy samples, were biologically relevant and included immunological pathways of skin disease (Table 1). Besides pathways related to more general immunological pathways such as antigen presentation and interferon signaling, Wnt/β-catenin signaling and Graft-versus-host disease signaling was among the IPA top canonical pathways. Wnt/β-catenin signaling has been shown to have relevance in the induction of inflammation in keratinocytes in psoriasis [34], whereas graft-versus-host disease has pathway similarities of dermatitis by the activation of the JAK pathway.

3.6. The gene expression profile from the tape strip samples can separate other AD datasets

In order to confirm the AD-specific gene expression profile obtained by the tape strip method in this study, we investigated how well it separated other transcriptomic data on AD. In the study by Tsoi et al. 2019 (PMID: 30641038, deposited in GEO as GSE121212 [35]), biopsies from 27 AD patients and 38 healthy controls were investigated. The SC tape strip derived gene expression pattern of the lesional/non-lesional contrast in this

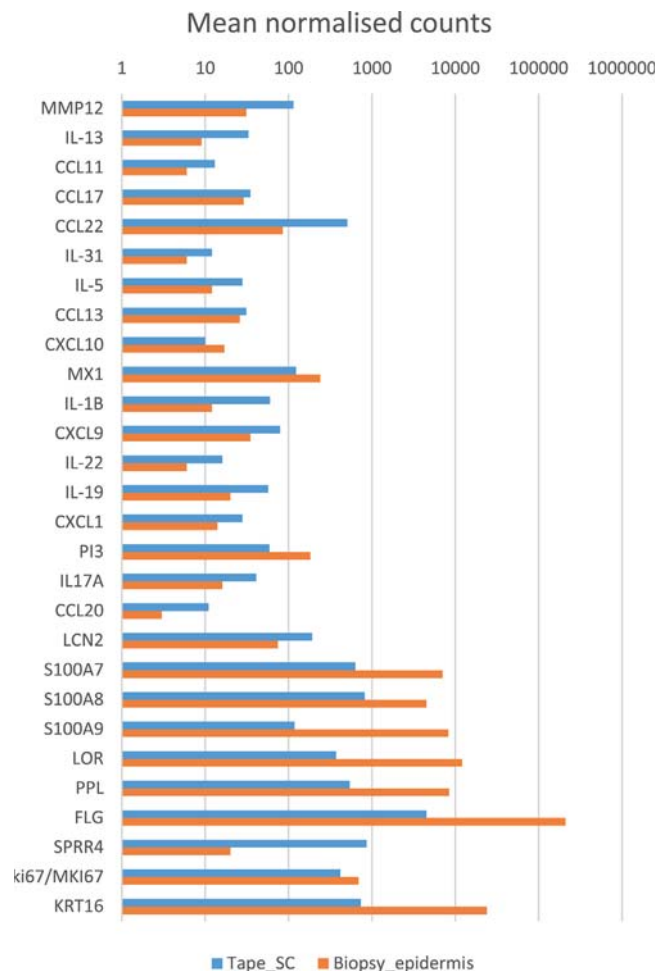

Fig. 3. Mean normalized counts (DESeq2) of selected biomarkers of AD for lesional tape strip SC and biopsy epidermis samples. The correlation coefficient for each gene is shown in parentheses.

study was able to both successfully separate healthy from atopic skin based on 126 DEGs ($p < 0.05$, >2-fc) and to cluster lesional from non-lesional samples, confirming the gene expression profile's biological relevance for AD in and independent dataset (Figure A.3). In addition, the gene expression profile of our L/NL signature from the tape strips successfully separated the dataset from Dyjack et al. [4] containing tape strip data from L and NL sites of 15 AD patients as well as the epidermal laser capture microdissection data from Esaki et al. 2015 (GSE120721) containing L and NL samples from 5 AD patients [36] (Figure A.4 and A.5).

The gene expression profile from the tape strip samples show differences between lesional and non-lesional skin histologies

SC tape strip derived gene expression profiles of the contrast between non-lesional and lesional AD skin showed clear relation to dermatologic disease and immunological as well as inflammatory functions. The gene expression differences were driven by genes known to be differentially expressed in lesional versus non-lesional skin, including S100A proteins, CCL22, and IL36 G [37] and can be seen in the IPA top network for the contrast being: "*Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease*" (Figure A.6).

3.7. Tape strip samples can be used to assess microorganisms of the skin

The use of random hexamer primers for RT-PCR as part of the library build enabled us to investigate the microbial fraction of our samples. The microbial results showed a statistical significantly ($p < 0.005$) larger fraction of the reads, being microbial from the tape samples (lesional and healthy, mean fraction of the total reads being microbial 6.4 %, range 0.02–30 %) as compared to the epidermal biopsy samples (lesional and healthy, mean fraction of the total reads being microbial 0.07 %, range 0.02−0.2%). The top 10 microbial species from tapes were skin relevant, with similarity to previously shown data from AD patients [38], with the most prominent species being *Cutibacterium acnes*, whereas those found in the biopsy samples were less skin relevant (e.g. *Choristoneura occidentalis granulovirus*) (Table A.5). The Shannon Weiner diversity index ranged from 0.61 to 3.29 (Table A.6). In Fig. 5, the relative abundance of the top 10 mostly expressed species is shown.

4. Discussion

We showed that RNA from two consecutive skin tape strips, kept at room temperature for several days before RNA extraction, could be used successfully for full transcriptome sequencing and has clear practical advantages in a clinical setting. The integrity of the obtained transcriptome was supported by several other datasets. Overall, analysis of SC RNA resulted in identification of well-known molecular markers of AD, including CCL17/TARC. Further, we showed that the skin microbiome could be assessed from tape strip samples.

Both RNA from the tape strip and skin biopsy samples could differentiate healthy from diseased skin, with the epidermal part of the biopsy showing the clearest pattern of differentiation. This is most likely explained by the increased immune activity found in this skin layer. For all sample types, healthy and diseased skin were characterized by genes related to keratinocytes, angiogenesis and inflammatory processes, which is in line with the pathogenesis of AD [39,40].

Based on the PCA representation of the dataset, we observed that PC1 clustered the samples primarily by the sampling method (biopsy vs. tape strip). Since the expression levels of more than 5000 genes differed between the two sampling methods, this suggests a "global" effect, which was found mainly to be due to differences in the levels of noncoding RNA, and RNA associated with structural genes such as keratins and genes related to ribosomes. Due to these methodological differences, care should be taken when choosing a skin sampling method.

The tape strip method collects cells from the SC of the skin, where no de novo synthesis of proteins or RNA occurs. Therefore, the RNA found in the skin cells collected by this method is most likely fragmented RNA originating from the deeper skin layers of epidermis or infiltrating immune cells.

Interestingly, we found that the average number of uniquely mapped reads was higher for samples obtained by tape stripping than those from the biopsy. Longer fragments could have explained this; however, we found the average input length to be similar among samples. Therefore, this difference is probably due to technical differences rather than biologically differences. Additionally, we found that the percentage of intronic reads was higher in tape strip samples compared to those in the biopsies, indicating that the RNA from SC contain less mature mRNA than that of the deeper layers. DeLuca et al. suggest that a high level of intronic reads is due to DNA contamination [41]. As the samples were extracted following the same protocol, DNA contamination should not differ between the sampling methods. Gaidatzis et al. suggested that the level of intronic reads is correlated with the transcriptional activity [42], but in this case, it is far more likely

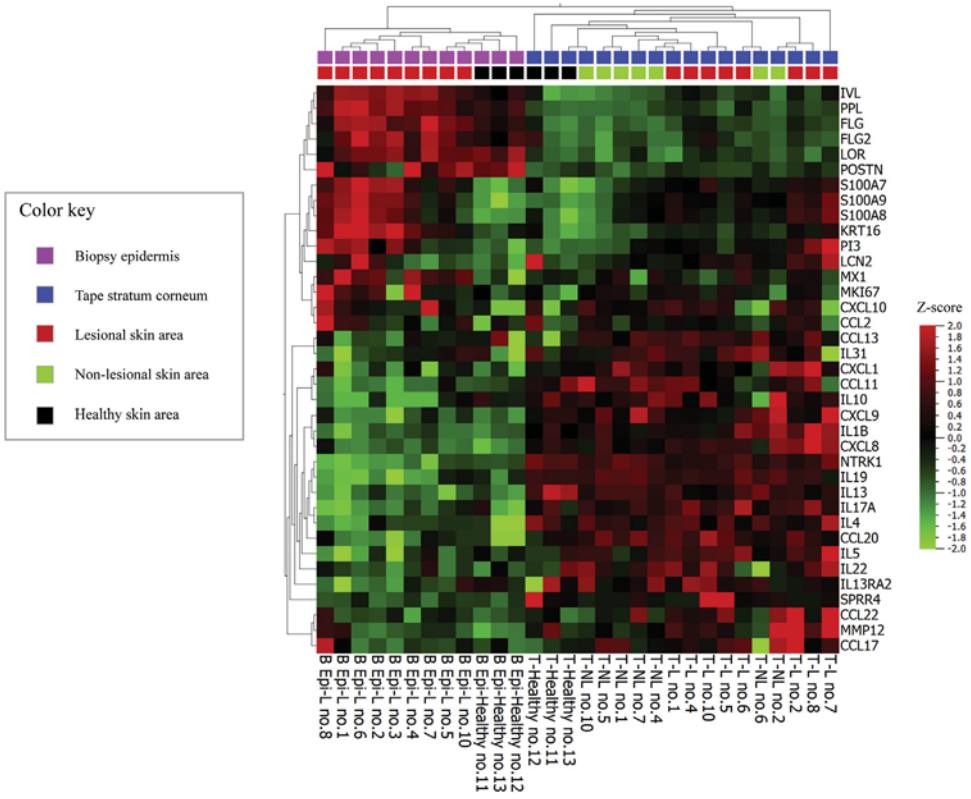

Fig. 4. Heatmap of 37 selected AD genes. AD-specific genes are detected both by biopsy epidermis (lesional and healthy) and tape stripping stratum corneum (lesional, non-lesional, and healthy skin areas).

Table 1
Top canonical pathways (IPA) of the gene expression profile contrast between lesional and healthy skin histologies. The table shows the top canonical pathways for both SC tape strip and epidermal biopsy samples. All pathways are biologically relevant for the immunology of skin disease. The p-value tell if there is a statistically significant overlap between the dataset genes and the genes that are regulated by a known regulator gene from IPA.

Lesional vs. healthy SC tape samples		Lesional vs healthy epidermal biopsy samples	
Top canonical pathway	p-value	Top canonical pathway	p-value
Communication between innate and adaptive immune cells	1.42E-08	Glucocorticoid receptor signaling	3.99E-08
Antigen presentation pathway	3.64E-07	Interferon signaling	7.80E-05
Graft-versus-host disease signaling	2.36E-06	Antigen presentation pathway	8.70E-04
Dendritic cell maturation	1.41E-05	Wnt/β-catenin signaling	6.94E-04
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	3.86E-05	Production of nitric oxide and reactive oxygen species in macrophages	1.74E-03

that the higher level of intronic reads for the tape samples was due to biological differences still to be explained, e.g. intron retention in SC due to the stop of enzymatic degradation of intronic reads as the corneocytes become anucleated.

Besides investigating the human transcriptome, the protocol used in this study random hexamer primers for library build enabled us to make additional investigations of the skin microbiome. This has previously been investigated on DNA level [43], however, here we show that the investigation can be performed in parallel with the transcriptomic analyzis of the human skin cells. A statistically significant higher number of microbial reads was found in the SC tape strip samples as compared to those found in the epidermal biopsy samples. Bacteria in the SC tape samples were skin relevant, whereas those identified in the epidermal biopsy samples were low in abundance and not skin relevant; therefore, the latter might be due to reagent contamination. Furthermore, the microbial diversity found in SC tape strip samples showed a clinically relevant AD pattern including elevated levels of *Staphylococcus aureus* and *Staphylococcus epidermidis* in AD skin [44]. It is important to note that our protocol was not optimized for microbial reads, and therefore, the number of microbial reads could most likely be optimized by using a targeted microbiome protocol.

In this study, only three young healthy controls were included. For an in-depth investigation of the differences between healthy and AD skin, more healthy controls must be included. However, despite the small number of controls, we showed a strong tendency towards differential transcriptional activity between healthy and diseased skin. By using commercially available kits specific for low input and degraded RNA, the sequencing protocol was optimized for tape strip samples, and therefore, it can be questioned if the full potential of the biopsy samples was obtained. Furthermore, the ammonium thiocyanate separation of epidermis and dermis may have degraded the RNA from the biopsy samples as suggested by the low RIN values for these samples. However, as expected, the RNA was less fragmented in the biopsy samples compared to that obtained from the tape strip

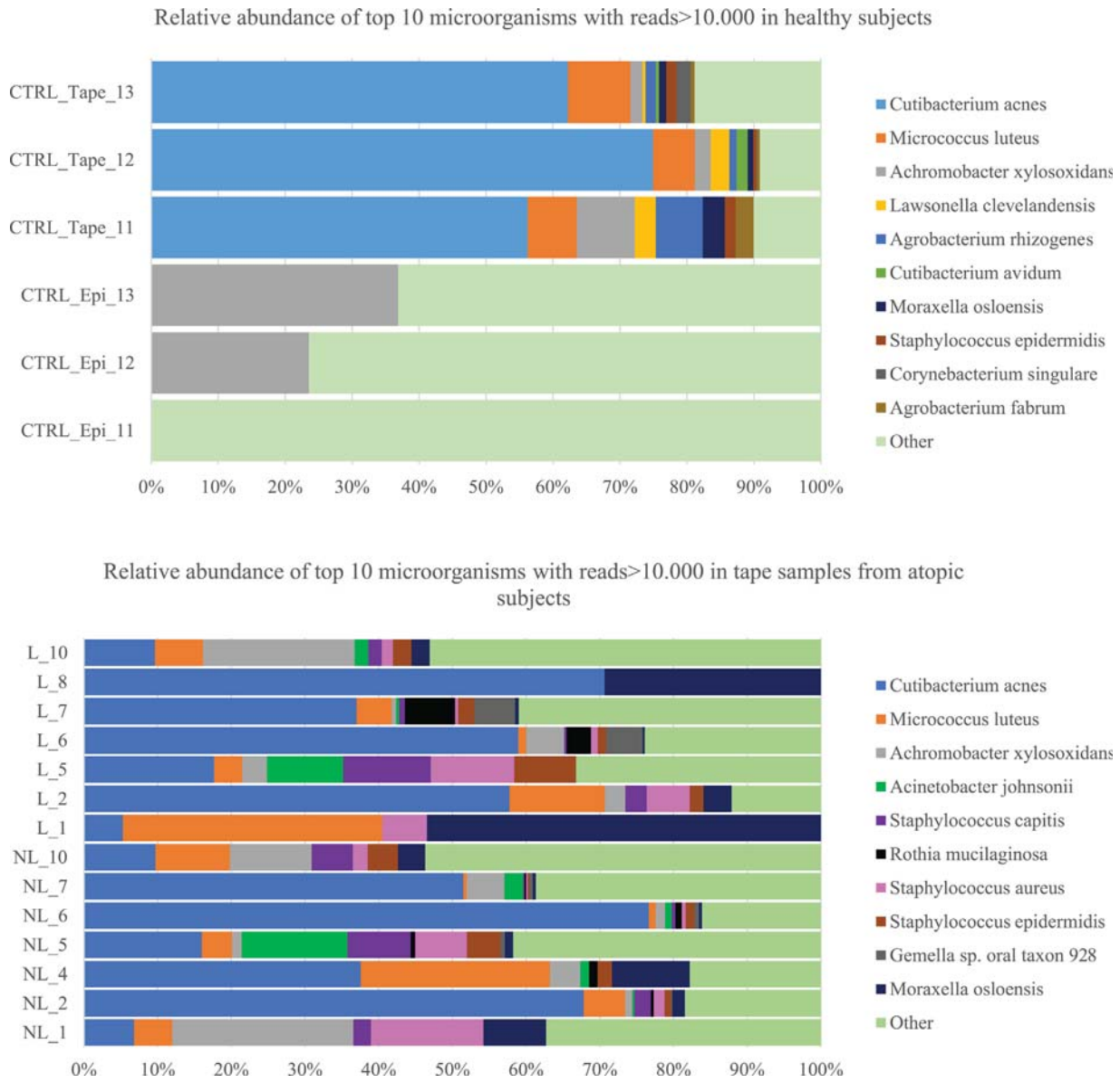

Fig. 5. Relative abundance of the top 10 microorganisms with "number of reads covered by the clade rooted at this taxon" over 10.000. Data is generated from whole transcriptome data assessed by Kraken. A) Relative abundance of top 10 species of epidermal biopsy samples and SC tape samples from healthy subjects. B) Relative abundance of top 10 microorganisms of lesional and non-lesional SC tape samples from AD patients.

samples. The patient group in this study was relatively small and heterogenous, and for future studies, investigating if the tape strip method can be used to asses different endotypes of AD, a larger group of patients is preferred. For future work an explorative investigation of the proteomic profile obtained by tape stripping, e.g. by mass spectroscopy, would further highlight the use of tape strips for an in depth investigation of the biomarkers of the skin.

We showed that the SC transcriptome could be determined by analyzing two tape strips kept at room temperature for several days making the method highly practical in a clinical setting as well as showing that there is potential for the tape method to be used for outpatient sampling. The difference between the transcriptome of SC and biopsies was mainly caused by structural genes. Known markers of AD were confirmed using our protocol, and we predict a significant potential of tape strips as a future, non-invasive clinical tool to identify subtypes of AD.

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Declaration of Competing Interest

Thomas Litman is employed both by KU and by LEO Pharma. The other authors declare no conflict of interest.

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Appendix A. NCBI GEO data accession: GSE160501

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10.2 Manuscript II: *The transcriptome of hand eczema assessed by tape stripping*

Title: The transcriptome of hand eczema assessed by tape stripping

Running title: The transcriptome of hand eczema

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Abbreviations

Allergic contact dermatitis (ACD)

Atopic dermatitis (AD)

Differentially expressed gene (DEG)

Gene ontology (GO)

Hand eczema (HE)

Hand eczema severity index (HECSI)

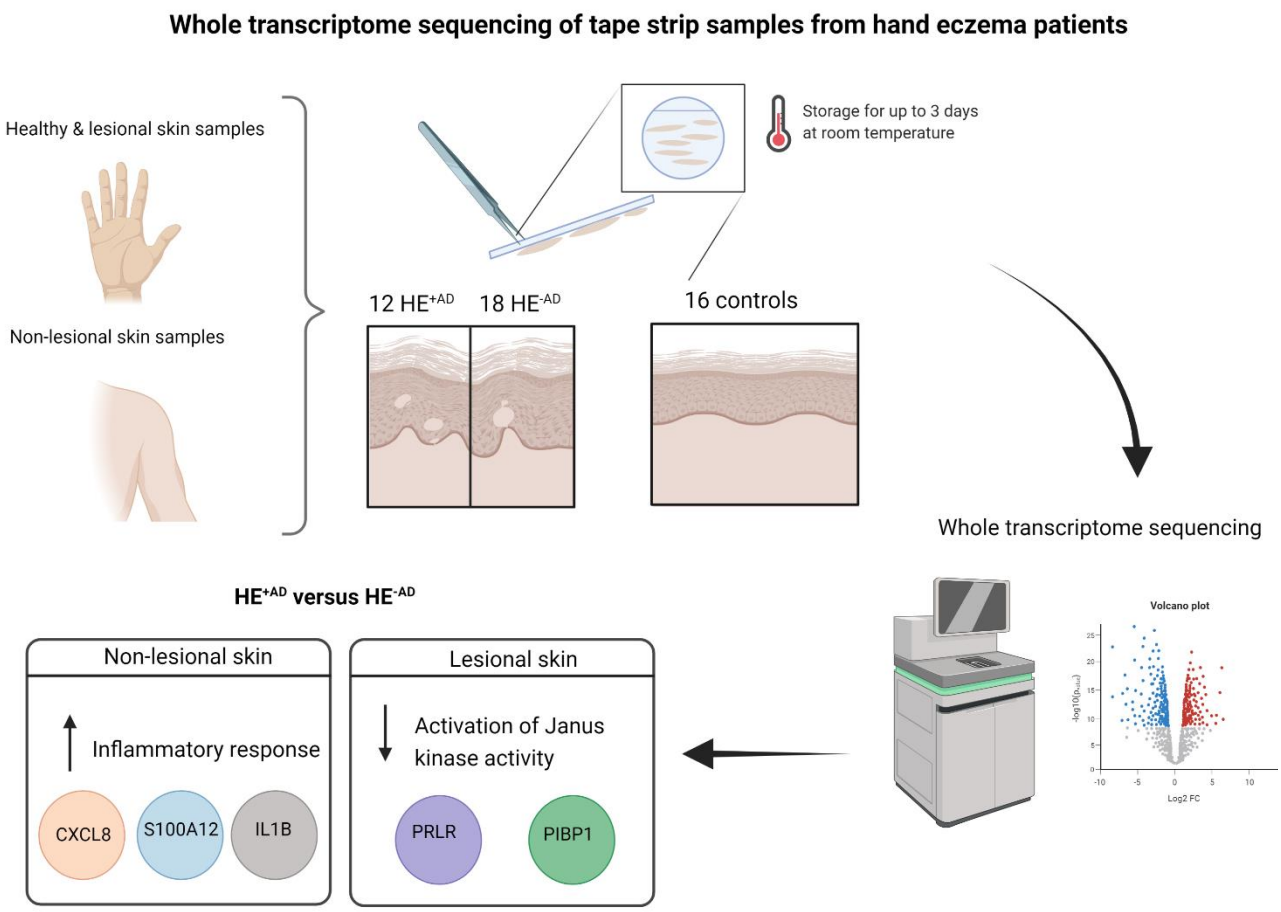
Hand eczema with atopic dermatitis (HE^{+AD})

Hand eczema without atopic dermatitis (HE^{-AD})

Irritant contact dermatitis (ICD)

Whole transcriptome sequencing (WTS)

Graphical abstract



Key findings:

- Whole transcriptome sequencing can be performed on RNA from tape strips from different localizations on the hands, from both hand eczema (HE) patients and healthy controls.

- The transcriptomic difference between HE with and without atopic dermatitis (AD) can be assessed by tape stripping and include an increase of inflammatory markers for HE patients with AD.

- Skin tape strip samples identify transcriptomic differences between irritant- and allergic contact dermatitis on the hands.

Abstract

Background: No biomarkers have been identified that can classify subtypes of hand eczema (HE). Although skin biopsies represent the golden standard for investigations of the skin, the invasive technique is not favorable when investigating skin from sensitive areas. Recent advances in the use of skin tape strips for molecular investigations enable non-invasive investigations of HE.

Objective: By using whole transcriptome sequencing (WTS), the molecular profile of HE according to different localizations on the hands, etiologies, and clinical/morphological subtypes was investigated.

Methods: Thirty adult, DanishHE patients, 12 with and 18 without concurrent atopic dermatitis (AD),as well as16 controls were included. Tape strip samples were collected from lesional, non-lesional, and healthy skin. Total RNA was extracted, and WTS was performed.

Results: The largest molecular difference of HE patients with and without AD was found in non-lesional skin areas and included a downregulation of *CXCL8*for HE patients without AD.Differences between allergic- and irritant contact dermatitis included promising epidermal biomarkers such as *EPHA1*.

Conclusion: Skintape strip samples could be used to assess the gene expression profile of HE on different localizations of the hands. The skin tape strip method identified new molecular markers that showed promising result for the identification of HE subtypes.

Key words: Atopic hand eczema, contact dermatitis, subtypes of hand eczema, tape stripping, transcriptomics

Introduction

Hand eczema (HE) is a prevalent disease with a 1-year prevalence of 9% in the general population.[1] It may affect quality of life, impact work ability,[2,3] and require treatment periodically or continuously, depending on severity and chronicity.[4]

HE may be a result of different etiologies[5], which complicates both effective treatment and prevention. HE can be a part of atopic dermatitis (AD), and/or environmental factors, such as exposures to allergens and/or irritants, may resultin allergic contact dermatitis (ACD) and/or irritant contact dermatitis (ICD) on the hands. The skin impairment of AD also makes the skin more vulnerable to irritants,sometimes leading to a mixed pattern of AD and ICD.

Diagnostic criteria exist for the different etiological subtypes. ACD can be diagnosed by a combination of patch testing and exposure analysis,[5] AD has distinct disease characteristics, such as early onset, and can be diagnosed by fulfillment of the HanifinRajka criteria,[6] while ICD depends on exclusion of ACD and exposure analysis demonstrating significant exposures to irritants. There are currently no biomarkers in use for supporting the diagnosis of these etiological subtypes in the routine investigation of patients with HE. In addition, subtypes exist, with so far unknown etiology.Some have distinct morphology including palmar hyperkeratotic HE and recurrent vesicular HE, whereas other have no apparent detectable causal factors.[5]Also, anatomical patterns exist, so that some patients primarily have a dorsal and others a palmar pattern.[7]

Recent advances in the use of skin tape strips to obtain valid skin samples now enable research within immunotypes and specific biomarkers.[8–12] In a previous study, we found that the stratum corneum transcriptome in AD could be assessed by tape stripping of the skin.[10]Based on a paired comparison with skin biopsies from the same patients, we identified established molecular markers of AD in the tape samples. In the current study, we are taking the next step by investigating if differences in the transcriptome can be detected by tape strippingof HE patients with different anatomical patterns, of different etiology, and main morphologies.

Materials and methods

Study population

Thirty adult, Danish, patients with HE were recruited from the Department of Dermatology and Allergy, Herlev-Gentofte hospital, Denmark, between March 2019 and September 2020. The

patients included eightpatients with current AD,four with a history of AD (HE^{+AD})and 18 with HE and no history of AD (HE^{-AD}).

Furthermore, 16 age-matched adult controls with no history of inflammatory skin diseases or other atopic diseases (asthma or hayfever) were included. All participants were asked to abstain from use of topical anti-inflammatory treatment and emollients for 24 hours before inclusion. Patients did not use any systemic therapies for their HE or AD. Other exclusion criteria included ongoing infections, pregnancy, lactation, or use of antibiotics, phototherapy, or self-tanners within four weeks of sample collection. Current AD was diagnosed by a physician at the Department of Dermatology and all patients with AD fulfilled the HanifinRajka criteria.[6] Clinical severity scores included the Hand Eczema Severity Index (HECSI)[13] for all patients and the Eczema Area and Severity Index (EASI)[14] for patients with concomitant activeAD. Furthermore, clinical-morphological subtypes of HE were evaluatedaccording to the classification by Menné et al.[5]All participants answered a detailed questionnaire including questions on use of emollients, and for the patients, the duration of their hand eczema.

Health care data relevant to the patients' etiological classification of HE was extracted from electronic health records (EPIC). The etiological classification of HE included irritant contact dermatitis (ICD), allergic contact dermatitis (ACD), atopic HE (AHE), and protein contact dermatitis (PCD). If the HE could not be etiologically classified according to these four sub-diagnoses or had more than one sub-diagnosis, the HE was grouped as 'etiologically unclassifiable' or 'mixed etiology' respectively. The ACD sub-diagnosis was given if the patient had at least one clinically relevant type IV allergy. The ICD sub-diagnosis was given if the patient had a clinically relevant exposure to irritants.[5] In Supplementary Table 1 ,an overview of the exposures to irritants and/or allergens relevant for the HE can be seen. No patients were diagnosed with PCD.

The characteristics of the study population can be seen in Table 1. Based on the questionnaire, 67% of the HE^{+AD} patients reported that their HE started before the age of two, whereas 89% of the HE^{-AD}patients reported that their HE started when they were 18years or older. A majority of patients reported that the eczema was present almost all the time (HE^{-AD} 92% and HE^{+AD} 75%), and more than half of the patients reported that the eczema had been present all the time within the last year (HE^{-AD} 56% and HE^{+AD} 58%). A full overview of the self-reported duration of HE can be seen in Supplementary Table 2.

The study was approved by the local ethics committee(H-16050507) and the Danish Data Protection Agency (HGH-2017-073), oral and written consent was gathered before inclusion, and the study followed the Helsinki declaration.

Skin samples

Skin samples of both lesional and non-lesional stratum corneum were taken from the patients by tape stripping.[15] The lesional tape strips were taken from alesion on the most affected area of the hands(palmar or dorsal aspects). The non-lesional skin sample was taken from the upper arm. From thecontrols, a healthy skin sample was taken from the hands (palmar or dorsal aspects). From each sample site, two consecutive standard D-squame tape strips (Monaderm, Monaco, France) were collected applying uniform pressure as previously described.[10,15]For RNA extraction,both tapes were used. The samples were stored and shipped at room temperature, and RNA was extracted within three days from sampling.

RNA extraction and whole transcriptome sequencing

RNA was extracted with the miRNeasy Micro Kit from Qiagen (Qiagen Denmark, Copenhagen, Denmark) following the manufacturer's instruction. RNA from the two tapes was pooled directly on the column after phase separation.

For whole transcriptome sequencing (WTS), the library build was performed with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian kit (Takara Bio Europe,Saint-Germain-en-Laye, France) following the manufacturer's recommendation without fragmentation.

Paired-end sequencing (2 x 100 bp) was performed on a NovaSeq 6000 (Illumina, California USA) using the NovaSeq 6000 S2 Reagent Kit v1 (Illumina, California USA). The sequencing resulted in an average sequencing depth of 82.7M reads per case.

Quality assessment and alignment of sequencing reads were performed as previously described.[10]

Normalization and differential expression analysis

Gene counts were normalized and analysed by the Bioconducter package DESeq2 (version 1.30.1) using 'apeglm' for LFC shrinkage.[16]A gene was considered as a differentially expressed gene (DEG) if an observed difference in normalized read counts between two experimental conditions had a Benjamini-Hochberg adjusted p-value <0.1 and a log2 fold change > 1 or < -1. For the visual

examination, data was transformed by the variance stabilizing transformation (vst) function of DESeq2, with “blind”set to “FALSE”.

Cluster, gene ontology (GO) and keyword analysis were conducted with STRING (Version 11.0, https://version-11-0.string-db.org/).

Data visualization including principal component analysis (PCA), heatmaps, and unsupervised hierarchicalclustering were performed in Qlucore Omics Explorer v. 3.6 (Qlucore AB, Lund, Sweden).

Statistics

The statistical analyses were conducted in R (R core team, version 4.0.4, http://www.R-project.org/).

Differential analyses were performed between skin areas in patients (lesional, non-lesional) and compared with controls (healthy skin), anatomical localizations on the hands (palm and dorsum), AD status (HE^{-AD} and HE^{+AD}), HE etiologies (ACD, ICD and AD), and clinical HE subtypes.

For the investigation of the gene expression differences between the etiologies, patients with a single etiology were included (ACD n=5, ICD n=5, and AD n=6) (Table 1). Patients grouped as “etiologically unclassifiable” or having a mixed etiology were not included in this data analysis.

The investigation of clinical HE subtypes included groups with at least three patients and only one clinical subtype, which was only fulfilled by: chronic fissured hand eczema and recurrent vesicular hand eczema.

For the statistical test between RNA parameters and individual biomarker levels,Wilcoxonrank sum test was performed.

Results

Whole transcriptome sequencing on RNA from tape strips from the hands

WTS was performed ina total of 76tape strip samples (a lesional and non-lesional sample from each HE patient and a healthy sample from each control) (Figure 1A). Seven samples were subsequently excluded due to quality issues as assessed by the FastQC reports (fourlesional and three non-lesional samples). With the removal of these, a total of 69 samples were included in the subsequent analyses (26 lesional, 27 non-lesional, and 16 healthy samples).

The mean number of protein coding read counts (having an open reading frame) was 2.7 million for all samples (median [IQR]: 0.7 million [0.4-3.2 million]), with no statistically significant difference ($p > 0.05$) between the skin areas (average;lesional: 2.2 million, non-lesional: 3.1 million, healthy: 1.1 million)(Supplementary Figure 1A).

The average input read length was the same across skin sites, however, the average mapped read length showed a statistically significant decrease ($p < 0.05$)from lesional to non-lesional and healthy skin sites, respectively (Supplementary Figure 1B and C). No difference was observed between the number of intronic reads according to skin areas (Supplementary Figure 1D).

Transcriptional differences between dorsum and palm were higher for eczema patients

The 16 control samples included six samples from the dorsum and 10 from the palm. The 26 lesional samples included seven samples from the dorsum and19from the palm (AD^{+HE}: 6 dorsal and 5 palmar; AD^{-HE}: 1 dorsal and 14 palmar).

For the healthy skin samples nostatistically significant difference in the number of protein coding reads between the localizations (dorsal and palmar aspects) on the hands were observed ($p=0.64$), however, for the lesional samples, a statistically significant higher number of protein coding reads were observed for the dorsal aspects of the hands ($p=0.035$) (Supplementary figure 2).

Only a few DEGs were detected between dorsum and palm (15 for healthy skin samples and 63 for lesional skin samples) (Figure 1B and C respectively), and the genes did not show enrichment for specific biological processes (data not shown). In addition,PCA did not show apparent clustering related to the dorsal- or palmar aspects of the hands for neither skin area (Supplementary Figure 3A and B respectively).

Tape strip samples show the immunology of lesions on the hands

The main transcriptomic differences between skin areas (healthy, non-lesional, and lesional) were found between lesional and non-lesional as well as healthy skin (PC1: 28%), whereas the non-lesional and healthy skin samples had similar overall gene expression profiles (Figure 2A, and Supplementary Figure 3).

When investigating the number of DEGs between skin areas, the largest difference in gene expression - based on numbers of DEGs between the skin areas - was found between lesionaland healthy skin areas (2 884 DEGs, 1 355 up and 1 529 down in lesional skin). The second largest difference was observed betweennon-lesional and healthy skin areas (1 012DEGs, 697 up, 315 down in non-lesional skin).The difference between patients'lesional and non-lesional skin areas included 620 DEGs (273 up, 347 down in lesional skin) (Figure 2B). Figure 2C shows the top 25 DEGs between healthy and lesional skin areas. Of these 25 genes, 10 were differentially expressed between all skin contrasts (healthy vs non-lesional, healthy vs lesional, and non-lesional vs lesional). The 10 genes enriched for gene ontologies important for inflammation including "cytokine activity" (GO:0005125) and "antigen processing and presentation of exogenous peptide antigen via MHC class II" (GO:0019886).

Looking at the genes common to the specific skin areas the genesassociatedwith healthy skin included *COL4A2, KRT14* and *S100A*genes(Supplementary Dataset 1), genes associatedwith to non-lesional skin included hair genes (*KRTAPs*) and *IL18 (*Supplementary Dataset 2), and genes associatedwith to lesional skin areas including*CXCL8, S100A8* and *CCL17* (Supplementary Dataset 3) (Figure 2B and Supplementary Figure 4).

The most prominent difference between HE^{+AD} and $HE^{-AD}$was at the non-lesional skin sites

Looking at the number of DEGs between HE^{+AD} and HE^{-AD} the greatest difference was observed for the non-lesional skin sites (211 DEGs) as compared to the lesional skin site (28 DEGs) (Figure 3A). The difference between non-lesional skin of HE^{+AD} and HE^{-AD}showed anincrease in several inflammatory markers including*S100A12, MMP9,CXCL8,* and *IL1B* and a decrease in *PRLR* and *PIBP1*related to the"activation of Janus kinase activity" (GO:0042976) for lesional the skin.

Next, we investigated if the overall transcriptomic difference between non-lesional and healthy skin was driven by the HE^{+AD} patients (Supplementary Figure 5).In general, the genes specific for the difference between non-lesional and healthy skin for HE^{+AD} and HE^{-AD} respectively, did not enrich

for immunologic pathways (data not shown), however, the specific genes for the HE^{+AD} non-lesional and healthy skin difference included known markers of AD and general inflammation such as *MMP12*, *CCL17*, *CCL27*, and several *S100A* genes.[17,18] The 315genes common tothe non-lesional and healthy skin difference of HE^{+AD} and HE^{-AD}included several genes important for the immunology of eczema lesions including *SPRR*- and *S100A* genes, *IL18*, and *CCL22*.[19] Furthermore, the common genes enriched for several immunological processes included interferon-gamma-mediated signaling pathway (GO:0060333) and antigen processing and presentation of exogenous antigen (GO:0019884).

The transcriptome differs between atopic hand eczema and irritant contact dermatitis

When investigating the difference between lesional skin sites according to the etiologies of AD, ACD, and ICD only, we saw that the greatest difference was found between AD and ICD (32 DEGs), followed by the difference between ACD and ICD (six DEGs). Only one (*NRK*),and not skin relevant gene, was differentially expressed between AD and ACD.

Of the 32 DEGs between AD and ICD, 25 genes were upregulated, and seven genes were downregulated in AD (Supplementary table 3). When investigating the gene ontologies related to the 25 upregulated genes, the top three GO enrichment was related to response to interferon-alphaand mast cell activation (cellular response to interferon-alpha; GO:0035457, response to interferon-alpha; GO:0035455, and mast cell activation; GO:0045576). For the two other differences, too few genes were differentially expressed to conduct gene enrichment analysis.

When looking at the six genes differentially expressed in our study between ACD and ICD we found markers that could have potential for the distinction between ACD and ICD, including *EPHA1* and *ACTN3*. The genes, however, did not distinguish e.g. ACD from AD and the mixed etiologies (Supplementary figure 6).

The tape strip samples detect gene expression differences between clinical subtypes of hand eczema

Next, we investigated if the tape strip samples detected gene expression differences according to the clinical subtypes of HE.

A total of 248 DEGs were detected between chronic fissured and vesicular eczema (240 upregulated and eight downregulated in chronic fissured eczema) (data not shown). The 240 upregulated genes did not correlate to any gene enrichment, however, the eightdownregulated genes enriched for epidermis development (GO:0008544), cornification (GO:0070268), and tissue development (GO:0009888), mainly due to the involvement of *KRT4, KRT78, RPTN*,and *EMP1*.

Discussion

Tape strip skin samples can be used to assess the gene expression profile of different localizations on the hands. We showed that the most notable differences between HE^{-AD} and HE^{+AD} were found in non-lesional skin areas. The tape strip skin samples detected transcriptomic differences between etiologies of HE as well as some clinical-morphological subtypes. There seems to be a potential use of non-invasive tape strip samples for detecting new biomarkers to facilitate the molecular investigation of the different subtypes of HE.

To our knowledge, the transcriptomic differences according to localizations on the hands have not yet been investigated in spite of apparent differences such as the epidermis of the palm being thicker than at other localizations on the hands.[20] We saw no difference in the number of protein coding reads from the tape strip samples of healthy skin from different localizations on the hands(palm and dorsum).In contrast, for lesional samples, a statistically significant higher number of protein coding reads was detected from the dorsal samples, but the transcriptomic profile of the palm as compared to the dorsum did not show differences related to specific biological processes from either skin area. This shows that the tape strip samples can be used to investigate the transcriptome of the hands independent of the localization.

When investigating the molecular markers related to the different skin areas (lesional, non-lesional, and healthy) we found that *S100A* genes were related to both lesional as well as healthy skin. *S100A* genes are known to be important for the inflammatory process of AD,[21–23] and have also been shown to be increased in lesions of HE,[24] however they are also expressed in epidermis of healthy skin.[25] Our findings suggest that care should be taken when interpreting the biological role of these molecular markers on hand skin, and further studies are needed to establish the role of different *S100A* genes . The genes related to lesional skin also included *CCL17*, one of the most reliable biomarkers of AD.[17,23] Though *CCL17* has also been shown to correlate to severity of AD in tape strip samples,[26] we did not see a correlation between *CCL17* and *HECSI* in this study(data not shown). Whether this is due to the scoring system or the biomarker level of *CCL17* from the hands needs further investigation.

A recent study by Kumari et al. investigated the protein expression of selected molecular markers of HE and their relation to treatment with alitretinoin.[27] In that study, they found CLDN1, LOR, FLG, KRT10,and TSLP to be related to the lesional skin of HE. In our study, we only rediscovered a

difference between healthy and lesional skin for*CLDN1*. If this is due to the difference in study population, sample type (biopsy versus tape stripping) or proteins versus mRNA is not known.

The largest difference between HE with and without AD was found at the non-lesional skin sites. Non-lesional skin areas of AD patients have previously been shown to have a distinct molecular pattern as compared to healthy skin.[28,29] We therefore tested if the difference between non-lesional and healthy skin was greater for the HE^{+AD} group. When investigating the difference between non-lesional and healthy skin for the two HE subtypes we could see that the HE^{+AD} group indeed showed common markers of AD, however the genes showing enrichment for inflammatory processes wereshared between the two HE subtypes. This indicates that in the HE^{-AD} patients, a general skin inflammation was observed even at skin areas distant from the active eczema on the hands.

In this study, HE^{+AD} was defined as HE with current or previous AD.[30] This definition includes historic AD in the HE^{+AD} group though it might not be relevant for their active HE. When considering the etiology of the current HE lesion, only relevant AD was considered, however, for only two HE^{+AD} patients their AD was not considered relevant for their HE lesion supporting the importance of the impaired skin barrier of AD patients.[31]

When investigating the patients' current etiologies, we found that the largest transcriptomic difference was between AD only and ICD only. This difference included an upregulation of genes that enriched for the biological process of response to interferon-alpha and mast cell activation, both important for an inflammatory process. This finding suggests that AD^{+HE} patients have a larger activation of mast cells as well as interferon-alpha.

A recent study investigated if machine-learning-driven biomarker discovery could differentiate ACD from ICD.[32] The study was based on applications of known allergens or irritants and was therefore not based on samples from a clinical setting. Furthermore, the skin samples were obtained by skin biopsies, and therefore included the deeper layers of epidermis and dermis. The study identified 21 genes that in combination made up 28 gene-sets that were able to distinguish the two etiologies. In our study we did not rediscover transcriptomic differences between the biomarkers identified in this study (Supplementary Table 4). This could be due to the differences between gene expression of full biopsy samples vs. stratum corneum tape samples where a major global difference is expected.[10]

Six genes were differentially expressed between ACD and ICD. These genes included *EPHA1*, which has been found to be an important marker for a normal epidermal differentiation.[33,34] Though the role of EPHA1 in contact dermatitis is not known, the results may have importance for the distinction between ACD and ICD. It should be noted, however, that the biomarkers found in this study had difficulty distinguishing ACD and ICD to the mixed etiologies. Therefore, to study this in detail, larger, more homogenous patient groups should be included to validate this based on the specific etiologies, as well as protein contact dermatitis, which was not included in the current study.

There are some limitations to our study; first, sample size, even though we aimed at fairly large groups for a study of this nature, the mixed etiologies and morphologies meant that the number of patients with some pure forms were limited. We defined HE^{+AD} as HE with current or previous AD.[30]This definition includes historic AD in the HE^{+AD} group though it might not be relevant for their active HE. However, when considering the etiology of the current HE lesion, only relevant AD was considered. We took the non-lesional sample distant from the lesional samples, this may have caused confounding effects, however it can be difficult to be sure that samples are truly non-lesional, if obtained from skin areas adjacent to the inflammatory area on the hands.

This study shows that the tape strip method is a valuable tool to investigate the transcriptomic differences between HE with and without AD independent of the localization of the lesion. The use of non-invasive skin sampling of the hands enables large-scale studies of HE with no harm to the patients. Furthermore, the non-invasive tape strip samples show potential for the investigation of the molecular profile related to different etiologies as well as clinical subtypes of HE.

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Tables

Table 1.Characteristics of study participants. The study included 12 hand eczema (HE) patients with a history of AD (HE^{+AD}), 18 HE patients with no history of AD (HE^{-AD}) and 16 age matched controls. Severity of the eczema was measured by the Hand Eczema Severity Index (HECSI) and Eczema Area and Severity Index (EASI).

	All patients n=30	HE^{+AD} n=12	HE^{-AD} n=18	Controls n=16
Sex, female (% female)	21 (70.0)	12 (100.0)	9 (50.0)	10 (62.5)
Age, median [IQR]	59.0 [44.3; 66.0]	59.0 [37.0; 66.0]	57.5 [48.5; 64.0]	59 [49.0; 64.0]
HECSI, median [IQR]	56.0 [34.0; 136.5]	58.5 [37.5; 144.5]	56.0 [33.0; 129,0]	-
EASI, median [IQR]	-	14.1[6.7; 22.4]	-	-
Etiologies of HE				
Atopic HE	6	6	0	-
Irritant contact HE	5	0	5	-
Allergic contact HE	5	1	4	-
Mixed etiology	7	5	2	-
Etiologically unclassifiable	7	0	7	-

Figures

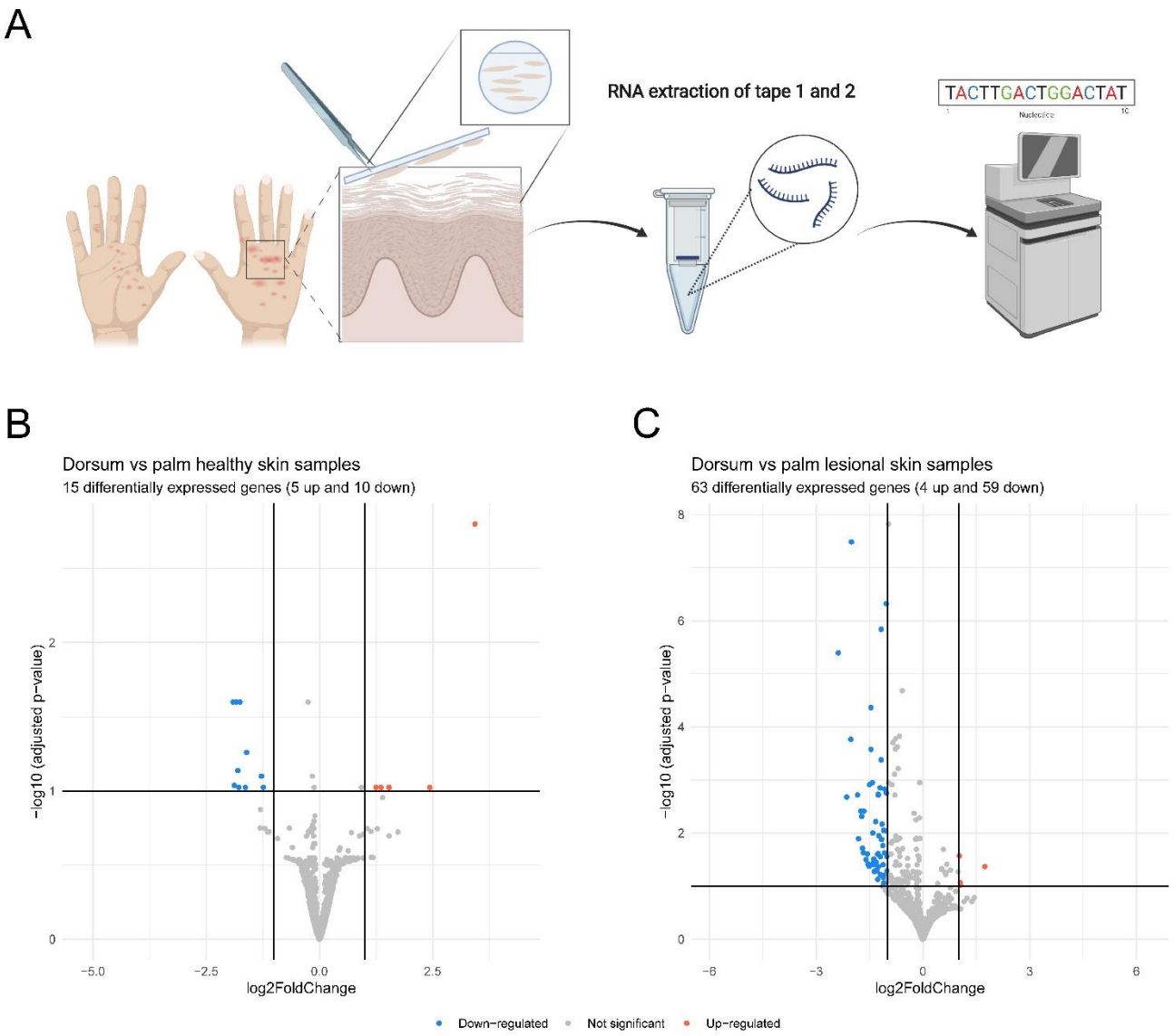
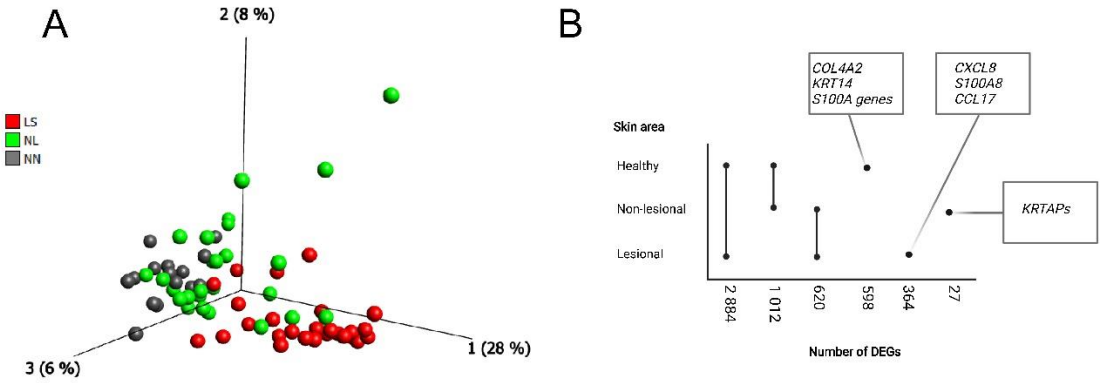


Figure 1. A) Tape strip samples were taken from the hands (a lesional from the patients and a healthy from the controls). Furthermore, a non-lesional tape strip sample was taken from the upper arm of the patients. Whole transcriptome sequencing was performed on RNA extracted from the first two tapes, shipped, and stored at room temperature. B) Differential analysis of dorsum versus palm of healthy skin samples resulted in 15 differentially expressed genes (5 up and 10 down) (adjusted p-value < 0.1, Fold change > 2). B) Differential analysis of dorsum versus palm of lesional skin samples resulted in 63 differentially expressed genes (4 up and 59 down) (adjusted p-value < 0.1, Fold change > 2). Created with Biorender.



C

Top 25 protein coding genes according to skin type

Genes	LSvsNN	NLvsNN	LSvsNL
BIRC3	**	**	**
BTN2A1	**	-	-
CCL22	**	**	**
CD74	**	**	**
ETV3	**	**	-
GOLGA6L2	**	-	**
HEPHL1	**	**	**
HLA-DRA	**	**	**
PDZD4	**	-	**
IK	**	**	-
IL36RN	**	**	**
ISY1	**	-	-
CNTD1	**	-	**
KYNU	**	-	-
LCE3A	**	**	**
MUC3A	**	**	-
RGS1	**	**	**
UBE2J2	**	-	-
SERPINB3	**	**	**
SERPINB4	**	**	**
PIGH	**	-	**
OGFR	**	-	-
TMSB4X	**	**	-
STAT6	**	-	-
VIM	**	**	-

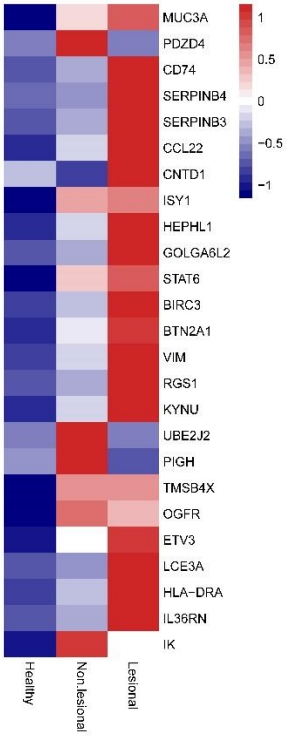


*Figure 2 A) The principal component analysis (PCA) shows that the largest difference (PC1) between skin areas (lesional (LS), non-lesional (NL) and healthy (NN) skin is found between lesional and healthy/non-lesional skin. B) A total of 2 884 genes are differentially expressed between lesional and healthy skin, 1 012 genes between healthy and non-lesional, and 620 genes between non-lesional and lesional skin areas. The diagram also shows that 364 genes were related to lesional skin, 598 to healthy skin, and 27 to non-lesional skin areas. C) The heatmap shows the mean normalized count of the different skin areas for the top 25 genes differentially expressed between lesional and healthy skin (defined by the smallest Benjamini-Hochberg corrected p-values and a fold change>2) scaled by gene. The table show the adjusted p-values (**=<0.001). Ten of the 25 genes are differentially expressed between all skin area differences. Created with Biorender.*

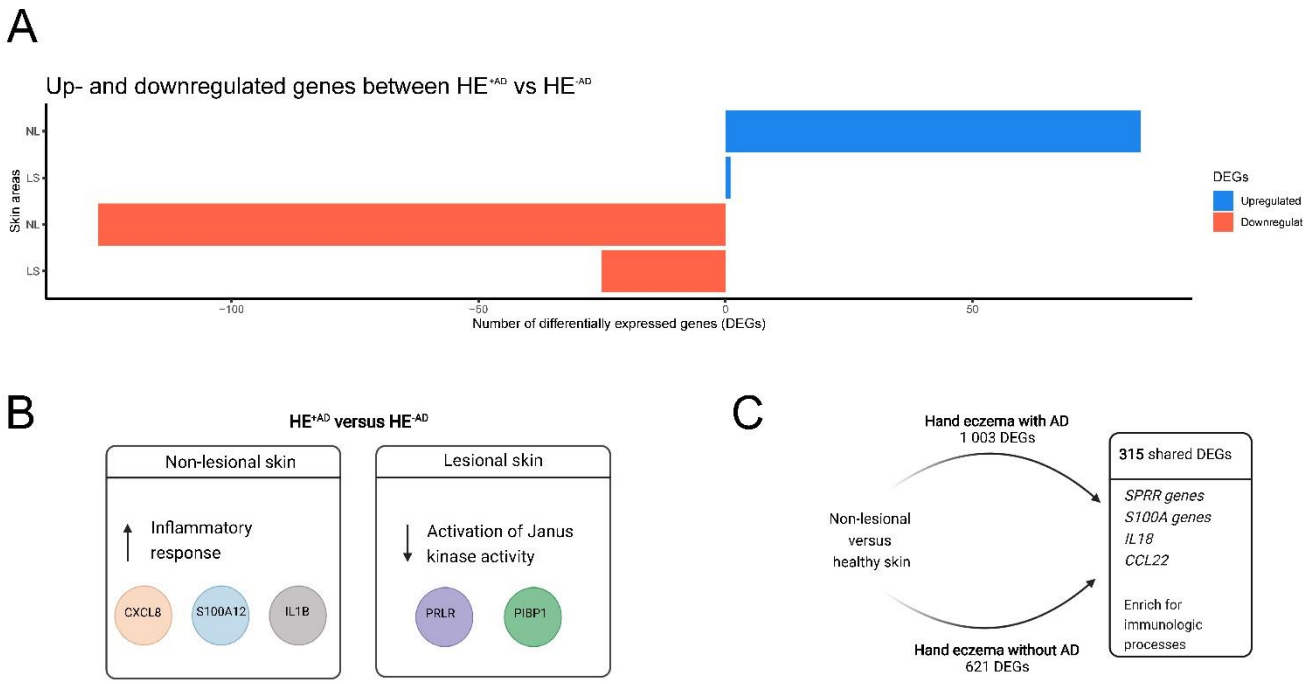


Figure 3A) Up- and downregulated genes of lesional and non-lesional skin of HE^{+AD} and HE^{-AD} respectively. B) Diagram showing the difference between HE^{+AD} and HE^{-AD} for non-lesional and lesional skin respectively. C) Schematic of the difference between non-lesional and healthy skin for HE^{+AD} and HE^{-AD} respectively, as well as the common differentially expressed genes (DEGs).Created with Biorender.com.

Appendices

Supplementary tables

Supplementary table 1. Known relevant type IV allergies and irritants as well as exposures. ACD= allergic contact dermatitis, ICD= irritant contact dermatitis.

Etiology	**Relevant type IV allergy**	**Relevant exposure to irritants**
ACD	MCI/MIMethylchloroisthiazolinone/Methylisothiazolinone	NA
ACD	Fragrance mix 1, fragrance mix2, tree moss, citral, and linalool	NA
ICD	NA	Gloves at work
ACD	Fragrance mix I, fragrance mix II, Hydroxycitronellal	NA
ICD	NA	Rubber gloves and hand sanitizers at work
ACD	Colophpnium, Asteraceae (sesquiterpene)	NA
ACD	Sesquiterpenelactone mix, and alpha-methylene-y-butyrolactone-mix	NA
ICD	NA	Occupational: Gardener
ICD	NA	Wet hands and use of gloves
ACD	Sesquiterpene, chrome, and nickel	NA
ICD	NA	Frequent domestic hand wash

Supplementary table 2. Questionnaire about age of onset and duration of hand eczema. HE^{-AD}(n=18) refers to hand eczema without atopic dermatitis and HE^{+AD} (n=12) refers to atopic hand eczema.

How old were you when the hand eczema first appeared? (n)			How often have you had eczema on your hands since then? (n)			Have you, during the previous 12 months, had hand eczema on any occasion? (n)		
	HE^{-AD}	HE^{+AD}		HE^{-AD}	HE^{+AD}		HE^{-AD}	HE^{+AD}
Under 6 years	0	8	Only one time and in less than two weeks	0	0	Yes, all the time	10	7
Between 6 and 14 years	0	2	Only one time lasting for two weeks or more	4	0	Yes, more than half of the time	5	3
Between 15 and 18 years	2	0	Several times	3	3	Yes, approximately half of the time	2	1
Over 18 years	16	2	Nearly all the time	11	9	Yes, less than half of the time	1	1

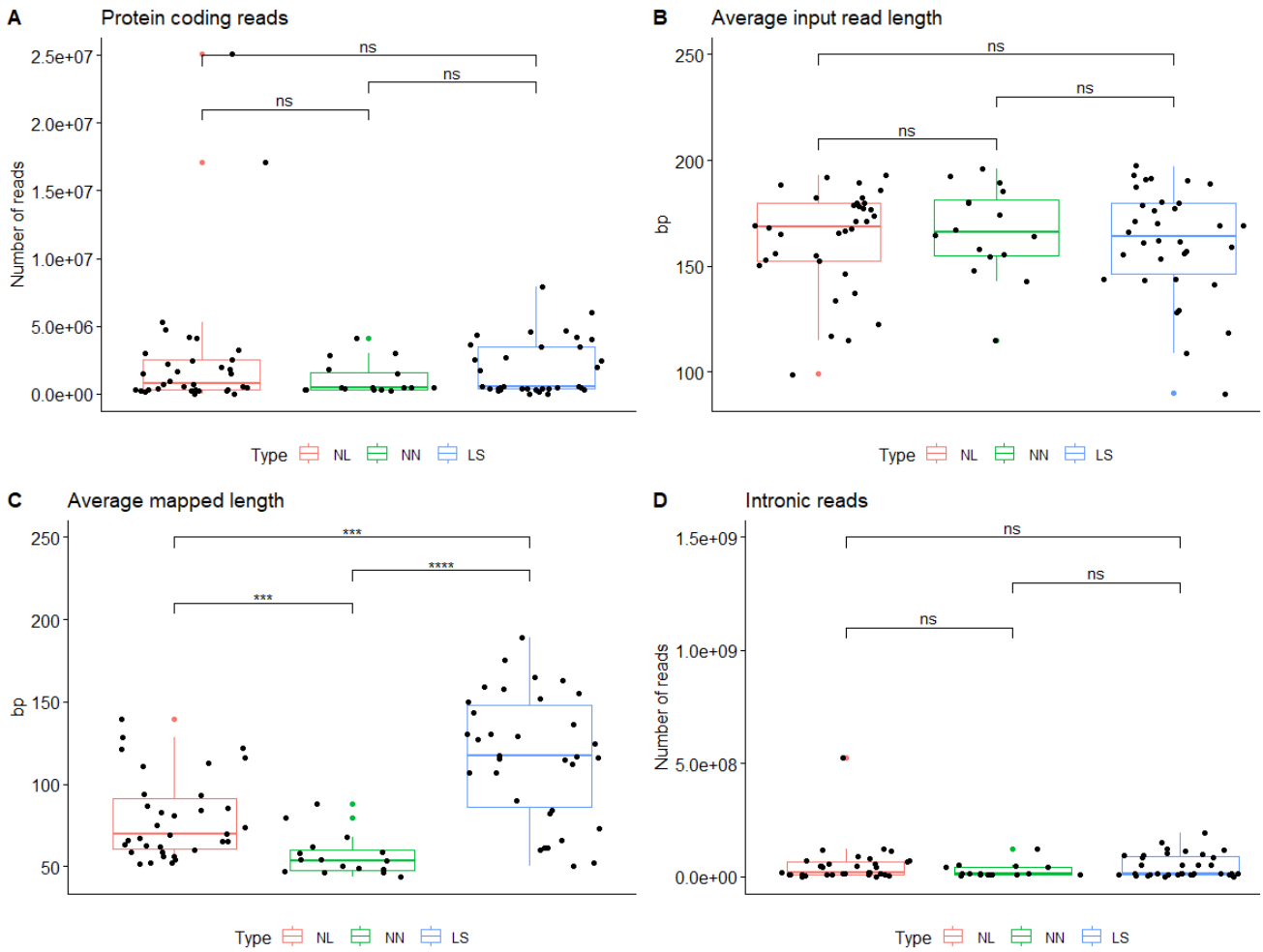
Supplementary table 3. Differentially expressed genes between atopic dermatitis and irritant contact dermatitis for lesional skin sites. A gene is considered differentially expressed if the adjusted p-value (Benjamini-Hochberg) is below 0.1 and the fold change is larger than 2.

Gene	baseMean	log2FoldChange	lfcSE	p-value	padj
ADGRA1	181.86	-2.97	0.70	8.35E-07	0.0015
ZNF787	109.39	-2.88	0.82	1.43E-05	0.0091
WISP1	81.20	-2.82	0.74	4.32E-06	0.0049
ATP6V1H	101.03	-1.90	0.60	4.48E-05	0.016
ACTN3	90.47	-1.39	0.41	2.71E-05	0.013
REEP2	74.52	-1.10	0.39	0.000149	0.029
FANCM	81.14	-1.00	0.46	0.00062	0.061
RSAD2	50.28	1.08	0.62	0.0018	0.099
UTP6	116.70	1.22	0.54	0.00068	0.062
GBP2	115.34	1.27	0.60	0.00093	0.073
FBXL5	51.13	1.33	0.59	0.00069	0.062
RHOH	87.52	1.38	0.51	0.00023	0.037
FFAR2	54.03	1.42	0.52	0.00018	0.034
LCP2	190.06	1.48	0.68	0.00072	0.063
SELL	45.030	1.54	0.76	0.0010	0.077
CSF3R	140.62	1.54	0.59	0.00026	0.040
TAGAP	73.69	1.55	0.87	0.0015	0.094
PLAUR	260.49	1.57	0.77	0.00094	0.073
RPTN	175.90	1.59	0.78	0.00089	0.072
SOD2	512.13	1.63	0.83	0.0011	0.078
FGF1	95.02	1.66	0.79	0.00086	0.071
AQP9	91.94	1.74	0.90	0.0012	0.082
DDX60L	130.88	1.82	0.51	1.55E-05	0.0093
IFIT2	51.52	1.88	0.83	0.00058	0.061
CASP4	49.59	1.95	0.69	0.00015	0.029
BCL2A1	83.61	1.97	1.01	0.0011	0.077
GBP1	88.07	1.97	0.89	0.00065	0.062
IFITM2	57.16	2.00	0.90	0.00067	0.062
GBP5	108.72	2.45	0.76	4.58E-05	0.016
IFIT3	51.070	2.49	0.82	7.66E-05	0.022
G0S2	356.53	2.53	0.90	0.00015	0.029
SEMG2	342.50	3.10	1.079	0.00011	0.028

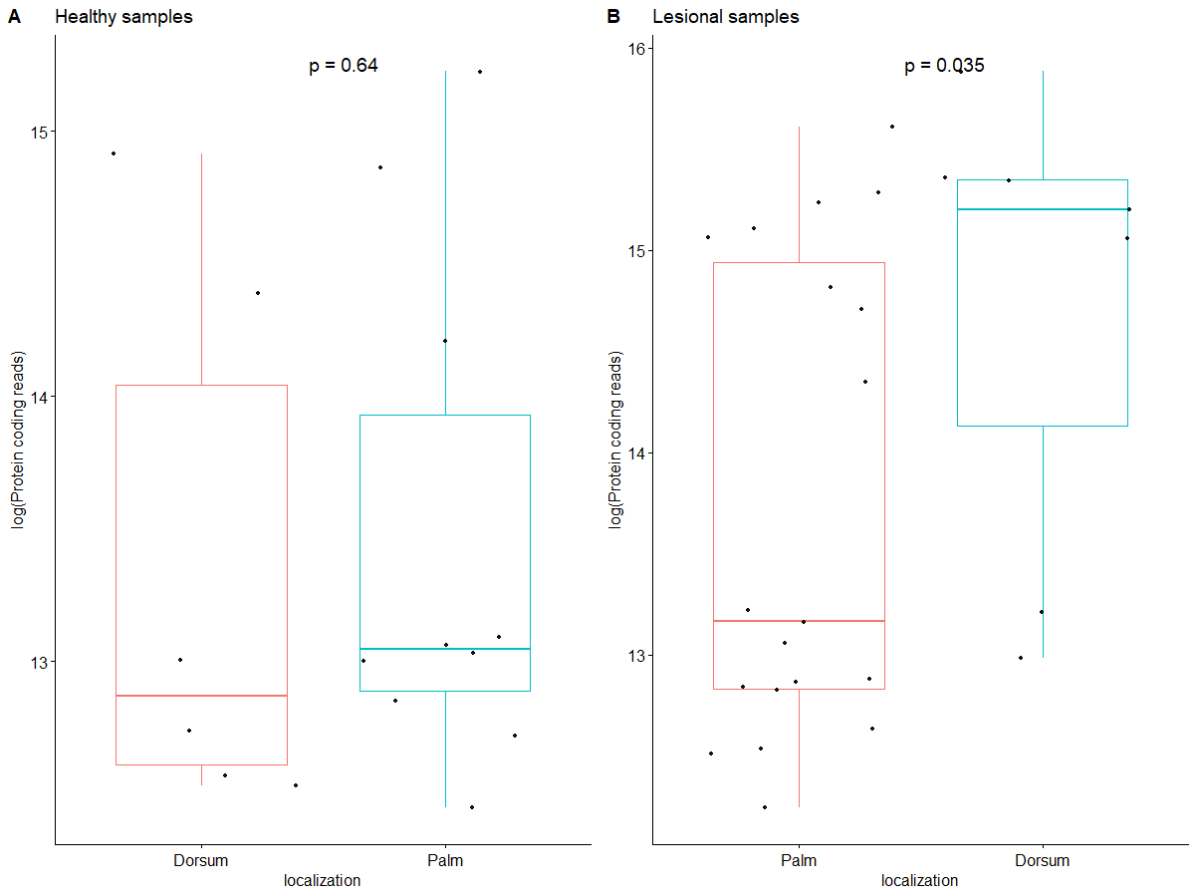
Supplementary table 4. Genes from V. Fortino, L. Wisgrill, P. Werner, S. Suomela, N. Linder, E. Jalonen, A. Suomalainen, V. Marwah, M. Kero, M. Pesonen, J. Lundin, A. Lauerma, K. Aalto-Korte, D. Greco, H. Alenius, N. Fyhrquist, Machine-learning–driven biomarker discovery for the discrimination between allergic and irritant contact dermatitis, Proc. Natl. Acad. Sci. U. S. A. 117 (2021) 33474–33485. https://doi.org/10.1073/PNAS.2009192117.

Gene	baseMean	log2FoldChange	lfcSE	p-value	padj
CD47	57.37	0.068	0.13	0.10	NA
BATF	26.03	0.0091	0.11	0.74	NA
FASLG	14.91	0.015	0.11	0.58	NA
RGS16	33.29	0.035	0.11	0.37	NA
SYNPO	130.44	-0.038	0.11	0.37	0.69
SELE	27.16	0.035	0.11	0.28	NA
PTPN7	73.21	-0.0030	0.102	0.93	NA
WARS	194.78	-0.019	0.11	0.20	0.57
PRC1	44.97	0.064	0.12	0.20	NA
EXO1	40.74	-0.010	0.10	0.78	NA
RRM2	39.40	0.0074	0.10	0.83	NA
PBK	13.22	0.0030	0.11	0.91	NA
RAD54L	28.68	-0.063	0.12	0.16	NA
KIFC1	3.17	0.0068	0.11	0.64	NA
SPC25	0.86	-0.0028	0.11	0.69	NA
TPX2	48.07	0.021	0.10	0.59	NA
DLGAP5	18.44	0.012	0.11	0.67	NA
CH25H	12.79	0.0086	0.11	0.70	NA
IL37	11.29	-0.0049	0.11	0.80	NA

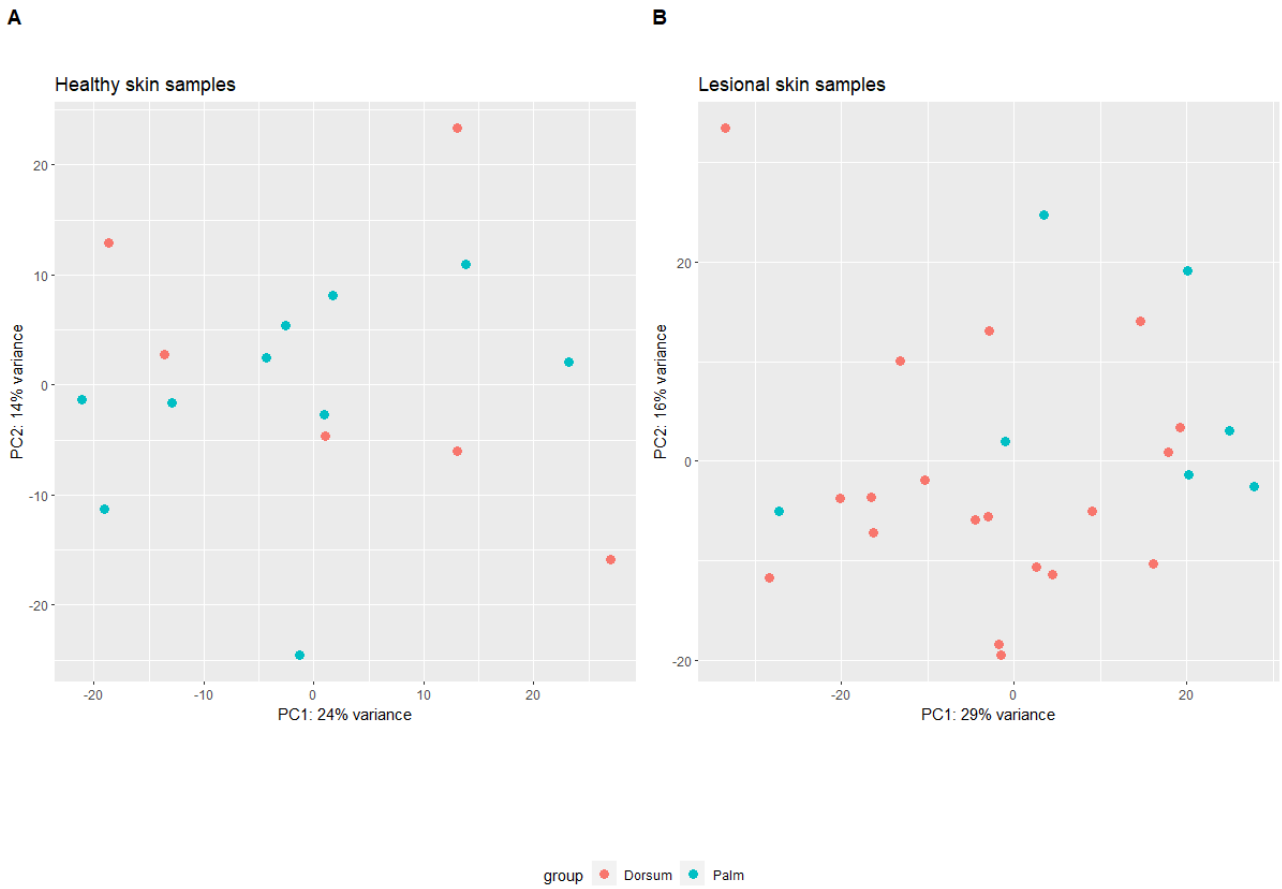
Supplementary figures



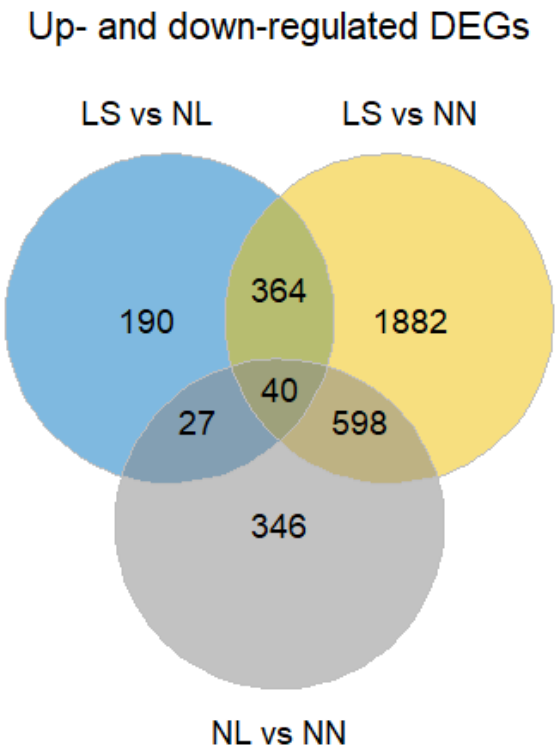
*Supplementary figure 1. The figure shows A) The number of reads having an open reading frame between skin areas (lesional: LS, non-lesional: NL, and healthy: NN). B) The average input read length according to skin area. C) the STAR Average mapped length according to skin area. D) The number of intronic reads between skin areas. Significance values are calculated by a Wilcoxon sum rank test (non-significant: ns, p<0.05: *,p<0.01: **, p<0.001: ***. p<0.0001: ****.*



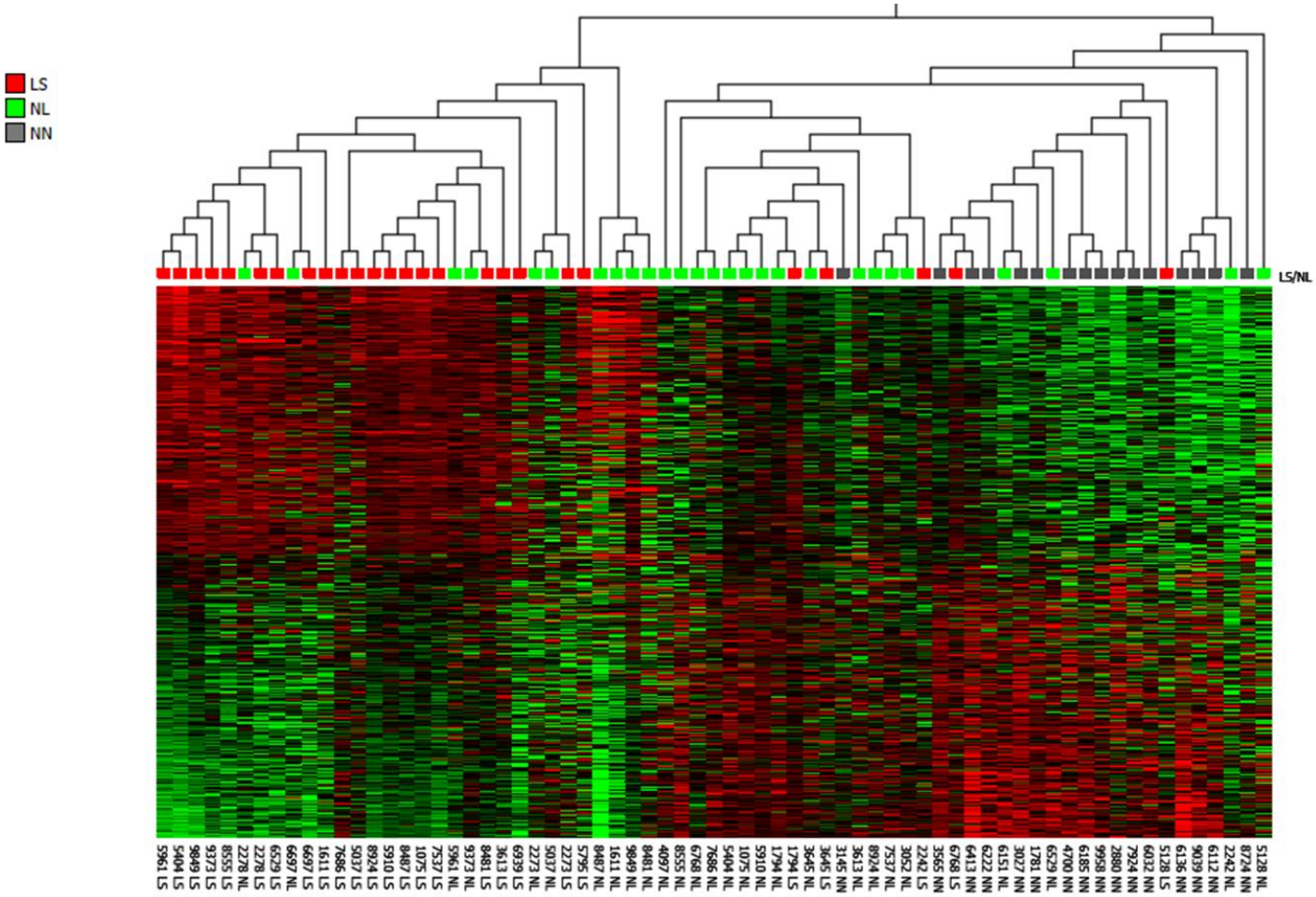
Supplementary figure 2.A) Number of reads in protein coding regions for healthy samples according to localization on the hands. No statistically significant difference (Wilcoxon sum rank test) was observed (p=0.645). B) Number of reads in protein coding regions for lesional samples according to localization on the hands. A statistically significant difference (Wilcoxon sum rank test) was observed (p=0.035)



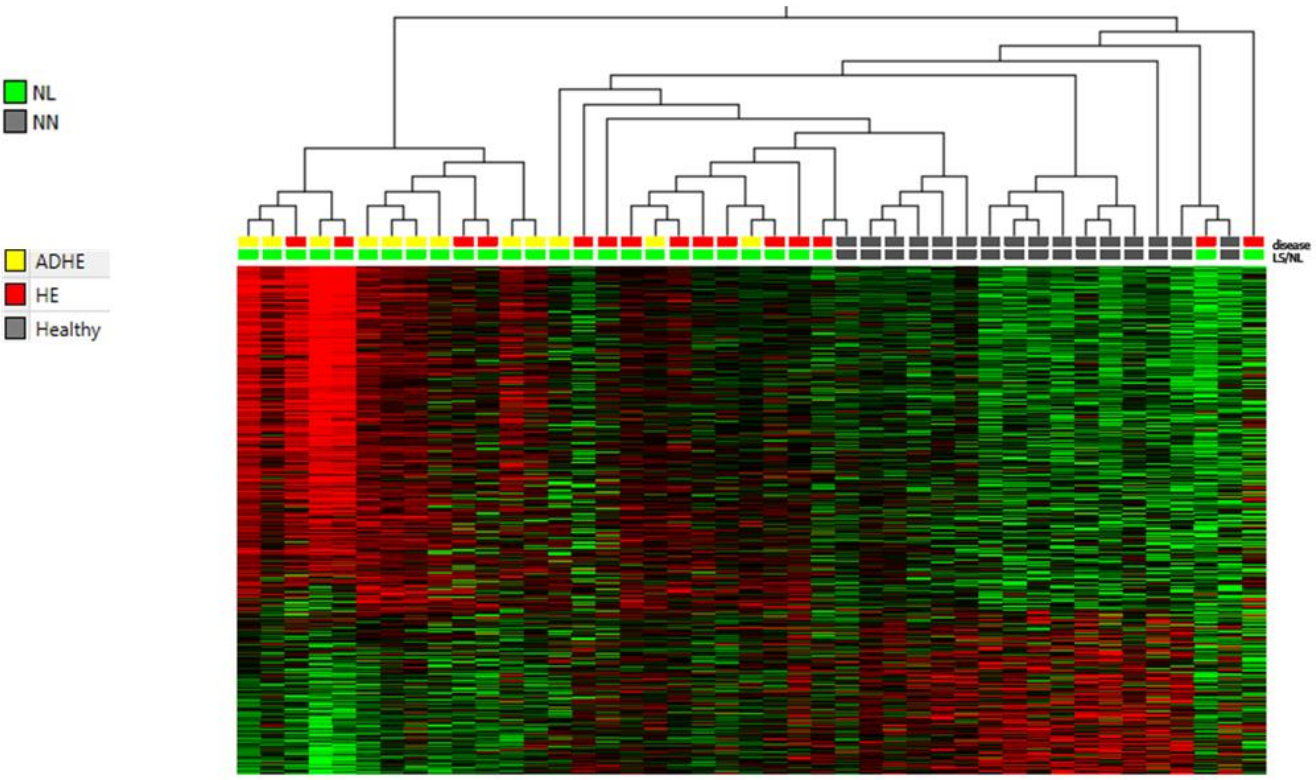
Supplementary figure 3. A) Principal component analysis (PCA) of the top 100 most differentially expressed genes of healthy skin on the hands do not show apparent clustering according to localization (dorsum and palm). B) PCA of the top 100 most differentially expressed genes of lesional skin on the hands do not show apparent clustering according to localization (dorsum and palm).



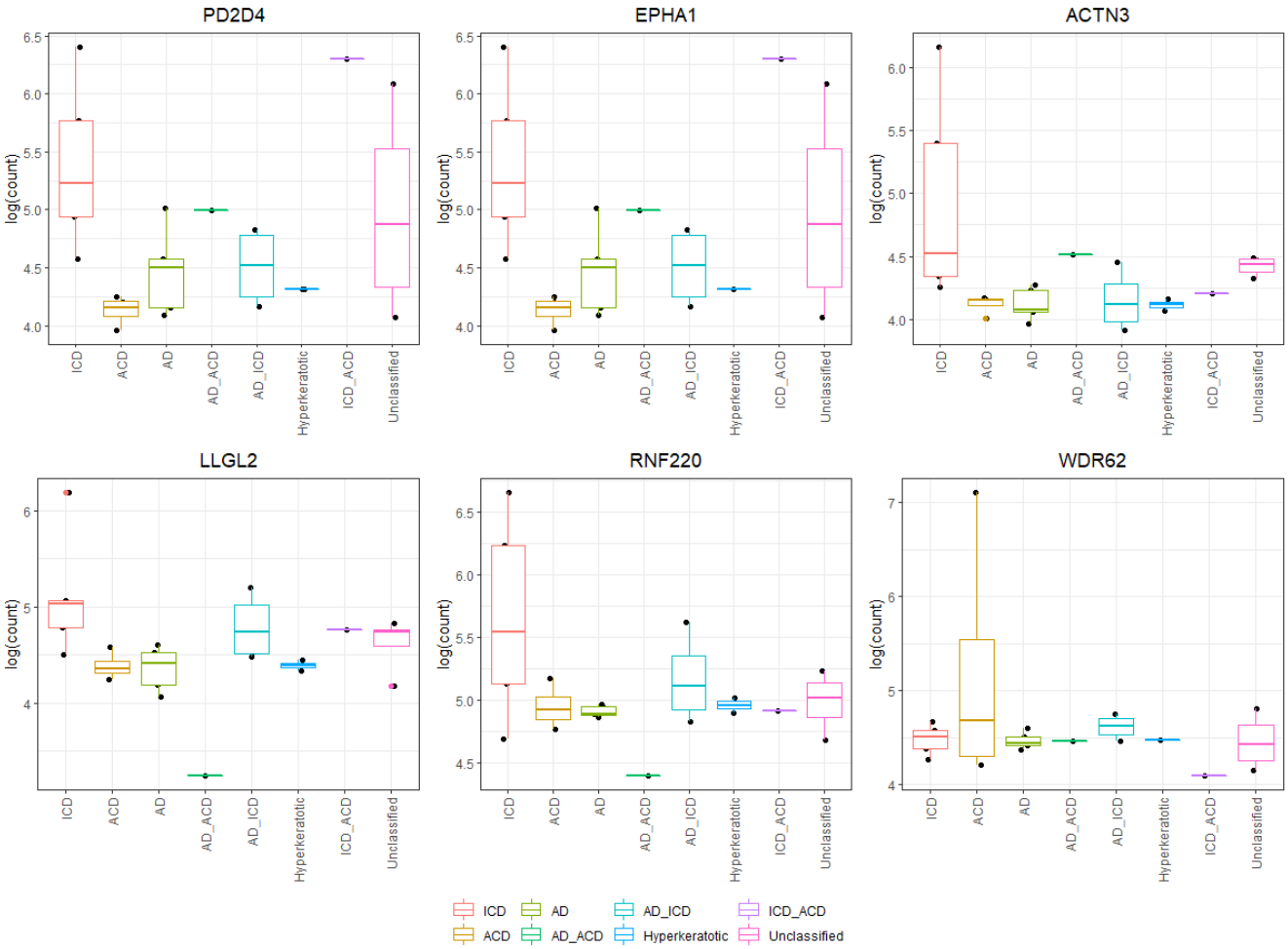
Supplementary figure 4. The figure shows A) A Venn diagram showing the number of differentially expressed genes between the different skin areas (lesional (LS), non-lesional (NL) and healthy (NN) skin). A total of 2 884 genes are differentially expressed between lesional and healthy skin, 1 012 genes between healthy and non-lesional and 620 genes between non-lesional and lesional skin areas. The diagram also shows that 364 genes were related to lesional skin, 598 to healthy skin and 27 to non-lesional skin areas



Supplementary figure 5 Unsupervised hierarchal clustering based on the3441 differentially expressed genes between all skin areas (lesional (LS), non-lesional (NL), and healthy (NN)) (FDR<0.1 and FC>2).



Supplementary figure 6 Unsupervised hierarchal clustering based on the 1009 differentially expressed genes between HE with and without AD for non-lesional skin samples (FDR<0.1, FC>2).



Supplementary figure 7. The six differentially expressed genes between lesional skin areas of irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Etiologies with an underscore refers to a mixed etiology.

10.3 Manuscript III:*The proteome of hand eczema assessed by tape stripping*

Title: The proteome of hand eczema assessed by tape stripping

Running title: The proteome of hand eczema

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Abbreviations

Atopic dermatitis	AD
Data-independent acquisition	DIA
Differentially expressed protein	DEP
Eczema Area and Severity Index	EASI
Hand eczema with atopic dermatitis	HE^{+AD}
Hand eczema without atopic dermatitis	HE^{-AD}
Hand eczema severity index	HECSI
High-performance liquid chromatography	HPLC
Liquid chromatography–mass spectrometry	LC-MS
Mass spectrometry	MS
Nano-scale liquid chromatographic	nLC
Principal component analysis	PCA

Abstract

Background: Hand eczema (HE) is a prevalent, often multifactorial and potentially debilitating skin disease.However, only few studies examined subtype-specific biomarkers, and no studies have yet investigated the proteome of HE using tape strips.

Objectives: To assess if the global protein expression of skin from the hands can be assessed by two consecutive tape strips and compare it to theprotein expression from skin biopsies. Furthermore, we assessed the protein expression in different subtypes of HE with a focus on HE with and without atopic dermatitis (AD).

Methods: Tape stripswere collected from lesional, non-lesional, and healthy skin of patients with HE (n=34) and healthy controls (n=13).Liquid chromatography–mass spectrometry was performed, and the global protein expression analysed. Comparison was made to previous published findings in *Skinatlas*based on skin biopsies.

Results: We identified 2,919 proteins by data-independent acquisition of stratum corneum-derived skin cells from tape strip samples.Of these 88.7%overlapped with those found in skin biopsies from *Skinatlas*.The global protein differences included an increased expression of immune-related markers and a decreased expression of structural barrier proteins for lesional skin. The difference between HE with and without concurrent AD was restricted to the lesional skin areas, and included an increased expression of FLG2 and LOR and a lower expression of KRT16 for HE with AD.No difference in the number of detected proteins between the dorsal- and palmar aspects of the hands was found for neither lesional nor healthy skin. Proteomic differences between the dorsal- and palmar aspect of the hands included a higher expression of KRT6A, KRT16 and KRT17 for the palmar aspects of lesional skin as compared to the dorsal aspect.

Conclusion: The protein expression of the hands can be assessed by tape strip samples. This method can be used independently of localization on the hands and shows potential for assessing the proteomic differences between subtypes of HE.

Key words: Atopic dermatitis, contact dermatitis, hand eczema, tape stripping, proteomics

Introduction

Atopic dermatitis (AD) is one of the most prevalent skin disease affecting up to 20 % of the European population, primarily affecting children[1–3]. It often begins in childhood and is an important risk factor for hand eczema (HE)[4]. The 1-year prevalence of HE is 9% in the general population, and it can have a major negative impact on the quality of life and work ability of the patients[5,6]. Apart from being a manifestation of AD, HE may be caused by contact with allergens and/or irritants, and in many cases HE is a result of different etiologies complicating the diagnosis and treatment of the disease[7].

Currently, the gold standard used to investigate the immunological print of a patient is a full skin thicknessbiopsy. However, the invasive technique causes scarring and comes with a small risk of infections. In addition, skin biopsies are not well suited for sensitive skin areas such as the hands[8].

Tape stripping is a non-invasive sampling technique that collect corneocytes[9]. The skin sampling technique has been reported to enable non-invasive investigations of the transcriptome as well as the proteome of the skin[10–16].

With the emergence of new treatments, the demand of knowledge about the subtype-specific immunology of HE is increasing. This can be investigated by quantifying the global protein expression e.g. with mass spectrometry-based techniques. To our knowledge no studies have performed mass spectrometry on tape strips from the hands.

Materials and methods

Study population

Details on the study population and sample collection have been previously reported (Sølberg et al. 2021[17]) in short,34 adult Caucasian patients with chronic hand eczema (lasting more than three months) and no use of systemic medication were recruited from the Department of Dermatology and Allergy at Herlev-Gentofte hospital, Denmark, between March 2019 and September 2020.

Furthermore, 13 age-matchedhealthy controls with no history of inflammatoryskin disease or other atopic diseases (asthma and hayfever) were included. HE patients included both patients with and without AD (HE^{-AD} (n=16) and HE^{+AD} (n=18)) (Table 1). The HE^{+AD} group included patients with active or a history of AD (14 with active AD and 4 with a history of AD)[7].

AD was diagnosed by a physician and fulfilled the Hanifin and Rajka criteria[18]. History of AD was assessed by self-reports from the patient. Severity of HE and AD was assessed by the same physician using the hand eczema severity index (HECSI)[19] and the Eczema Area and Severity Index (EASI)[20], respectively.

Table 1. Study subject demographics

	HE^{+AD} n=16	HE^{-AD} n=18	Healthy n=13
Gender female (% female)	13 (81.3)	9 (50.0)	5 (61.5)
Age (median [IQR])	57.5 [43.0; 64.0]	58.0 [45.0; 65.0]	58.5 [42.8; 65.3]
HECSI (median [IQR])	56.0 [33.0; 129.0]	56.0 [33.0; 128.3]	-
EASI (median [IQR])	11.8 [8.9; 22.3]	-	-
Dorsal samples	9	1	4
Palmar samples	7	17	9

Exclusion criteria included use of topical treatment such as moisturizers and topical corticosteroids within 24 hours of sample collection, as well as ongoing infections, pregnancy, lactation or use of antibiotics, phototherapy or self-tanners within 4 weeks of sample collection.

The study was approved by the local ethics committee (H-16050507) and the Danish Data Protection Agency (HGH-2017-073), oral and written consent was obtained before inclusion and the study followed the Helsinki declaration.

Sample collection

Skin samples were obtained from stratum corneumby tape stripping. From each sample site 10 consecutive standard D-squame tape strips (D-100, Monaderm, Monaco, France) were collected using a uniform pressure as previously described[9,12]. The samples were stored at -80°C. From the patients a lesional skin sample was collected from the most affected area (palmar and dorsal aspects). The non-lesional skin samples were taken from the upper arm. From the healthy subjects a skin sample was taken from the hands. Proteins were extracted from the fifth and sixth consecutive tape.

Sample preparation

A detailed protein extraction protocol can be found in Supplementary Materials. Briefly, proteins were lysed directly on the tape strips using a 20% 2-2-trifluoroethanol solution (TFE) containing DTT (5mM) followed by centrifugation. Tape strip was removed from the buffer and remaining material wad boiled and sonicated. 2-2-chloroacetamide (CAA) was applied to a final concentration of 25mM followed by overnight enzymatic digestion (37°C, 1200 rpm) using trypsin (0.5µg/µl) and lysine (0.5µg/µl) in an enzyme to protein ratio of app. 1:100. The following day samples were stage-tipped (2 layers of SDB-RPS) using a wash buffer containingtrifluoroacetic acid (TFA). The elution buffer contained acetonitrile (ACN) and ammonium hydroxide. After elution samples were dried using a vacuum concentrator (45 minutes, 45°C) and resuspended in A* buffer (2% ACN and 0.2% TFA) directly into a MS-plate (#AB-1300, Thermo Scientific, Life Technologies Europe, Roskilde, DK).

LC-MS

Liquid chromatography–mass spectrometry (LC-MS) was performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an EASY nLC 1200 ultra-high-pressure system (Thermo Fisher Scientific) using an in-house packed 50 cm column, 75 µm inner diameter with1.9 µm ReproSil-Pur C18-AQ (Dr Maisch GmbH, Ammerbuch, Germany). The analytical column was heated to 60°C and the peptides (500ng) were separated using a gradient of buffer A (0.1% formic acid) and buffer B (80% acetonitrile, 0.1% formic acid). The gradient ranged from 2 to 25% B in 90 min and stepped up to 40% in 15 min followed by a 5 min wash at 95% B and finally to 2% B for 3min at 300 nl per minute. The mass spectrometer was operated in Data Independent Acquisition mode (DIA) where the full MS resolution was set to 120.000 at 200 m/z and full MS ACG target was 300% with an IT of 50 ms. Mass range was set to 300-1650. AGC target for fragment spectra was set to 1000%. Thirty-three windows with variable width were used (Supplementary Table 1). The resolution was set to 30.000 and the IT to 54ms. Normalized collision energy was set to 30% and all data were acquired in profile mode using positive polarity.

Data analysis

The mass spectrometry data were analysed using the Spectronaut software (version 2.0.1.0,Biognosys AG, Schlieren, Switzerland). A library-based data-independent acquisition (DIA) workflow was used to quantify protein abundance.A previously described comprehensive spectral library of 10,701 skin proteins from abdominal healthy skin biopsies was used for protein identification (*Skin Atlas*)[21].Default parameters were used for analysisexcept ”Normalization strategy” that was set to “Local normalization”. For further analyses samples with a Qvalue<0.01 were included.

Subsequent sample normalization, filtering and imputation was performed in Perseus (version 1.6.15.0 , Maxquant)[22]on the MS2 quantities. For data analysis protein counts were log2 transformed. Due to expected differences according to skin area (lesional, non-lesional and healthy) normalization by median subtraction were conducted per skin area after filtering using cutoff value of 70% (at least in one group). Imputation was performed using a width of 0.15 and a downshift value of 1.8.

For protein enrichment analyses STRING (Version 11.0, https://version-11-0.string-db.org/) were used.

Statistics

The statistical analyses were conducted in Perseus (version 1.6.15.0 , Maxquant) and R (R core team, version 4.0.4, http://www.R-project.org/).

To investigate the proteomic differences between localizations on the hands (palm and dorsum), skin areas (healthy, non-lesional and lesional) as well as HE with and without AD differential analyses (student's t-test) were performed on log2 transformed data (Supplementary Fig. 1). A protein was considered as a differentially expressed protein(DEP) if it had a fold change>2 and a Benjamini-Hochberg adjusted p-value<0.1 (FDR).

To investigate the global proteomic differences principal component analyses (PCA) and hierarchal cluster analysis were performed.

Results

MS-based proteomic analysesyield 2,919 proteins from tape strips

Label free MS-based proteomics were performed on tape strip skin samples from the dorsaland palmar aspects of the hands of patients with HE andhealthy controls (total n=81) (Fig. 1a).

We quantified 2,919 proteins with signal intensities spanning more than eight orders of magnitude(Fig.1b).We filtered with quantitative values in >70% of the samples in at least one skin area group for comparisons between the clinical groups (1,515 proteins).

High abundant proteins included epidermal-specific proteins such as CALML5, S100A7 and several keratins, whereas low abundant proteins were generally expressed in most tissues includingand enriched for general cell mechanisms including protein- and peptide transport (GO:0015833 and GO:0015031)(Fig. 1b and Supplementary Table2).

To comparethe proteomic profile obtained from tape stripsin our study with skin biopsies, we investigated how many proteins were detected both in stratum corneum of skin biopsies (*Skinatlas*)[21] as well as in our tape strip samples.The proteomic profile of stratum corneum from skin biopsies included 8,778 proteins representing structural and immunologic proteins. Of these, 88.7 % (2,588) of the overall 2,919proteins detected in our tape samples overlapped with those found in stratum corneum by *Skin Atlas* (Fig. 1c). The proteins identified both in tape strips and skin biopsies from *Skinatlas* included important skin proteinssuch as keratins, SERPINS, S100A proteins, and members of the IL-1 family of cytokines -IL-18, and IL-1A (Supplementary Table 3). The proteins only detected instratum corneum fromthe tape strip samples of this study included structural components of epidermis such as KRT2, KRT82 and LOR as well as several hair and immune related proteins including KRTAPs, CCL22 and HLA proteins, however, these proteins were found in the deeper skin layers in *Skinatlas* (Supplementary Table 4).

No statistically significant differences were observed in the number of detected proteins between dorsum and palm for neither healthy nor lesional skin samples (p=0.15 and 0.43 respectively) (Table 1, Supplementary Fig.2).The greatest proteomic difference between dorsum and palm was found for the lesional skin areas (331 DEP, Supplementary Table 5) as compared to the healthy skin areas (91 DEP Supplementary Table 6) (Fig. 2a). The difference included a higher expression of the palm and sole specific KRT9[23] for the palmar aspects of both healthy and lesional skin, and a lower expression of HRNR for the dorsal aspects of the hands of both healthy and lesional skin.

Furthermore, we found a higher expression of KRT6A, KRT16, and KRT17 for the palmar aspects of lesional skin, all proteins key for early barrier damage as well as palmoplantar keratodermaand hyperkeratotic HE (Fig. 2b)[24–26]. For the dorsal aspects of lesional skin a higher expression of LOR and FLG2 was found.

The proteomic profile of hand eczema is driven by immunologic markers of lesional skin areas

To investigate theproteomic differences between skin areas (healthy, non-lesional and lesional) we performed a PCA. The main proteomic differences were found comparing lesional skin to non-lesional-and healthy skin (PC1: 31.27%)(Supplementary Fig.3). This wasalso reflected in the differential expression analysis (Table 2).

The proteomic differences between lesional and healthy skin included a higher expression of immune-related proteins, such as HLA proteins,and proteins related to the complement pathway. Lesional skin also showed a higher expression of the CD44 antigen, as well as the alarmins KRT6A, KRT16 and KRT17[25,27].In addition, the pro-inflammatory IL-1 family member IL-18 was upregulated, and we found a lower expression of the IL-1 family inhibitor IL-1R2 (Supplementary Table 7)[28].

The T-complex protein Ring Complex (TRiC) chaperonin subunits (TCP1, CCT2-CCT8),known to be involved in protein folding of misfolded cytoskeleton proteins[29],showed increased expression of all the subunits of the complex for lesional skin as compared to healthy skin. Significantly lower expressed proteins in lesional versus healthy skin included structural proteins such as FLG2 and loricrin as well as the anti-inflammatory cytokine IL-37 (Fig.3a).

In similarity to the lesional skin, we found a higher expression of IL-18 for non-lesional skin as compared to healthy skin. Furthermore, we found a higher expression of the pro-inflammatory cytokine IL-34. Lower expressed proteins included a large network of proteins related to cornification (GO:0070268) including FLG and several keratins in non-lesional skin (Supplementary Table 8).

In lesional skin compared to non-lesional skin, we found a higher expression of several S100A proteins (S100A8, S100A9 and S100A12) as well as HRNR and a lower expression of IL-34, IL-36G and IL-37 as well as LOR for lesional skin.Whereas IL-37 as known to suppress inflammation, IL-34 and IL-36G are pro-inflammatory cytokines (Supplementary table 9).

Table 2. The table shows the number of differentially expressed proteins (DEP) between the different skin areas. A protein is differentially expressed when it has a FC>2 and a Benjamini-Hochberg adjusted p-value<0.1.

Contrast	Total number of DEP	Higher expressed DEP	Lower expressed DEP
Lesional vs healthy	908	563	345
Non-lesional vs healthy	411	276	135
Lesional vs non-lesional	851	519	332

The greatest difference between hand eczema with and without atopic dermatitis is found between lesional skin sites

To assess the differences between HE with and without AD we investigated which proteins were differentially expressed between lesional and non-lesional skin sites, respectively. Whereas no proteins were statistically significantly differentially expressed between the non-lesional skin sites, 189DEP were detected between lesional skin sites (Fig. 3c, Supplementary table 10).The DEP between HE^{+AD} and HE^{-AD} included a higher expression of the structural proteins FLG2 and LOR and a lower expression of KRT16 for HE^{+AD}(Fig. 3d). Furthermore, we found a higher expression of the serine proteases SERPINB6 and SERPINB9 known to be involved in the acute inflammation of skin in AD^{+HE}, as well as the AD related SPINK5[30–35].

Discussion

We described the proteomic profile of the palmar and dorsal aspects of the hands from major subtypes of chronic hand eczema based on only few tape strip samples.

Here we show that the proteomic profile of different subtypes of HE (HE^{+AD} and HE^{-AD}) from both the palmar and dorsal aspects of the hands can be investigated from only two consecutive tape strips.

We successfully quantified 2,919 proteins, with 1,515 being proteins present in >70% of the samples in at least one skin area group (healthy, non-lesional, and lesional). The 2,919 proteins are to our knowledge the highest number of proteins detected from tape strips[36–38]. Of these, 88.7% overlapped with those detected from stratum corneum samples in *Skinatlas*[21], underlining the quality of the proteome obtained from tape strip samples. Of the proteins 325 proteins were only detected in our tape strip samples. These included several structural proteins such as LOR and KRT2 as well as the immune related proteins CCL22 and several HLA proteins. These proteins were, however, detected in other layers of epidermisand the difference is therefore methodological.

To our knowledge this is the first study investigating the proteome of different localizations on the hands (dorsum vs palm). We did not find any difference in the number of obtained proteins for neither healthy nor lesional skin between localizations, though structural differences exist in the thickness of epidermis of the dorsum and the palm.We found a lower expression of HRNR for the dorsal aspect of the hands of both healthy and lesional skin. Interestingly, a lower expression of FLG but a higher expression of FLG2 of the dorsal aspect of lesional skin on the hands was detected compared to the palmar aspects.The role of FLG2 on the hands is not well described and whether the difference in FLG expression of this study is due to localization of skin lesions on the hands or differences according to HE subtypes therefore need to be further investigated.For the palmar lesions a higher expression of the alarmins KRT6A, KRT16 and KRT17 was found. These alarmins are related to palmoplantar keratoderma as well as hyperkeratotic HE and show the potential for the tape strip samples to detect differences between some subtypes of HE[26,38].

The main proteomic difference between skin areas (healthy, non-lesional and lesional skin) was found to be between lesional and healthy/non-lesional skin, as healthy and non-lesional skin had almost similar proteomic profiles. The proteomic profile of lesional vs healthy skin showed a higher expression of immune-related proteins as well as a lower expression of structural and anti-

inflammatory proteins of lesional skin. This is in line with previous studies of AD and HE, and show the common inflammatory picture related to skin inflammation[39–43]. In this study we show an upregulation of the TRiC chaperonin subunits (TCP1 and CCT2-CCT8). This is in line with a recent study by Sobolev et al. showing an increased expression of TRiC for psoriatic skin lesions. Our finding thereby supports the theory that an upregulation of TRiCmight be due to an increased need for protein folding in lesional skin[44].

Interestingly,we found a lower expression of the pro-inflammatory cytokines IL-34 and IL-36G for lesional skin as compared to non-lesional skin. Though many inflammatory cytokines are known to be expressed even in non-lesional skin of AD patients it is not clear why these pro-inflammatory cytokines were higher expressed in non-lesional skin as compared to lesional skin[45].

The etiology of HE is important for the pathogenesis. The etiologies of HE includes contact dermatitis (irritative and allergic), protein dermatitis and atopic HE. We found an increased expression of the CD44 antigen in lesional skin as compared to healthy skin. CD44 is expressed on many mammalian cells including keratinocytes and has many immunoregulating functions including the activation of T cells. CD44 has previously been shown for lesions of contact dermatitis, especially in irritant contact dermatitis lesions[27]. Our finding of a general increased expression of CD44 in lesional skin show the importance of contact dermatitis for HE lesions independent of etiology. In line with thiswe found an upregulation of IL-18 which is important for the migration of antigen presenting cells and the subsequent production of antigen-specific T cells[46].

The proteomic difference between HE with and without AD was found exclusively for lesional skin sites and included mostly structural rather than immunological proteins. For HE with AD we found a higher expression of FLG2. This is in contrast with previous findings where a lower expression of FLG2 has been found for AD patients[47,48]. Whether this finding is due to methodological or biological differences between skin areas need to be further investigated.

There are some limitations to this study. Non-lesional samples were taken distant from the lesional samples, which might have caused a confounding effect of localization. It might, however, be difficult to obtain non-lesional samples adjacent to an active lesion on the hands. This study included HE patients with a history of AD as being HE^{+AD}. Though the AD might not be active at the present time, several studies have shown a general skin impairment of AD patients[49,50]. Furthermore, AD is a major risk factor for the development of HE[4,51].

In this study we show that tape strips can be used to assess the proteome of HE with and without AD independent of localization on the hands. The use of tape stripping, which is non-invasive sampling technique, will enable the possibility to conduct large-scale proteomic studies in the future. Furthermore, the tape strip samples show potential in the investigation of subtypes of HE, and future studies investigating the proteomic profile related to different morphologies and etiologies of HE might improve the clinical diagnosis and treatment of the disease.

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Figures

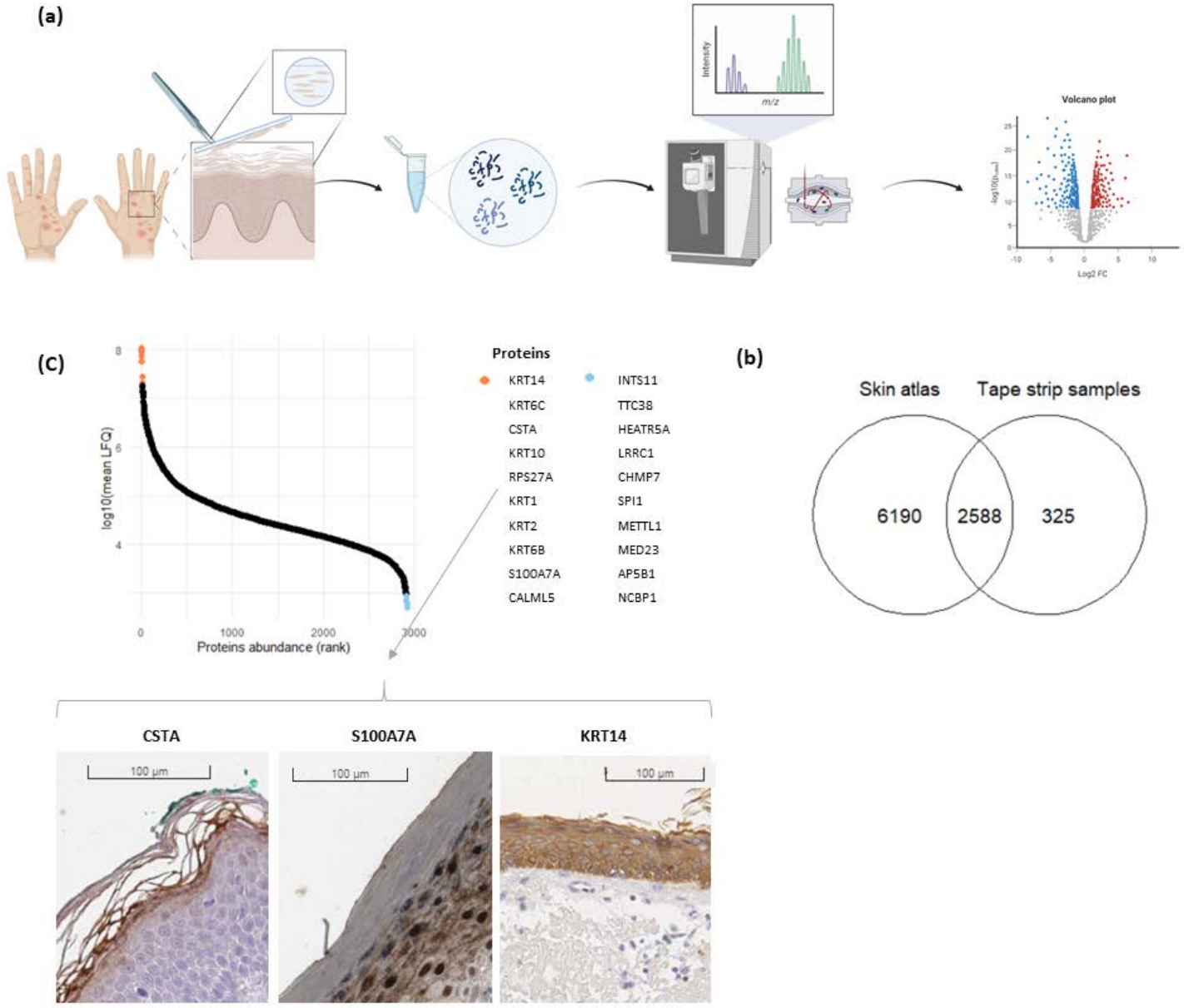


Figure 1. (a) For the investigation of the proteomic profile of stratum corneum of hand eczema, skin samples from different localizations on the hands were collected by tape stripping. The proteomic profile was obtained by label-free mass spectrometry proteomics using the Skin Atlas library. Concurrent data analysis included differential analysis and unsupervised hierarchal clustering. (b) The mean rank of proteins based on LFQ intensities show skin specific proteins in high, as well as low abundant proteins across all samples. The histological staining's show high abundance of selected proteins in keratinocytes of epidermis. (c) 2588 proteins were common between our tape strip samples and those detected stratum corneum of skin biopsies from the Skin Atlas project. Created with BioRender.com. Histology pictures from http://www.proteinatlas.org.

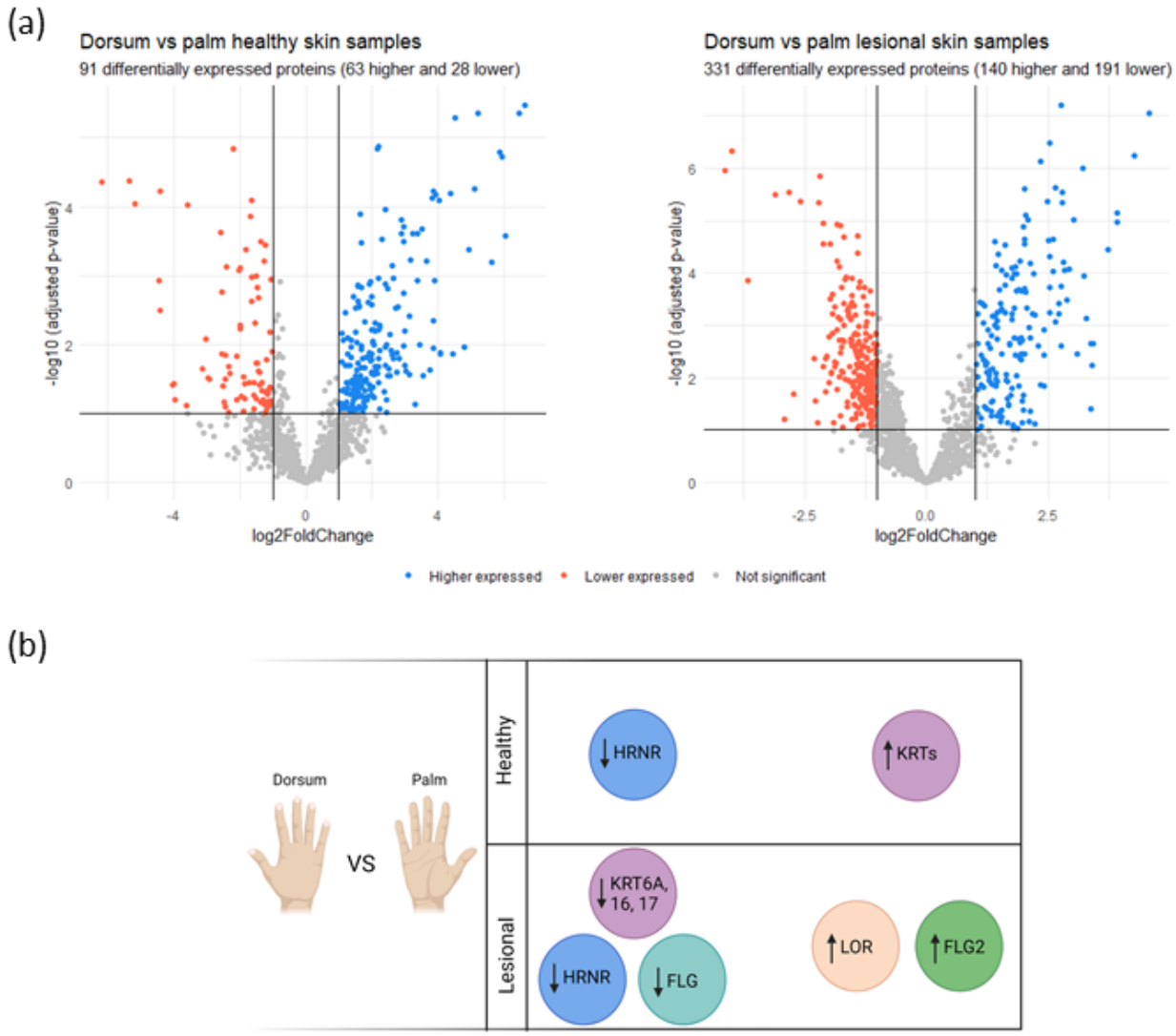


Figure 2. (a) Differential analysis of dorsum versus palm of healthy and lesional skin samples resulted in 91 and 331 differentially expressed proteins respectively (adjusted p-value < 0.1, Fold change > 2). B) The differentially expressed proteins between dorsum and palm included a lower expression of HRNR and a higher expression of keratins (KRTs) for healthy skin and a lower expression of HRNRN and FLG but a higher expression of LOR and FLG2 for lesional skin. Created with BioRender.com.

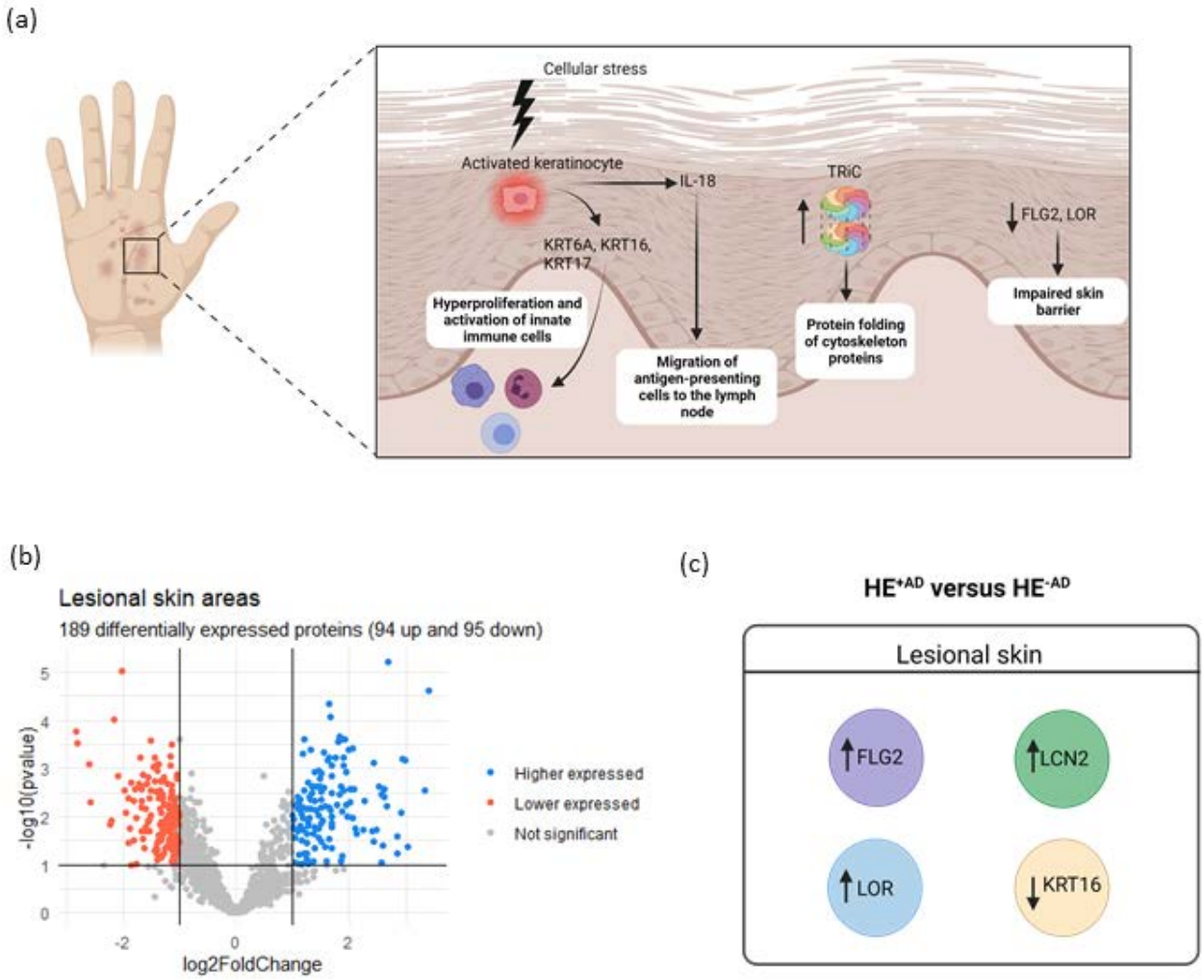
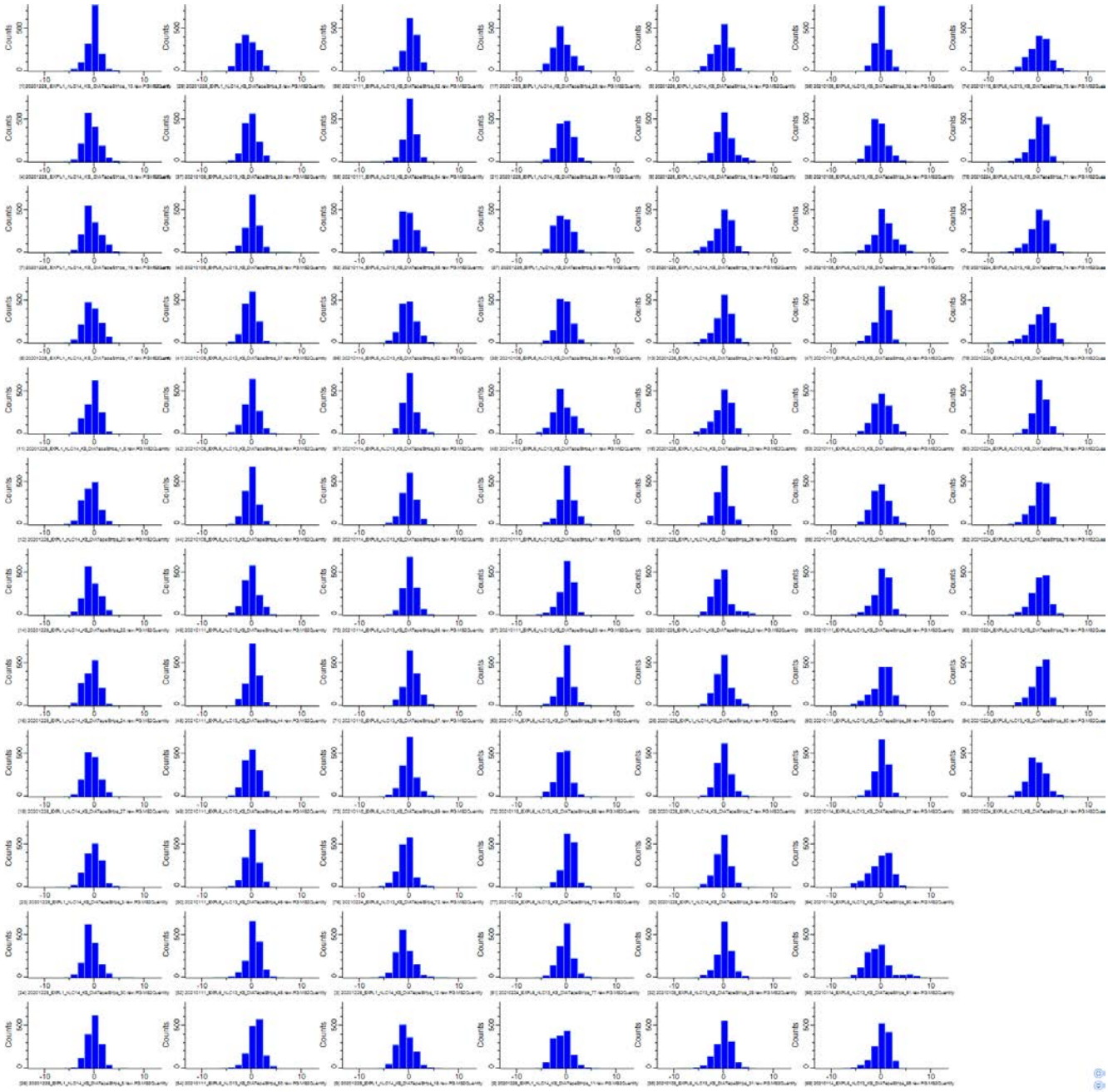
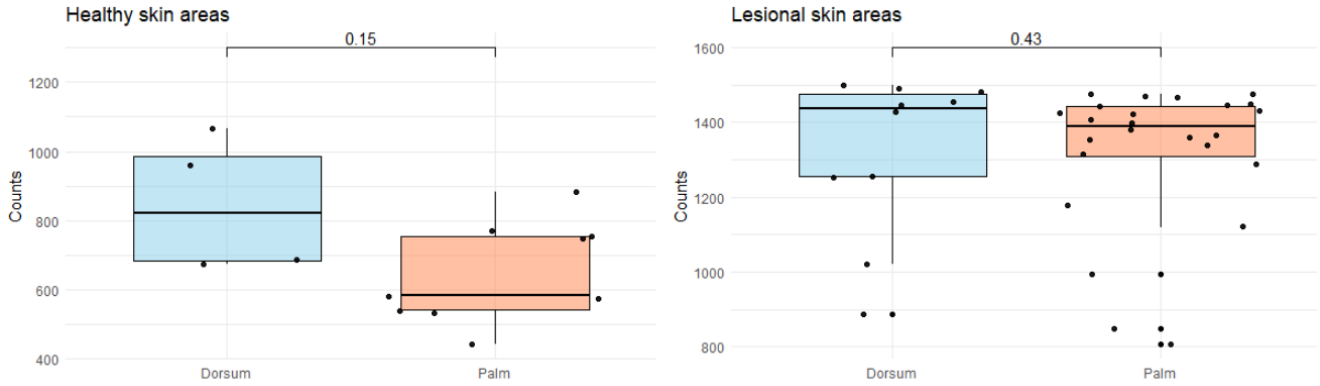


Figure 3. (a) The illustration shows some of the differences between lesional and healthy skin samples. (b) The volcano plot show that 189 (94 up and 95 down) differentially expressed proteins (DEP) were detected between lesional skin of HE with AD (HE^{+AD}) and HE without AD (HE^{-AD}) (FDR=0.1, FC>2). (c) The illustration shows some of the proteins higher- or lower expressed in HE^{+AD}. Created with BioRender.com.

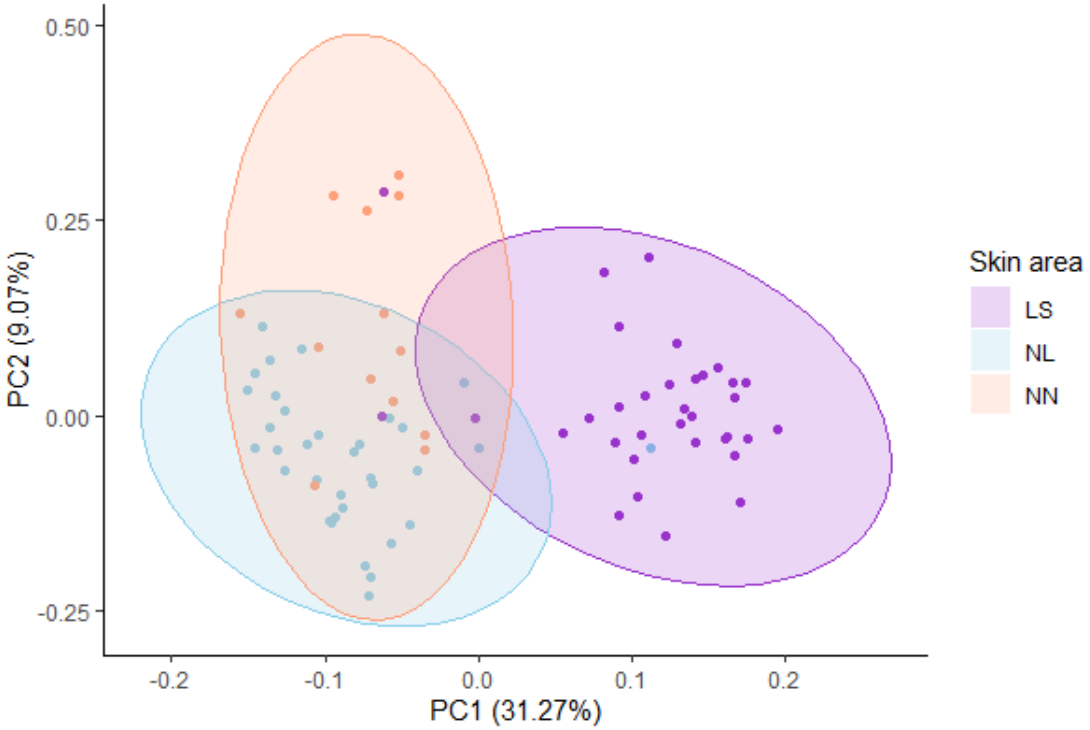
Supplementary figures



Supplementary figure 1Histogram of log2(x) transformed and normalized LFQ intensity (x-axis) vs. protein counts (y-axis).



Supplementary figure 2. No significant differences between localizations on the hands were observed between the number of detected proteins (Wilcoxon ranked-sum t-test) for neither healthy nor lesional skin areas.



Supplementary figure 3. Principal component analyses (PCA) of all quantified proteins show that the largest difference was observed between lesional (LS) and non-lesional (NL) as well as healthy (NN) skin areas (PC1) whereas minor differenceswere observed between non-lesional and healthy skin areas.

Supplementary tables

Supplementary table 1

TFE protein extraction from skin tape strips

Materials

- Mili-Q water
- 2-2-2- trifloroethanol (TFE) (50%)
- 1M DTT
- 100mM Tris pH 8.5
- CAA 550mM
- Lysine-C? (0.5 µg/µl)
- Trypsin (0.5 µg/µl)
- TFA (1%, 10%, and 100%)
- ACN (100%)
- Ammonium hydroxid (AH) (25%)
- Stagetips (2 layers of SDB-RPS in a 200 µl pipette tip).

Solutions

Make fresh solutions. Make buffers for day 2 during day 1 to reduce lab time on day 2.

Solution	Recipe
Lysis buffer	20% TFE, 5mM DTT in 100mM Tris Buffer
Enzyme solution	100 µl Mili-Q water + 4 µl Lysine-C(0.5 µg/µl) + 4 µl Trypsin (0.5 µg/µl)
Wash buffer I	99% Isopropanol + 1% TFA
Wash buffer II	0.2% TFA in Mili-Q water
Elution buffer	80% ACN + 1% Ammonium hydroxid
A*	2% ACN + 0.2% TFA

Day 1

1. Add 400 µl *lysis buffer* to each tube and vortex for 1 min. If cells from several tapes are to be pooled then transfer the lysis buffer to the next tube and vortex again.
2. 95 ℃ at 10 min without shaking. Cool down samples on ice.
3. Sonicate samples 15 min on the biorupter at high intensity (15 30/30s).
4. Spin samples down.
5. Add CAA to a final concentration of 25mM.
6. Vortex and spin down.
7. Incubate 20 min, **dark** at room temperature.
8. Add 324 µl *enzyme solution* (for app. 300 µl sample).
9. 37 ℃ 1200 rpm overnight.

Day 2

1. Add 1% volumen 100% TFA (e.g. 3.6µl 100%TFA for 360µl sample).
2. Spin down at high speed for 5 minutes (tabletop centrifuge).
3. Stage tip as follows on a PCR plate/tip box.
    4. 200 µl sample.
    5. Centrifuge 750 G for 5 min
    6. Repeat with the remaining sample.
    7. 200 µl *wash buffer I* (ISO+TFA).
    8. Centrifuge 750 G for 5 min
    9. 200 µl *wash buffer II* (0.2% TFA).
    10. Centrifuge 750 G for 5 min
    11. Transfer stage tips to a new PCR plate for elution.
    12. 60 µl *elution buffer*.
    13. Centrifuge 750 G for 5 min
1. Speedvac until completely dry 45 min at 45℃.
2. Add 6µl A* for the tape samples and 12 µl A* for the biopsy sample. Put on a lid.
3. 10 min 37℃ 1000rpm.
4. Spin down a few seconds.
5. Measure the concentration on nanodrop.

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